Oral Microbial Diversity in Health: An Ethnic Comparative Study.

Stephanie Jay Udoh BSc. (Hons)

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Abstract

This work involved two key phases: an *in-vitro* study and a clinical trial (*in-vivo*) study. The *in-vitro* study compared the antimicrobial properties of an oral formulation containing 5% potassium nitrate and 0.24% sodium fluoride to 1% chlorhexidine using a flat-bed perfusion model. The *in-vitro* objectives were 1. SIFT-MS analysis of volatile organic compounds before and after treatment, 2. assessing biofilm bacterial load before and after treatment, 3. assessing antibacterial susceptibility and bacterial cell membrane permeability effects of the oral formulation. The clinical trial (*in-vivo*) phase explored ethnic-specific oral microbiota profiles within a healthy population, particularly focusing on Black African and White European ethnic groups. The *in-vivo* objectives included 1. comparing volatile organic compound levels in mouth air between the two ethnic groups, 2. analyzing the variation in tongue and subgingival plaque microbiota within low-risk individuals of both ethnicities, and 3. scrutinizing the differences in tongue and subgingival plaque microbiota between Black African individuals with low risk and high risk for developing type 2 diabetes.

The *in-vitro* study results showed that the 5% potassium nitrate and 0.24% sodium fluoride toothpaste demonstrated significant reductions in volatile organic compound levels ($p < 0.01$), diminished biofilm density ($p < 0.01$), and increased bacterial cell membrane damage ($p < 0.001$) when compared to the 1% chlorhexidine formulation within the flat-bed biofilm model. In the *in-vivo* study, notable similarities emerged in the oral microbiota of both ethnic groups, revealing comparable profiles in health across various analytical dimensions. While subtle variations were detected, factors such as diet, oral hygiene practices, and lifestyle choices likely influenced these differences. Additionally, the analysis spotlighted unique bacterial taxa, including *Dialister* species and *Candidatus Nanosynbacter lyticus*, highlighting distinctions between low and high-risk groups.

The study concludes that the tested oral formulation may present an effective alternative to Chlorhexidine. It reveals striking similarities in oral microbiota between White European and Black African individuals, with minor differences possibly influenced by diet and oral hygiene practices. Further research is vital to validate these findings, explore the formulation's clinical applications, and understand the implications of microbial variations for oral and systemic health in diverse racial groups.

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Writing a thesis is a solitary journey, but the road to completion is made easier by the support and encouragement of others. I would like to express my deepest gratitude to all those who have supported me throughout this process.

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ACE	Abundance-Based Coverage Estimator
AGE	Advanced Glycation End Product
ANOSIM	Analysis of Similarity
ANOVA	Analysis of Variance
BHI	Brain Heart Infusion
BMI	Body Mass Index
BOP	Bleeding on Probing
BSA	Bovine Serum Albumin
C5aR	C5a Receptor
CAL	Clinical Attachment Loss
CHX	Chlorhexidine
COPD	Chronic Obstructive Pulmonary Disease
CRP	C-reactive Protein
DGGE	Denaturing Gradient Gel Electrophoresis
Del-1	Developmental Endothelial Locus 1
DCs	Dendritic Cells
DM	Diabetes Mellitus
eNOS	Endothelial Nitric Oxide Synthase
ET1	Endothelin 1
FAA	Fastidious Anaerobe Agar
G+ve	Gram positive
G-ve	Gram negative
Hb1Ac	Glycated Haemoglobin
HMDS	Hexamethyldisilazane
HOMD	Human Oral Microbiome Database
HOMIM	Human Oral Microbe Identification Microarray

HOMINGS	Human Oral Microbe Identification using Next-Generation Sequencing
IFN	Interferon
IL	Interleukin
ICAM-1	Intracellular Cell Adhesion Molecule-1
KEGG	Kyoto Encyclopedia of Genes and Genomes
KNO_3	Potassium Nitrate
LDA	Linear Discriminant Analysis
LEfSe	Linear Discriminant Analysis Effect Size Analysis
Mϕ	Macrophages
MBC	Minimum Bactericidal Concentration
MCP-1	Monocyte Chemoattractant Protein-1
Mfa1	Minor Fimbria Subunit Protein
MIC	Minimum Inhibitory Concentration
MIM	Multiple Ion Monitoring
MMP	Matrix Metalloproteinase
MTG	Metagenomics
MTT	Metatranscriptomics
NaF	Sodium Flouride
NF-kB	Nuclear Factor-Kb
NMDS	Non-Metric Multi-Dimensional Scaling
NO	Nitric Oxide
OH	Oral Health
OCP	Osteoclast Precursors
OPG	Osteoprotegerin
OTU	Operational Taxonomic Unit
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PCoA	Principal Coordinates Analysis

PD	Phylogenetic Diversity
PEEK	Poly-Ether Ether Ketone
PH	Periodontal Health
PICRUSt	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States
PKC	Protein Kinase C
PGE2	Prostaglandin E2
PPD	Periodontal Pocket Depth
PRR	Pattern Recognition Receptor
PSD	Polymicrobial Synergy and Dysbiosis
QAS	Quaternary Ammonium Salts
RP	Refractory Periodontitis
RAGE	Receptor for Advanced Glycation End Products
RANKL	Receptor Activator of Nuclear Factor κB Ligand
ROS	Reactive Oxygen Species
SEM	Scanning Electron Microscopy
SerB	Serine Phosphatase
SIFT-MS	Selected Ion Flow Tube Mass Spectrometry
SIM	Selected Ion Monitoring
SLS	Sodium Lauryl Sulphate
TLR2	Toll-Like Receptor 2
TNF	Tumour Necrosis Factor
Tregs	T Regulatory Cells
VOCs	Volatile organic compounds
VCAM-1	Vascular Cell Adhesion Molecule-1
VFAs	Volatile Fatty Acids
VSCs	Volatile Sulphur Compounds
YLD	Years Lived With Disability

Chapter 1 Introduction

1.1 Periodontal disease: overview and epidemiology

Periodontal disease is a common oral infection affecting the tissues that surround and support the teeth. Existing literature confirms that periodontal diseases have been recognized and treated for at least 5000 years, with gingivitis and periodontitis being identified as the most common periodontal diseases (Nazir *et al.,* 2020). For example, approximately 50% of the population in all age groups exhibit reversible gingival inflammation in the United States (Taylor and Borgnakke, 2008). Additionally, moderate or severe periodontitis, with destruction of periodontal attachment tissues is much less common than gingivitis yet still a common chronic disease, affecting approximately 5–15% of any population (Albandar *et al.,* 1999; Burt, 2005). According to Tonetti *et al.* (2017), gingivitis and periodontitis are inflammatory conditions caused by the formation and persistence of microbial biofilms on the hard, non-shedding surfaces of teeth. Gingivitis which is characterized by bleeding, swollen subgingival plaques, and pain, is the first manifestation of the inflammatory response to the biofilm and is usually reversible (i.e. if the biofilm is disrupted, gingivitis resolves), but if biofilm persist, gingivitis becomes chronic and progresses to periodontitis, commonly known as subgingival plaque disease (Fig 1.1.) (Michaud *et al.,* 2017).

Periodontitis is a multifactorial disease with the microbial dental biofilm or dental plaque as its initiator (Michaud *et al.,* 2017). Furthermore, it develops over time with accumulation of dental plaque, bacterial dysbiosis, formation of periodontal pockets, subgingival plaque recession, and is typically characterized by non-reversible tissue destruction resulting in progressive loss of periodontal ligament, connective tissue and alveolar bone, which can ultimately lead to tooth loss (Michaud *et al.,* 2017). The clinical features of periodontal disease include signs and symptoms such as colour alterations, volume and texture of the gingivae, bleeding upon probing (BOP), increased periodontal pocket depth (PPD) due to reduction of resistance to probing of soft marginal gingival tissues, clinical attachment loss (CAL), gingival recession, alveolar bone loss, root furcation exposure, increased tooth mobility and drifting (Rahman, 2023).

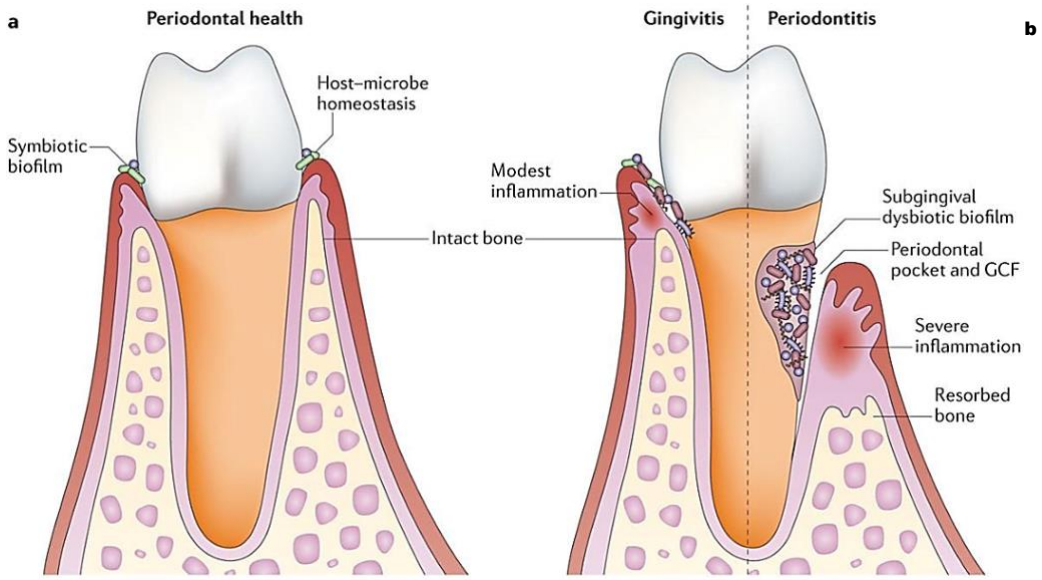


Figure 1.1 Comparison of the anatomical structure of the periodontium in health (a) and (b) in gingivitis & periodontitis (Mostajo, 2017).

Globally, periodontal diseases accounted for 3.5 million years lived with disability (YLD) in 2016 (Nazir *et al.,* 2020). Moreover, a review by Jin *et al.* (2016) suggests that there was a 57.3% increase in the global burden of periodontal disease from 1990 to 2010. According to Kassebaum *et al.* (2014), periodontitis is the 6th most prevalent oral condition worldwide with a prevalence of 30-35%. It has also been reported by the Global Burden of Disease study (2016) that severe periodontitis affects 11.2% of the global adult population and is one of the major causes of tooth loss which can cause compromised mastication, altered speech, low self-confidence, and poorer quality of life in general. Additionally, findings from a study by Listl *et al.* (2015) reported that in 2010, worldwide loss of productivity due to severe periodontitis was estimated to be US$54 billion per year.

Development and progression of periodontal disease in an individual are personalised by a number of endogenous and exogenous risk factors (Genco and Borgnakke, 2013). Assessment, knowledge and proper managements of these factors can facilitate the prevention and management of the disease (Bartold, 2018). Some of these risk factors can be lifestyle factors, such as smoking and alcohol consumption. They also include diseases and unhealthy conditions such as diabetes mellitus, obesity, metabolic syndrome, osteoporosis, and low dietary calcium and vitamin D (Mihaela *et al.,* 2017). These risk factors are modifiable and their management is a major component of the contemporary care of many periodontal patients (Reynolds, 2014). Genetic factors also play a role in periodontal disease and allow one to target individuals for prevention and early detection (Bartold, 2018). The role of genetic factors in aggressive periodontitis is clear. However, although

genetic factors (i.e., specific genes) are strongly suspected to have an association with chronic adult periodontitis, there is as yet no clear evidence for this in the general population (Genco and Borgnakke, 2013). Other risk factors include age, sex and socioeconomic status (Reynolds, 2014).

Periodontal disease inequalities exist among different age groups, and the severity of the disease increases with advancing age. In the United Kingdom, it is estimated that 9% of the population aged over 16 years have severe periodontitis with the global prevalence expected to increase in coming years due to growth in the aging population (Rahman, 2023). In an epidemiological study by Tadjoedin (2017), it was found that the highest prevalence of chronic periodontitis was shown in the elderly population (82%), followed by adults (73%) and adolescents (59%). Furthermore, a review of 75 studies reported that the prevalence of severe periodontitis increases with age and peaks at the age of 40 years and then remains stable in older age, hence exhibiting a high burden of disease in the elderly population (Kassebaum *et al.,* 2014). Nazir *et al.* (2020) suggests that the high prevalence of periodontal disease in older populations may be attributed to poor oral hygiene, lack of government financing for oral health services, and lack of oral health promotion programs and policies aimed at the older population in various countries around the world.

Periodontal disease has become well established as a public health problem since it is highly prevalent and causes disability and social inequality. It is also known that periodontitis is associated with reduced quality of life, masticatory dysfunction, and is a major factor in the increase in costs of oral health care (Tonetti *et al.,* 2015). Furthermore, research has associated periodontal disease with other common systemic conditions such as diabetes, cardiovascular disease, adverse pregnancy outcomes, rheumatoid arthritis, and chronic obstructive pulmonary disease (Rahman, 2023). Despite being a global public health problem and the dramatic increase in the burden of periodontal disease during the last decades, limited periodontal data exists in the WHO oral health data bank. For instance, Nazir *et al*. (2020) reports that out of 193 countries of the United Nations, periodontal disease data of only 20 countries for adolescents, 27 countries for adults, and 18 countries for older persons have been maintained by the WHO. This presents some challenges as oral health programs aimed at preventing periodontal disease require robust epidemiological data, and the allocation of health resources to provide treatment for periodontal disease cannot be achieved in the absence of updated and reliable data. While periodontal disease can generally be prevented, it has been reported that patients with periodontal disease

usually seek oral care when the disease reaches an advanced stage because its early stages are usually asymptomatic. Due to this, early diagnosis and treatment are crucial for the maintenance of periodontal health in the population (Nazir *et al*., 2020).

1.2 The oral microbiome and periodontitis

1.2.1 An introduction to the oral microbiome

In the human body, there are existing complex communities of microorganisms that form the human microbiome (Ethan *et al.,* 2021). These organisms educate the immune system and contribute their genome to its host, providing the host with traits that did not originally evolve with the human body (He *et al.,* 2015). According to Turnbaugh *et al.* (2007), the microbiome in every human body can be classified into core phylotypes which are important for maintaining homeostasis required for health, and variable phylotypes which provides the genetic diversity that is unique to each individual. Clinically, the implication of unique microbiomes between individuals may explain why disease can manifest and progress differently (Zarco *et al.* 2012).

The oral cavity contains several distinct microbial habitats such as the teeth, gingival sulcus, tongue, attached gingivae, cheek, lip, hard palate and soft palate (Graves *et al.,* 2019). Moreover, it is recognised as one of the most clinically relevant microbial habitats as it is colonised by a personalised set of microorganisms including bacteria, archaea, viruses and fungi (Graves *et al.,* 2019). The oral microbiome refers to the ecological community of commensal, symbiotic or pathogenic microorganisms established within biofilms on hard and soft tissues in the oral cavity (He *et al.,* 2015). It is dynamic due to the direct and continuous communication with the external environment and has been linked to the two most common diseases in the human mouth including periodontitis. Approximately 700 bacterial species have been identified in the human oral cavity, with over 400 of these species isolated from the periodontal pocket and the remaining 300 from other oral sites such as the tongue, oral mucous membranes, carious lesions and endodontic infections (Udoh *et al.,* 2022).

It is widely accepted that oral microorganisms cause diseases mainly by a synergistic way, and the ecologic interactions within the oral community play a crucial role in determining local homeostasis or a transition to a state of disease (Ethan *et al.,* 2021; Griffen *et al*., 2012;

Hajishengallis and Lamont, 2012; Marsh, 2005; Socransky *et al.,* 1998). With appropriate oral hygiene, the oral microbiome typically exists in a symbiotic or commensal state where relationships among microbes benefit the host or maintain the biodiversity within the oral cavity at no expense to the host (Stone and Xu, 2017). In health, commensal organisms may prevent diseases by enhancing colonization resistance by pathogen exclusion; decreasing the virulence of a pathogen by degrading virulence factors or hindering its ability to multiply; aiding in development of host defences by priming a healthy immune system; and preventing detrimental immune responses by maintaining balance within the host (Belibasakis *et al.,* 2019). Moreover, progression to disease occurs once this symbiotic balance is lost between host and microbiome. An ecological shift could occur either due to a change in relationship between microbes and host, an increase in relative abundance of certain species or its acquisition of virulence factors (Ethan *et al*., 2021).

1.2.2 Changing concepts in periodontitis

In 1998, Socransky *et al.* proposed that oral diseases could be better understood by focusing on the consortia of organisms rather than individual pathogens. They describe how bacteria tend to be grouped in clusters according to nutritional and atmospheric requirements. Their work identified five sets of bacteria or complexes that were consistently found together in periodontitis (Fig 1.2.). They suggested that the most pathogenic group comprised of three key species including *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola*, which collectively formed the "red complex". Moreover, this complex depended on earlier colonisation of the periodontal pocket by a complex consisting of somewhat less pathogenic organisms called the "orange complex". Most periodontal sites harbour either all or none of the species belonging to the same complex, while individual species or pairs of species are detected less frequently than expected, reinforcing the hypothesis of a community theory rather than germ theory (Socransky *et al.,* 1998; Caldwell *et al.,* 1997). Socransky *et al.,* (1998) established interrelations between the complexes as well, suggesting that the microbiota linked to the red cluster are seldom detected in the absence of the orange complex. Additionally, it was found that in higher detected amounts of the orange complex, the greater the colonisation by the red complex members. Moreover, yellow and green complexes showed a similar preference for each other and a weaker relation with the orange and red complexes, while the purple complexes showed rather loose relations with all the clusters. These relations could be explained by mechanisms of antagonism, synergism and environmental selection. Clinically, this was evidenced by

bleeding sites, with yellow and green complexes being associated with shallow pockets (PD <3 mm), while orange and red ones related to increasing periodontal indices and more advanced lesions (Socransky *et al.,* 1998; Socransky *et al.,* 1991). These bacterial complexes (Figure 1.2.) were based on microbial analysis of subgingival plaque samples using DNA-DNA checkerboard hybridisation assays.

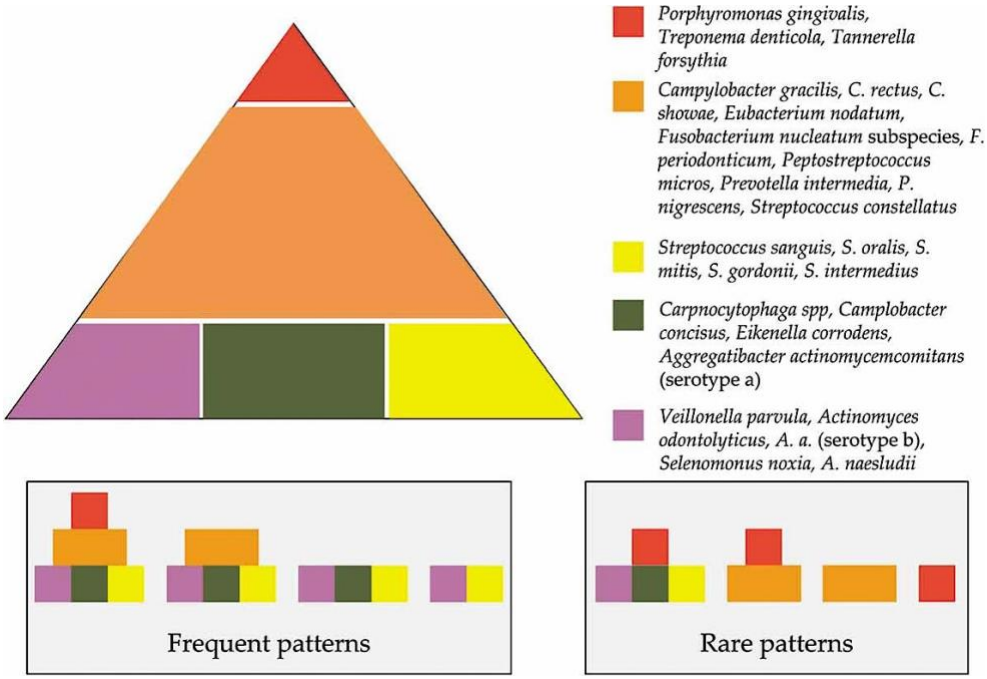


Figure 1.2 The relationship between subgingival species based on a modified adaptation of Socransky's work (Abdulkareem *et al.,* 2023). The figure depicts a pyramid structure representing the hierarchical arrangement of the species. The base of the pyramid represents the early colonizers, which are followed by the orange complex. The orange complex acts as a bridge between the early colonizers and the red complex, which dominates the biofilm during the advanced stages of periodontitis. This visual representation highlights the progressive shift in microbial composition as periodontitis advances.

The convenience and appeal of the complex or cluster concept led to widespread adoption. However, as high-throughput sequencing approaches for microbe detection were adopted, newer concepts emerged due to broader insights into bacterial evolution, epidemiology and pathogenesis. First, several studies (Ximenez-Fyvie *et al.,* 2000; Mayanagi *et al*., 2004; Diaz *et al.,* 2006) showed that red complex organisms such as *P. gingivalis* can be found in the absence of disease. Moreover, contrary to the dogma of Gram-negative bacterial dominance in periodontitis, it appears that Gram-positive anaerobic species exhibit a significant increase in deep diseased sites relative to health sites and can be detected in greater abundance than Gram-negative species (Kumar *et al.,* 2005). It is now also

understood that the presence of bleeding sites is associated with increased total bacterial biomass rather than a distinct bacteriome (Abusleme *et al.,* 2013).

Another revolutionising concept that has emerged is the keystone pathogen hypothesis, a key feature of the polymicrobial synergy and dysbiosis model (Hajishengallis *et al.,* 2012). This concept explores the convergence of a synergistic and dysbiotic microbial community rather than specific "periopathogens" to establish a disease-provoking microbiota that overwhelms the host. These keystone pathogens are thought to act as initiators by elevating the virulence of the entire community, even at low abundance levels (Ethan *et al.,* 2021). For example, the disruption to the host innate immune and inflammatory response caused by *P. gingivalis* via the complement pathway, which triggers a dysbiotic community within the periodontium (Bostanci *et al*., 2012). However, it is important to note that the presence of a keystone pathogen may not always lead to conversion to dysbiosis as studies show that most disease-associated taxa are present in health, albeit in lower populations (Abusleme *et al.,* 2013; Hajishengallis *et al.,* 2012). Conversely, most health-associated taxa have also been commonly detected in periodontitis, suggesting suppression of these species rather than loss, during the establishment of periodontitis (Abusleme *et al.,* 2013; Hajishengallis *et al.,* 2012). Although, these concepts have established the existence of a global dysbiosis during periodontitis, with ecological shifts in a broad and synergistic community structure, rather than shifts in membership by single or several select pathogens (Ethan *et al.,* 2021).

1.3 Oral microbial biofilms in periodontal health and disease

A biofilm is defined as an assemblage of microbial cells that is irreversibly associated with a surface and enclosed in a matrix of primarily polysaccharide material (Mosaddad *et al.,* 2019). Figure 1.3 shows a classical biofilm lifecycle in the oral cavity which includes bacterial attachment, biofilm growth and maturation and biofilm dispersal (Kuang, Chen and Xu, 2018). Biofilm-associated organisms tend to exhibit differences from their planktonic counterparts including physiological properties, susceptibility to anti-microbial agents, interaction with host tissues and immunological response (Sbordone and Bortolaia, 2003). Biofilms may form on a wide variety of surfaces in the oral cavity which provide an ideal environment for the colonisation and growth of bacteria (Mosaddad *et al.,* 2019). Colonisation of the oral microbiome has mostly occurred on tooth surfaces which leads to

biofilm (dental plaque) formation; however, other sites including the tongue have attracted great attention due to its colonization by microbes associated with halitosis (He *et al*., 2015).

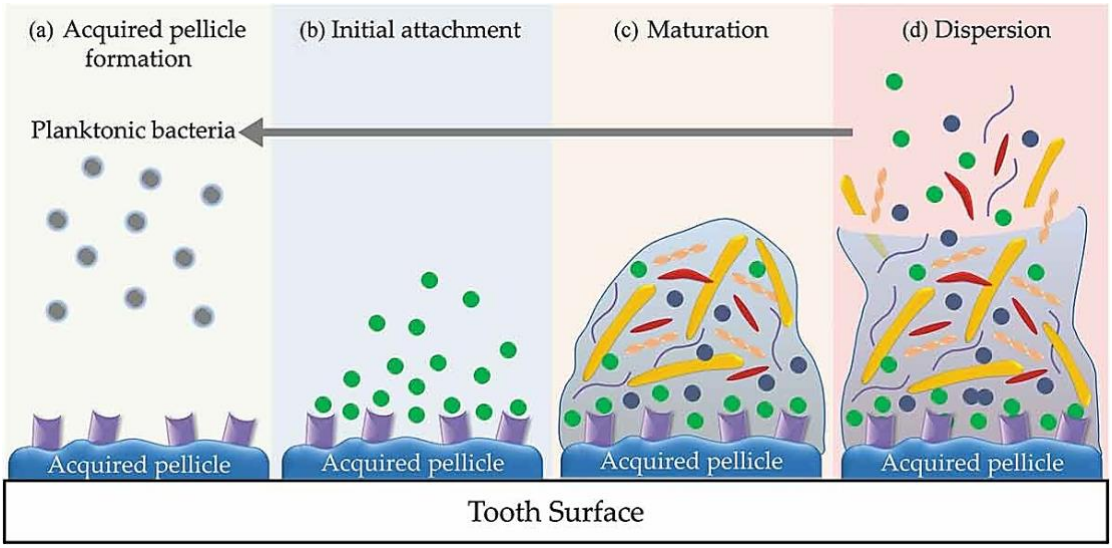


Figure 1.3 The process of biofilm formation and development in the oral cavity (Abdulkareem *et al.,* 2023). (a) Acquired pellicle formation; (b) Initial attachment of early colonizers; (c) Maturation of biofilm and coaggregation of bacteria; (d) Dispersion of bacteria.

Dental biofilm on the tooth surface is initiated by an acquired pellicle (Fig 1.3), a proteinaceous conditioning film comprising of albumin, lysozyme, glycoproteins, phosphoproteins, lipids and gingival crevice fluid, which promotes bacterial adhesion to the tooth surface (Graves, Corrêa and Silva, 2019). Moreover, dental plaque can be classified as supragingival or subgingival plaque, depending on the location (Fig 1.4) (He *et al.,* 2015). The subgingival plaque is typically more anaerobic and is situated below the gingival margin, while the supragingival plaque located above the gingival margin (Graves, Corrêa and Silva, 2019). The composition of the supragingival plaque varies with the balance between periodontal health and disease.

In periodontal health, the microflora at the subgingival plaque's grove is scanty with Gram-positive streptococci dominating the population (Fig 1.4). Moreover, healthy gingivae is related to a very simple supragingival plaque arrangement including (1-20) layers or predominantly Gram-positive bacteria including *Streptococcus spp., Actinomyces viscosus*, and *Parvimonas micra* (previously *Peptostreptococcus micros*), and also Gram-negative species like *Campylobacter gracilis*, *Fusobacterium nucleatum*, *Prevotella intermedia*, and *Veillonella* (Mosaddad *et al.,* 2019).

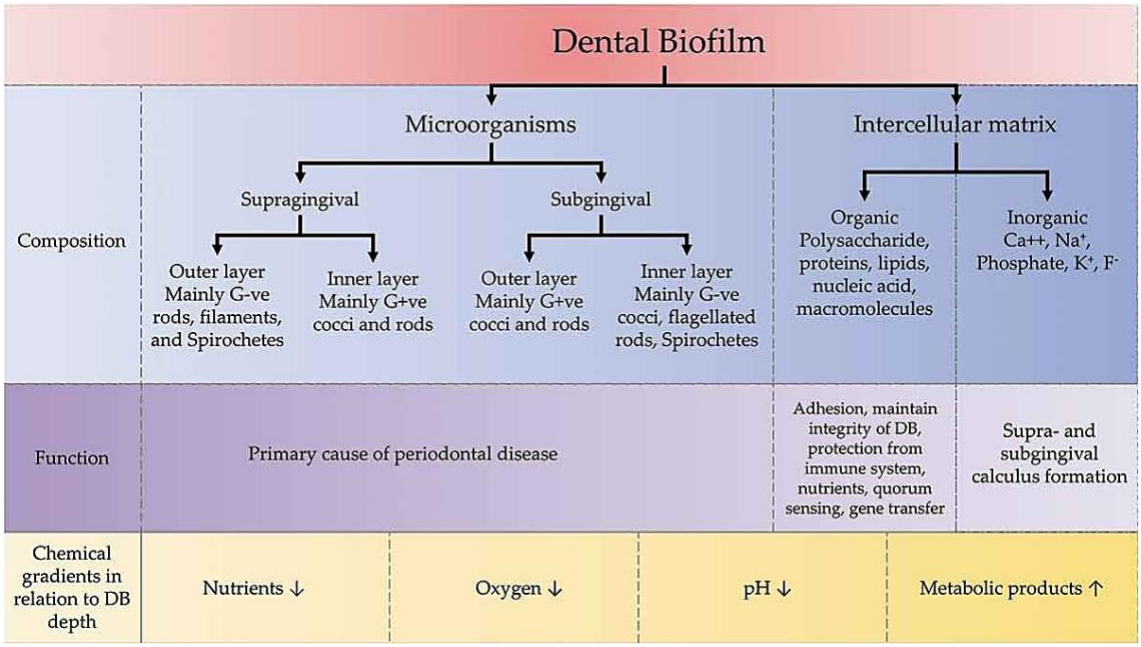


Figure 1.4 Components of dental biofilm, their respective functions, and the relationship between chemical gradients and the depth of the biofilm (Abdulkareem *et al.,* 2023). (DB) dental biofilm, (G+ve) Gram-positive, (G-ve) Gram-negative.

The first or early colonizers are organisms that can withstand the high oxygen concentrations and resist the various removal mechanisms of the oral cavity such as swallowing, chewing, nose-blowing, salivary, nasal mucosa, and crevicular fluid outflow (Manji, Dahlen and Fejerskov, 2018). Their proliferation enables the subsequent adhesion of other bacterial species, which are incapable of attaching to hard surfaces of teeth and can only attach to already existing microorganisms (Mosaddad *et al.,* 2019). This is so-called "Secondary Colonization". The increase in the number of plaque layers creates a nutritional and atmospheric gradient, decreasing the oxygen level and favouring the growth of anaerobes within the matrix (Wang *et al.,* 2017; Kalia *et al.,* 2017). Clinical gingivitis is accompanied by the evolution of a more organised dental plaque. Such biofilms are characterized by multiple cell layers (100-300), with bacterial stratification arranged by metabolism and aerotolerance (He *et al.,* 2015; Mosaddad *et al.,* 2019). In addition to the Gram-positive cocci, rods, and filaments associated with healthy gingivae, the number of Gram-negative cocci, rods, and filaments increases, and anaerobic bacteria such as *F. nucleatum*, *C. gracilis*, *B. forsythus*, *Capnocytophaga* spp. become evident (Kononen *et al*., 2015).

Gingivitis typically precedes periodontitis; however, the progression from gingivitis to periodontitis is not an automatic or universal occurrence observed in every patient or at every site. This transition depends on three primary factors: host sensitivity, pathogenic

bacteria, and protective bacteria (Mosaddad *et al*., 2019). Some pathogenic bacteria possess virulence features such as pili, fimbriae and blebs that allow adhesion and colonisation, and host defences are impaired through a number of mechanisms that initiate tissue damage and impedes tissue recovery (Beall *et al.,* 2018). The biofilm associated with periodontitis exhibits a complex composition, comprising multiple layers. Notably, the structure of the bacterial community differs slightly between the dynamic and decomposing phases compared to the remission period. Moreover, evidence suggests that for pathogenic potential to be realised within the periodontal ecosystem, activities of a keystone pathogen such as *P. gingi*valis are required (Mosaddad *et al.,* 2019).

1.4 Polymicrobial synergy and dysbiosis (PSD) in periodontitis

Dysbiosis is an imbalance in the relative abundance of microbial species within an ecosystem associated with the disease (Hajishengallis and Lamont, 2016). In periodontitis, dysbiotic microbial communities are thought to exhibit synergistic virulence, this increases resistance to the host immune response and exploits tissue-destructive inflammation. This in result fuels the feedback loop of escalating dysbiosis and inflammatory bone loss, potentially leading to tooth loss and systemic complications (Fig 1.5) (Hajishengallis, 2014). Although the tooth-associated biofilm plays a crucial role in the initiation and progression of periodontitis, it is primarily the host inflammatory response that inflicts the irreversible damage on the periodontium (Hajishengallis *et al.,* 2012). Hajishengallis and Lamont (2012) suggest that a number of core requirements are required for a potentially pathogenic community to arise including (i) Bacterial constituents will express the relevant adhesins and receptors to allow assembly of a heterotypic community (ii) Individual members of the community will be physiologically compatible or at least non-antagonistic (iii) Combined activity of dysbiotic community will resist host innate and acquired immune responses and contribute to inflammation. Moreover, the host-microbe homeostasis can also be disrupted by various risk factors (Fig 1.5) including congenital or acquired host immunodeficiencies or immunoregulatory defects, systemic diseases, environmental factors and epigenetic modifications in response to environmental changes (Hajishengallis, 2014).

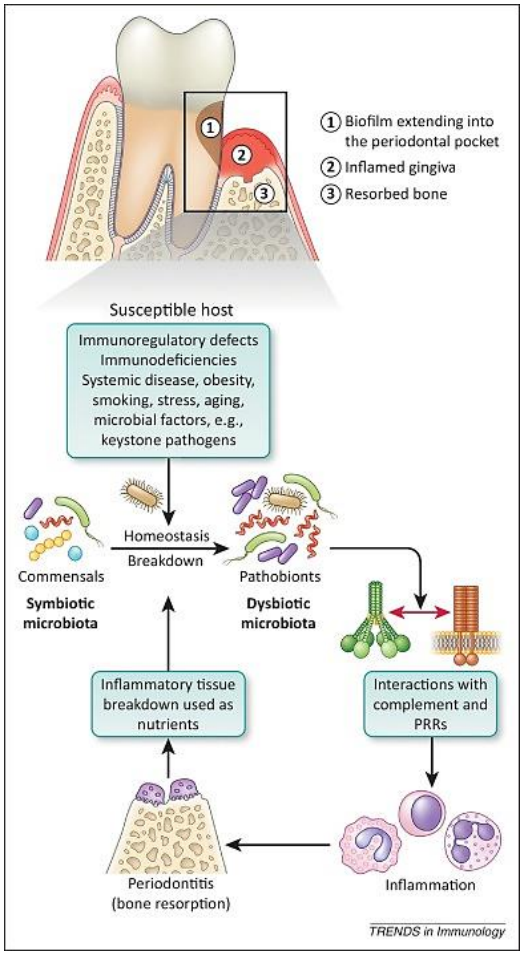


Figure 1.5 The PSD model of periodontal disease (Hajishengallis and Lamont 2016).

Periodontal health relies on maintaining a controlled inflammatory state that facilitates a harmonious relationship between the host and the microbial community within the periodontium. However, defects in the immunoinflammatory status of the host or predisposing conditions and environmental factors can shift the balance towards dysbiosis. The presence of keystone pathogens, aided by accessory pathogens can similarly tip the balance toward dysbiosis even in hosts without apparent predisposing genetic or environmental factors (at least in mice). The dysbiotic microbiota elicits inflammation primarily through crosstalk signalling between complement and pattern recognition receptors (PRRs). This inflammation has two major and interconnected effects: firstly, it induces destructive inflammatory processes in the periodontal tissues, including the characteristic bone loss associated with periodontitis. Secondly, it generates nutrient-rich conditions through the breakdown of tissue, such as the release of tissue breakdown peptides and other products, which further promote dysbiosis and contribute to the ongoing destruction of the periodontal tissues. This establishes a self-perpetuating pathogenic cycle. It is important to note that host susceptibility not only influences the transition from a symbiotic to a dysbiotic microbiota but also determines the predisposition of the host to develop inflammation severe enough to cause irreversible tissue damage. In theory, there may be individuals who can tolerate the conversion of a symbiotic microbiota into a dysbiotic state without experiencing periodontal bone loss. These individuals would be susceptible to dysbiosis but not to the accompanying destructive effects on periodontal tissues.

1.5 Pathogenicity of *P. gingivalis* in periodontitis

Porphyromonas gingivalis has been identified as a keystone pathogen in periodontitis by several studies (Sufaru, Martu and Solomon, 2022; Derveau *et al*., 2012; Hajishengallis, 2012; Hajishengallis *et al.,* 2011), highlighting its capacity to manipulate the host response, rather than a direct inducer of inflammation. Specifically, the Gram-negative anaerobe subverts the innate immune signalling including the communication between the complement system and Toll-like receptors (TLRs) (Hajishengallis, Darveau and Curtis, 2012). *P. gingivalis* can impair host defences in ways that alter growth and development of the entire microbial community (Fig 1.6), thereby triggering a destructive change in the normally homeostatic relation with the host (Hajishengallis, 2014). A study by Hajishengallis (2011) in mice showed that *P. gingivalis,* which comprises less than 0.01% of the total bacterial count (similar to its low abundance in human periodontitis-associated biofilms), failed to induce dysbiosis and periodontitis in mice lacking the cellular receptors necessary for the bacterium to subvert leucocyte defences. Furthermore, in non-human primates, specific immunization with a gingipain-based vaccine resulted in a reduction of indigenous *P. gingivalis* cells, accompanied by a decrease in the overall subgingival bacterial load and protection against bone loss (Hajishengallis, Darveau and Curtis, 2012). These studies provide compelling evidence of *P. gingivalis* as a keystone pathogen in periodontitis, capable of modulating the oral microbiota through direct effects independent of the host. Conversely, accessory pathogens represent a subset of organisms that synergistically support or enhance the virulence of keystone pathogens, such as *P. gingivalis* (Hajishengallis and Lamont, 2016). For instance, *S. gordonii* serves as an attachment substratum for *P. gingivalis* colonization, facilitated by the interaction between the minor fimbria subunit protein (Mfa1) and specific domains on the surface proteins SspA/B of streptococci (Daep *et al.,* 2011). Moreover, when *P. gingivalis* is grown in communities containing *F. nucleatum* and *S. gordonii,* it exhibits differential expression of over 500 proteins, indicating significant phenotypic changes in response to these accessory pathogens, thereby increasing its pathogenicity (Hajishengallis, 2014).

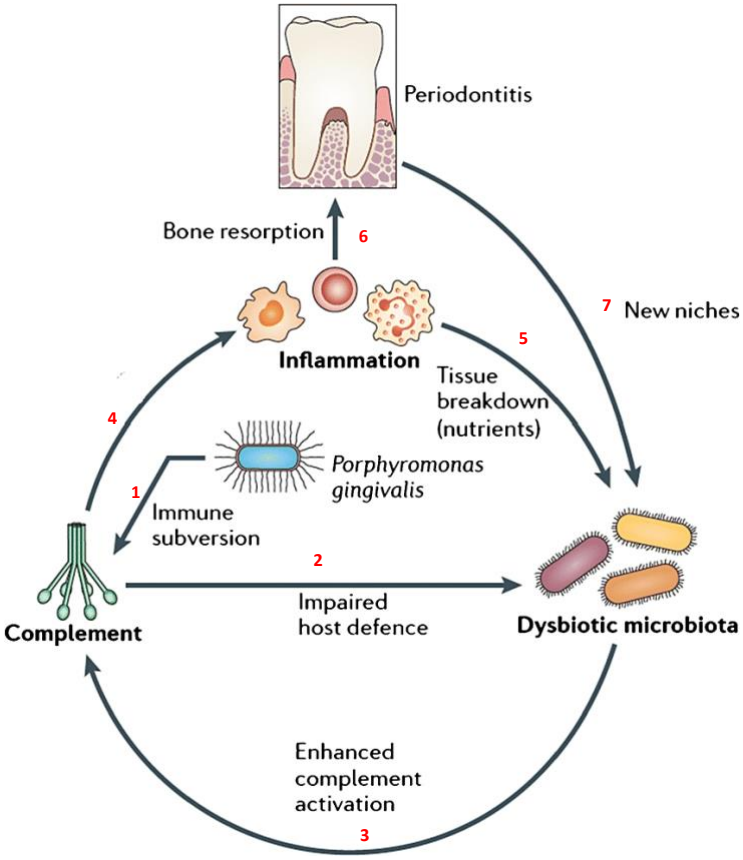


Figure 1.6 *P. gingivalis*–induced dysbiosis and periodontal disease (Hajishengallis *et al.,* 2012).

P. gingivalis subverts the complement (step 1) and impairs host defence (step 2), leading to overgrowth of oral commensal bacteria, which causes complement-dependent inflammation (steps 3 and 4). The destruction of inflamed tissue (step 5) benefits the growth of bacteria by creating a gingival inflammatory exudate that is rich in nutrients, including broken-down host proteins and hemin, which is a crucial source of iron. These changes in the environment are effectively utilized by proteolytic and asaccharolytic bacteria, leading to shifts in the composition of the bacterial community. Furthermore, the inflammation-induced resorption of bone (step 6) provides new opportunities for the dysbiotic microbiota to colonize (step 7). These combined modifications ultimately result in the development and continuation of periodontal disease.

P. gingivalis contains arginine-specific cysteine proteinases called gingipain which exhibits complement C5 convertase-like activity, generating high levels of C5a locally to activate a C5a receptor (C5aR) on leucocytes (Liang *et al.,* 2011). The signalling of C5aR is known to interact with Toll-like receptor 2 (TLR2), which is concurrently activated by surface ligands of *P. gingivalis*. This interaction between C5aR and TLR2 leads to an amplified inflammatory response but reduces the ability of leukocytes to effectively eliminate the bacterium (Wang *et al.,* 2010). Additionally, *P. gingivalis* can inhibit the activation of antimicrobial pathways in leukocytes that are dependent on Toll-like receptor 4 (TLR4). It achieves this by expressing an atypical form of lipopolysaccharide that contains 4-acyl-monophosphorrylated lipid, a

potent antagonist of TLR4 (Jain and Darvaeu, 2010). Moreover, *P. gingivalis* utilizes a secreted enzyme called serine phosphatase (SerB) to hinder the synthesis of interleukin-8 (IL-8) by epithelial cells. This delay in IL-8 production postpones the recruitment of neutrophils, facilitating *P. gingivalis'* initial colonization of the periodontium (Bainbridge *et al.,* 2010). In the healthy periodontium, the synthesis of IL-8 by the junctional gingival epithelium, located near the tooth surface, plays a critical role. This synthesis creates a gradient that attracts neutrophils into the gingival crevice (Hajishengallis *et al.,* 2011). However, wild-type *P. gingivalis* disrupts this process and subverts the recruited leukocytes, potentially allowing uncontrolled growth of other bacterial species within the biofilm. This phenomenon aligns with observations showing an increase in the overall bacterial count after *P. gingivalis* colonization in mouse models of periodontal disease (Hajishengallis *et al.,* 2011). Uncontrolled bacterial growth triggers a heightened complement-dependent destructive inflammatory response (Fig 1.6), leading to the generation of various tissue-breakdown products, including degraded proteins and hemin. These products serve as nutrients for *P. gingivalis,* further promoting alterations within the biofilm and contributing to the establishment of a disease-associated microbiota (Hajishengallis, Darveau and Curtis, 2012).

Recent research indicates that *P. gingivalis* has the potential to modulate the adaptive immune response (Fig. 1.7). Specifically, the interaction between *P. gingivalis* and dendritic cells induces a cytokine profile that promotes T helper (Th)17 polarization while inhibiting the Th1 lineage (Jauregui *et al*., 2013; Gaddis *et al.,* 2013; Moutsopoulos *et al.,* 2011). Furthermore, *P. gingivalis* inhibits the production of Th1-recruiting chemokines by gingival epithelial cells and interferon (IFN)g by T cells (Jauregui *et al.,* 2013; Gaddis *et al*., 2013). Based on these findings, it can be hypothesized that *P. gingivalis*, as a keystone pathogen, can manipulate T cell development to promote Th17-mediated inflammation while impairing effective Th1-dependent cell-mediated immunity, which promotes immune clearance of *P. gingivalis*.

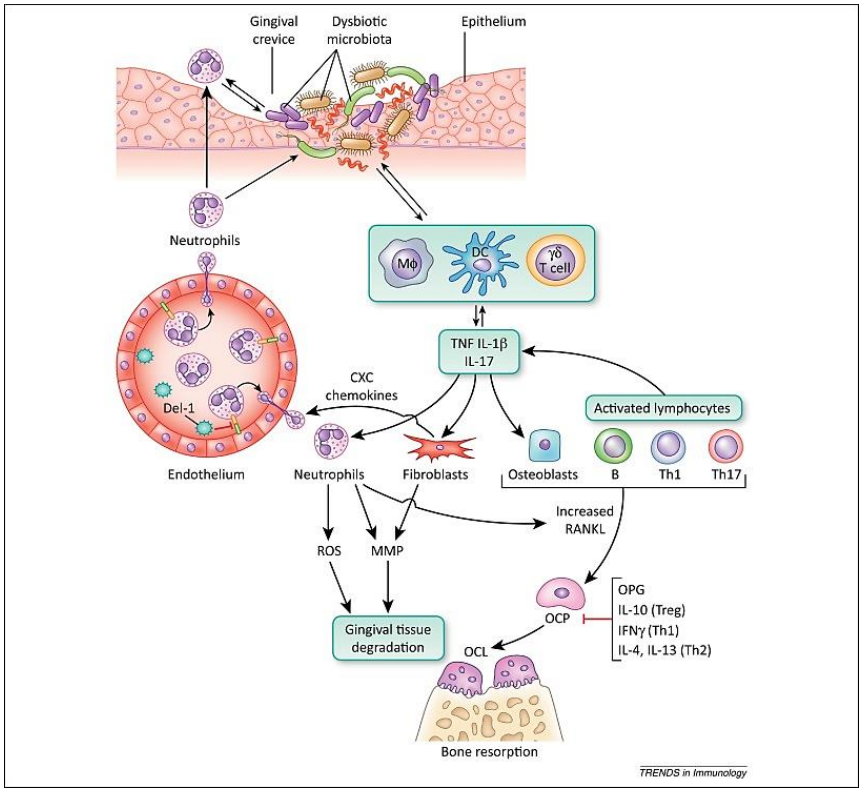


Figure 1.7 Inflammatory mechanisms leading to bone loss in periodontitis (Hajishengallis, 2014).

Recruited neutrophils to the gingival crevice fail to control a dysbiotic microbiota, which can thus invade the connective tissue and interact with additional immune cell types, such as macrophages (Mϕ), dendritic cells (DCs), and gamma delta (γδ) T cells; a subset of innate-like lymphocytes. These immune cells produce proinflammatory mediators such as tumour necrosis factor (TNF), interleukin (IL)-1β, and IL-17, which are known to promote bone resorption. Additionally, they regulate the differentiation of T helper (Th) cell types, further contributing to and exacerbating the inflammatory response. IL-17, a signature cytokine of Th17 (although also produced by innate cell sources), exerts its effects on innate immune and connective tissue cells, including neutrophils, fibroblasts, and osteoblasts. Through these interactions, IL-17 induces the production of CXC chemokines, which recruit neutrophils in a manner dependent on developmental endothelial locus 1 (Del-1). It also promotes the expression of matrix metalloproteinases (MMPs) and other tissue-destructive molecules, such as reactive oxygen species (ROS). Furthermore, IL-17 enhances the expression of receptor activator nuclear factor κB ligand (RANKL) by osteoblasts, which drives the maturation of osteoclast precursors (OCPs). Activated lymphocytes, specifically Th1 and Th17 cells, play a significant role in pathological bone resorption through the RANKL-dependent mechanism described above. Osteoprotegerin (OPG) acts as a soluble decoy receptor that inhibits the interaction between RANKL and its functional receptor (RANK) on OCPs. The ratio of RANKL to OPG increases with the escalation of inflammatory activity. Moreover, activated neutrophils express membrane-bound RANKL, which allows them to directly stimulate osteoclastogenesis if they are in close proximity to the bone. The anti-inflammatory cytokine IL-10, produced by T regulatory cells (Tregs), along with interferon (IFN)γ from Th1 cells and IL-4 plus IL-13 from Th2 cells, can suppress osteoclastogenesis.

1.6 Periodontitis and systemic diseases

Epidemiologic studies have associated periodontitis with over 43 systemic diseases (Scannapieco *et al.,* 2016; Cullinan *et al.,* 2013; Bartold *et al.,* 2012). The majority of these studies have found relatively modest odds ratio to specific systemic diseases including cardiovascular disease, respiratory disease, cancer and diabetes (Slots, 2022). Cardiovascular diseases are a set of diseases that include coronary artery disease, congestive heart failure, cardiac arrhythmias, valvar heart disease and stroke (He *et al*., 2015). Slots (2022) highlights that it is a major cause of disability according to the CDC and is the leading cause of death in the US. Given its high economic and social impact, the correlation between cardiovascular and periodontal disease has attracted attention of various researchers. A study showed that both prevalence and incidence of cardiovascular disease are significantly increased in patients with periodontitis (Bahekar *et al.,* 2007). Moreover, an association between edentulousness and serum antibodies against *P. gingivalis* and *A. actinomycetemcomitans* with coronary heart disease was observed in a study with 1163 men (Bahekar *et al.,* 2007). Although various epidemiological studies have suggested an association between periodontitis and cardiovascular disease, the impact of the oral infection on cardiovascular diseases has remained unclear (Slots, 2020).

Periodontitis has also been linked to both chronic obstructive pulmonary disease (COPD) and pneumonia. Pneumonia, which involves an infection in the airways, significantly contributes to morbidity and mortality, particularly in immunocompromised patients, and affects people of all ages. (Winning and Linden, 2015). COPD is characterised by progressive airflow obstruction and inflammation in the airways. Lung infections in COPD can result from various causes: microorganisms infecting the lower respiratory tract through inhaled infectious aerosols, the spread of infection from nearby areas, and the transmission of pathogens from sites outside the lungs (Bui *et al.,* 2019). Furthermore, pneumonia may be linked with periodontitis, as the oral cavity often harbours many potential opportunistic pathogenic bacteria. Several of these oral pathogens have been implicated in causing lung infections including *A. actinomycetemcomitans, Actinomyces israelii, Capnocytophaga spp, Chlamydia pneumoniae, E. corrodens, F. nucleatum, Fusobacterium necrophorum, P. gingivalis* (Bui *et al.,* 2019). Studies investigating a link between COPD and periodontitis remain preliminary, with only a limited number of studies investigating the impact of established chronic periodontitis on acquired lung infections (Winning and Linden, 2015).

The increased occurrence of cancer development in individuals with chronic inflammatory conditions has prompted investigations into potential associations with periodontitis. However, studies linking periodontitis to cancer face challenges due to confounding factors such as smoking and socioeconomic status (Mihaela *et al.,* 2017). Nevertheless, periodontitis has been identified as a potential risk factor for various cancers, including oral cancer. A meta-analysis study including 3183 subjects showed that patients with periodontal disease have an increased susceptibility to oral cancer (Yao *et al.,* 2014). Moreover, oral pathogens, specifically *P. gingivalis* and *F. nucleatum*, have been shown to positively correlate with development of oral cancer, suggesting that they could be biomarkers for early stages of the disease, or even targets for prevention of oral cancers in humans (Gallimidi *et al.,* 2015; Ha *et al*., 2015; Inaba *et al.,* 2014). More recent studies found a positive correlation between periodontal disease and pancreatic, lung, head and neck cancers, however, further studies with long-term follow up are still needed in this area (Michaud *et al.,* 2017).

1.7 Periodontitis and diabetes: a two-way relationship

1.7.1 Diabetes: overview and epidemiology

Diabetes Mellitus (DM) is a group of metabolic disorders characterised by elevated blood sugar levels – hyperglycaemia (Myers, 2018). Statistics show that DM is the ninth major cause of death worldwide with an estimated number of 5 million deaths in 2017 and global costs of $825 billion annually (Zheng, Ley and Hu, 2018). Epidemiological studies suggest that 425 million people are living with diabetes worldwide and about half of that population are undiagnosed (Cho *et al.,* 2018). In the UK, approximately 4.7 million people are living with diabetes, with about 1 million people thought to be undiagnosed (NHS, 2023). Furthermore, Diabetes UK (2023) reports that the NHS spends about 10% of its entire budget (£14 billion) yearly on diabetes.

Type 2 diabetes accounts for more than 90% of patients with diabetes and is linked to complications such as periodontitis that cause profound distress to patients and burdens health care systems (Chatterjee, Khunti and Davies, 2017). Moreover, incidence rates for type 2 diabetes continues to rise globally, especially in developing countries, with a prediction to reach 642 million people globally by 2040 (Sanz e*t al*., 2019). Additionally, mortality rates may vary between developed and developing regions due to the presence

of an effective and structured healthcare system in many developed regions such as the UK. Whilst the disease can be managed effectively in these regions, healthcare systems in some developing regions such as Nigeria continue to struggle due to several factors including unfavourable government policies, affordability of healthcare, etc. (Buowari, 2013). Although epidemiological data on periodontitis in West African regions is lacking, a study by Nazir (2017) reports higher prevalence rates in developing African regions compared to developed regions such as the UK. Currently, more than 14 million people in sub–Saharan Africa have diabetes, and research has predicted a two-fold increase by 2035 (Sundufu, Bockarie and Jacobsen, 2017). Studies have shown that the adverse effects of the disease are largely associated with hyperglycaemia (Fig. 1.8) which has negative impacts on multiple body systems as well as contributing to complications throughout the body including cardiovascular disease, renal disease, periodontitis and neuropathy (Sanz *et al*., 2018).

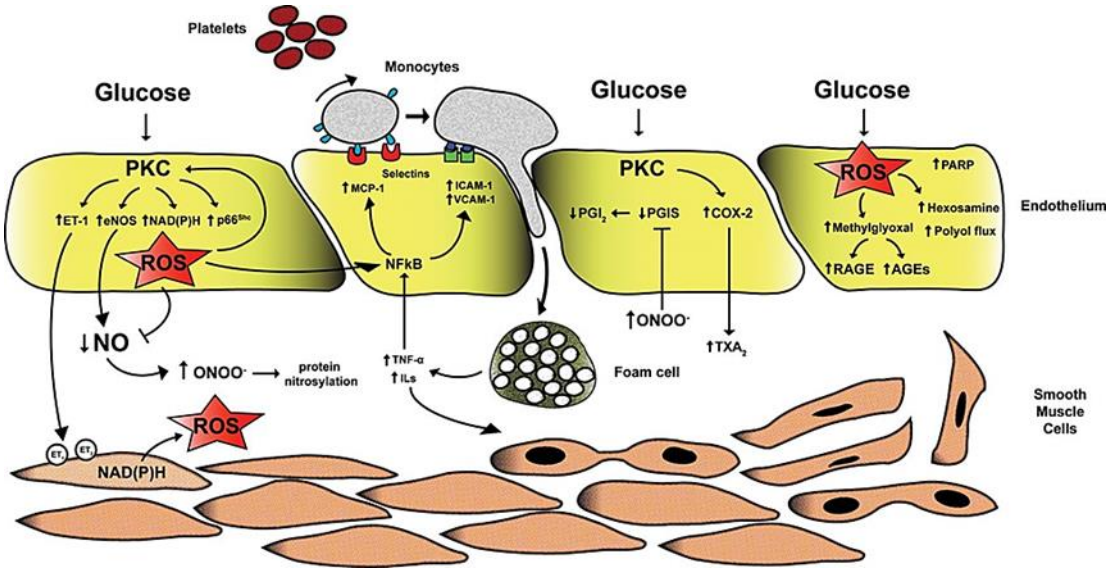


Figure 1.8 Molecular mechanisms of hyperglycaemia-induced vascular damage in type II diabetes (Paneni et al., 2013). High intracellular glucose concentrations trigger several cellular mechanisms including the polyol pathway, advanced glycation end products (AGEs), protein kinase C (PKC) activation, and NF-kB-mediated vascular inflammation. Once activated, PKC is responsible for different structural and functional changes in the vasculature including alterations in cellular permeability, inflammation, cell growth, extracellular matrix expansion, angiogenesis, and apoptosis. Moreover, PKC activation leads to ROS production by NADPH oxidase and p66Shc adaptor protein. Furthermore, increased oxidative stress quickly inactivates NO leading to the formation of the protein nitrosylation-responsible pro-oxidant ONOO−; reduced NO availability is also due to deregulation of PKC-dependent eNOS. Undoubtedly, PKC triggers enzyme up-regulation thus facilitating eNOS uncoupling and resulting in further accumulation of free radicals. Hyperglycaemia, on the other hand, reduces the activity of eNOS blunting phosphorylation at Ser1177. Along with the lack of NO, glucose-induced PKC activation causes increased synthesis of ET-1 favouring vasoconstriction and aggregation of platelets. Additionally, pro-inflammatory genes MCP-1,

VCAM-1, and ICAM-1 are up regulated by NF-kB signalling activation as a result of the accumulation of superoxide anion, thus leading to monocyte adhesion, rolling, and diapedesis with formation of foam cells in the sub-endothelial layer. Also, ROS increase the synthesis of glucose metabolite methylglyoxal resulting in the activation of AGE/RAGE signalling and the pro-oxidant hexosamine and polyol pathway flux. **Key:** Nuclear factor-κB (**NF-kB**); Protein kinase C (**PKC**); Endothelial nitric oxide synthase (**eNOS**); Endothelin 1(**ET1**); Reactive oxygen species (**ROS**); Nitric oxide (**NO**); Monocyte chemoattractant protein-1 (**MCP-1**); Vascular cell adhesion molecule-1 (**VCAM-1**); Intracellular cell adhesion molecule-1 (**ICAM-1**); Advanced glycation end product (**AGE**).

1.7.2 Periodontitis and type II diabetes: bidirectional relationship

Periodontitis is usually slowly progressing and asymptomatic; thus, many patients are unaware until the condition has progressed into advanced stages (Casanova Hughes and Preshaw, 2014). Studies show that periodontitis is a highly prevalent disease globally, with its mildest form having a prevalence of 45-50% in adults and its severe form estimated to affect 10-15% of adults in most populations (Sanz *et al*., 2018). Moreover, current literature unequivocally confirms type II diabetes as a major risk factor for periodontitis, with the prevalence of periodontitis estimated to be two to three times higher in diabetics than in an otherwise healthy population (Agarwal and Baid, 2023). A bidirectional relationship has been established between both diseases (Fig 1.9), where type II diabetes is associated with an increased prevalence and severity of periodontitis, and severe periodontitis with compromised glycaemic control (Polak and Shapira, 2018). This has important implications for the patients and healthcare institutions such as the NHS in terms of impact on quality of life, life expectancy, morbidity and healthcare costs (Romano *et al.,* 2021). Most studies have investigated the role of inflammatory mechanisms in the link between periodontitis and type II diabetes; however, there are gaps in current literature surrounding the relationship between type II diabetes and the oral microbiota, which this research aims to investigate.

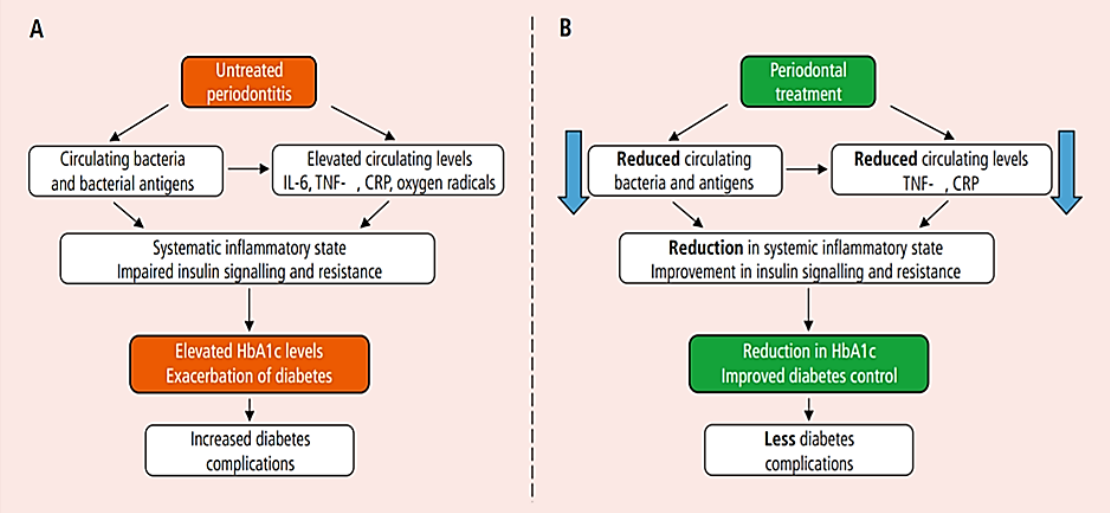


Figure 1.9 Schematic representation of the two-way relationship between type II diabetes and periodontitis (Preshaw and Bissett, 2019).

Type II diabetes increases inflammation in periodontal tissues and the inflammatory response is characterised by dysregulated host-derived mediators of inflammation and tissue breakdown (Polak and Shapira, 2018). **A:** In untreated diabetes, circulating bacteria, bacterial antigens, as well as pro inflammatory mediators and cytokines contribute to an upregulated systemic inflammatory state. This impairs insulin signalling and increases insulin resistance, leading to elevated HbA1c levels and increased diabetes complications. **B:** Periodontal therapy influences the reduction of periodontal inflammation, leading to a decrease in levels of circulating bacteria, antigens, cytokines and inflammatory mediators. This reduces the systemic inflammatory state, improving insulin signalling, while reducing insulin resistance. In turn, there is a reduction in HbA1c, improved diabetes control and less diabetes complications. **Key:** C-reactive protein **(CRP);** Glycated haemoglobin **(Hb1Ac);** Interleukin 6 **(IL-6);** Tumour necrosis factor-alpha **(TNF- α).**

Both type I and type II diabetes are associated with elevated levels of systemic markers of inflammation (Graves, Ding and Yang, 2020). The elevated inflammatory state in type II diabetes contributes to microvascular and macrovascular complications, and the hyperglycaemia results in the activation of pathways that increase inflammation, oxidative stress and apoptosis (Palwankar *et al.,* 2021). The most commonly implicated mediators of inflammation involved include T cell regulatory cytokines (IL-12, Il-18), pro-inflammatory mediators such as IL-1 β, IL-6, prostaglandin E2 (PGE2), TNF- α and metalloproteinase (MMP), all found in the salivary and gingival crevicular fluid (Sharma *et al.,* 2016). The poorly controlled diabetes in the presence of local factors such as dental plaque releases free radicals leading to less antioxidant activity, triggers the host cell neutrophils, lymphocytes and macrophages to release these inflammatory mediators and cytokines (Preshaw and Bisset, 2019). The release of these cells stimulates the release of matrix metalloproteinases,

leading to the breakdown of collagen and bone loss proceeding to periodontitis (Palwankar *et al.,* 2021). Also, the chronic hyperglycaemia induced by diabetes results in deposition of advanced glycation end (AGE) products in the periodontal tissues, and interaction with their receptor (RAGE – receptor for advanced glycation end products) results in local cytokine release and increased inflammation within the periodontal tissue (Polak and Shapira, 2018). In turn, local production of cytokines as well as the periodontal bacteria and their products contribute to upregulated systemic inflammation. As a result, the insulin signalling pathway is impaired and insulin resistance is increased, thus exacerbating diabetes (Preshaw and Bissett, 2019). Following treatment of periodontitis, there is a significant reduction in this process, leading to less complications associated with diabetes (Preshaw and Bissett, 2019).

Although not fully understood, studies show that there is a clear relationship between type II diabetes and periodontitis (Agarwal and Baid, 2023). Moreover, evidence has long established the existence of a bidirectional relationship between these two conditions (Fig. 1.9) with periodontitis severity being associated with hyperglycaemia, and in turn, elevated serum levels of glycated haemoglobin (HbA1c) being associated with severe periodontitis (Sanz *et al*., 2018). Furthermore, HbA1c measurement has proved to be a good indicator of the level of glycaemic control as it indicates the amount of glycated haemoglobin in the blood (Preshaw and Bisset, 2019). Treatment of periodontitis in patients with diabetes has been shown to reduce HbA1c by 3-4 mmol/mol (0.3-0.4%) after 3-4 months, such reductions are clinically relevant, as any reduction in HbA1c equates to reduced risk in diabetes complications (Agarwal and Baid, 2023). Moreover, to further support the existence of a bidirectional relationship, research has shown that there appears to be a lower risk of periodontitis in individuals with well controlled diabetes, having HbA1c levels ≤ 7% (53 mmol/mol) (Casanova, Hughes and Preshaw, 2014). Several meta-analyses also demonstrate a significant effect of periodontal therapy on glycaemic regulation in type 2 diabetics (Sanz *et al.,* 2018; Polak and Shapira, 2018; Madianos and Koromantzos, 2017). For example, Polak and Shapira (2018) highlight studies between 2013 and 2016 with data suggesting that periodontal treatment reduces serum TNF- α and CRP levels in type 2 diabetics as well as circulating bacteria and bacterial antigens which play a role in the inflammatory state of the body thus leading to improved insulin signalling and evidently HbA1c levels. Therefore, periodontal therapy may prove to be a promising treatment for slowing disease progression in type 2 diabetics; thus, emphasising the importance of identifying useful detection and treatment techniques for periodontal disease.

1.8 The oral microbiome and ethnicity

The expanding realm of oral microbiome research has unveiled the significant impact of ethnicity on the composition and diversity of oral microbial communities. Research demonstrates that individuals from different racial and ethnic backgrounds host distinct microbial profiles, suggesting that ethnicity influences the structure and diversity of the oral microbiota (Li *et al.,* 2014; Mason *et al.,* 2013; Yang *et al.,* 2019). Research suggest that the oral microbiome's composition is shaped by a myriad of factors, including diet, lifestyle, and genetic predispositions and ethnicity (Li *et al.,* 2014). This reflects the nuanced interplay between genetic backgrounds, cultural practices, and environmental exposures. Such studies underscore the existence of ethnicity-specific bacterial signatures, highlighting the microbial ecosystem's diversity across populations (Mason *et al.,* 2013). This microbial diversity is crucial for oral health, influencing the risk of diseases like periodontal conditions and dental caries. Recognizing ethnicity-specific signatures enhances our comprehension of oral microbial ecology and underscores the importance of personalized dental care and disease prevention strategies. The prevalence of specific microbial profiles in certain ethnic groups suggests potential for targeted interventions to modulate the oral microbiome, thereby mitigating disease risks. These findings further contribute to precision medicine, advocating for treatment strategies tailored to an individual's genetic, lifestyle, and environmental context.

However, attributing the oral microbiome's variability solely to ethnicity might simplify the complexity of microbial communities. Evidence suggests that environmental factors, such as diet and oral hygiene, alongside socioeconomic status, significantly shape the oral microbiome, possibly outweighing genetic factors. For instance, lifestyle factors, including tobacco use and alcohol consumption, have been identified as key influences on the microbiome, irrespective of ethnicity (Hayes *et al.,* 2018). The use of natural toothpaste like Miswak has also been shown to alter the oral microbiome, indicating that habits may have a more significant impact than ethnic background alone (Al-Mutairi *et al.,* 2020). Furthermore, the variability in the oral and gut microbiome across populations often reflects lifestyle and environmental differences rather than ethnic genetics (Balakrishnan *et al.,* 2021). These insights reveal that while ethnicity-specific microbial signatures are evident, the role of environmental and lifestyle factors in shaping the oral microbiome is profound. Acknowledging these factors can lead to more effective public health strategies that transcend ethnic distinctions.

This discussion proposes that despite the observed ethnic variations in the oral microbiota, the core microbial composition between groups, such as Black African and White European populations, shows considerable similarity. This suggests that the oral microbiome's formation results from a complex array of influences, including genetics, environment, diet, and lifestyle, with ethnicity being just one of the many factors. The presence of significant microbial overlap across ethnicities (Human Microbiome Project Consortium, 2012; Gupta *et al.,* 2017) supports this view. By conducting a comparative analysis of oral microbiota across different ethnicities, this thesis aims to elucidate ethnic-specific microbial signatures and their health implications, paving the way for future research and healthcare policies that recognize the diversity of population needs.

1.9 SIFT-MS as a diagnostic tool for periodontal disease using volatile breath biomarkers.

Volatile organic compounds (VOCs) are low-molecular-weight organic compounds that have high vapour pressure at room temperature, and are detectable in exhaled breath, saliva, and other bodily fluids (Gupta, 2016). Studies have shown that the human body generates some of these compounds endogenously via metabolic pathways, whilst others are derived from exogenous origins (Casas-Ferreira *et al.,* 2019). Similarly, bacteria produce a VOC profile specific to their species which can potentially serve as a diagnostic marker for certain diseases (Behera *et al.,* 2019). Due to this, identification of volatile markers associated with particular diseases has become a very important field of research as understanding the nature and role of these VOCs can provide valuable insights into the pathogenesis, diagnosis, and monitoring of periodontal disease. In periodontal disease, VOCs originate from various metabolic processes and interactions within the oral cavity, reflecting the dynamic nature of the oral microbiome and host response (De-Geest *et al.,* 2016). The production and release of VOCs in periodontitis are influenced by bacterial metabolism, immune system activity, tissue destruction, and oxidative stress (Thorn and Greenman, 2012). One group of VOCs associated with periodontitis is the volatile sulphur compounds (VSCs) generated by the putrefaction of sulphur-containing amino acids (i.e. cysteine, cystine and methionine). These VSCs include hydrogen sulphide (H_2S), methyl mercaptan (CH_3SH), and dimethyl sulphide ($(CH_3)_2S$) (Greenman *et al.,* 2012). VSCs are mainly

generated by anaerobic bacteria, such as *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*, present in periodontal pockets. Their foul odour contributes to halitosis (bad breath), a common symptom in periodontal disease (Figueiredo *et al.,* 2002). While VSCs are not specific to periodontitis, their levels can correlate with disease severity and can serve as screening tools for periodontal assessment.

Furthermore, the production of volatile fatty acids (VFAs) has been linked to periodontitis. VFAs, such as propionic acid and butyric acid, result from the bacterial fermentation of dietary carbohydrates and proteins (Qiqiang, Huanxin and Xuejun, 2012). They contribute to the acidic environment in periodontal pockets and can exacerbate tissue damage and inflammation. Elevated levels of VFAs have been detected in the breath, saliva, and gingival crevicular fluid of individuals with periodontitis, suggesting their potential as diagnostic markers (Qiqiang, Huanxin and Xuejun, 2012). Indoles and amines are additional classes of VOCs that have been investigated in relation to periodontitis. These compounds play a role in the complex interplay between microbial metabolism, inflammation, and tissue destruction observed in the disease (Thorn and Greenman, 2012). Indoles are aromatic compounds that arise from the breakdown of tryptophan, an essential amino acid, by certain bacteria present in the oral cavity (Thorn and Greenman, 2012). One prominent example is indole itself, which has been detected in breath and saliva samples of individuals with periodontitis. Indole and its derivatives, such as indoleacetic acid and indole-3-acetaldehyde, have been found to contribute to the malodour associated with halitosis in periodontal disease (Thorn and Greenman, 2012). Moreover, indole has been implicated in modulating host immune responses and bacterial virulence factors, potentially influencing the pathogenesis of periodontitis (Fiore and Murray, 2014). However, further research is needed to unravel the specific roles and clinical implications of indoles in periodontal disease.

Amines, on the other hand, are nitrogen-containing compounds that can be produced by microbial metabolism or derived from host cells (Thorn and Greenman, 2012). These compounds include putrescine, cadaverine, trimethylamine, and their derivatives. Elevated levels of amines have also been reported in breath and saliva samples of individuals with periodontitis (Thorn and Greenman, 2012). These amines can originate from the breakdown of amino acids, such as lysine and arginine, by certain bacteria in the oral microbiome (Thorn and Greenman, 2012). Amines have also been associated with the malodour observed in halitosis, contributing to the unpleasant smell characteristic of periodontal disease.

Furthermore, amines can act as inflammatory mediators and affect tissue homeostasis, potentially contributing to the progression of periodontitis (Nguyen *et al.,* 2019). However, further research is needed to understand the specific mechanisms by which amines influence periodontal pathogenesis and their potential diagnostic or therapeutic implications. It is worth noting that the presence and concentrations of indoles and amines can vary depending on individual variations in oral microbiota composition, disease severity, and other factors (Thorn and Greenman, 2012). Similarly, volatile alcohols have also been implicated in periodontitis and have received attention in VOC research related to the disease (Thorn and Greenman, 2012). These alcohols, including, propanol, butanol, and pentanol, are produced through microbial metabolism and other biochemical processes in the oral cavity (Thorn and Greenman, 2012). While their specific roles and contributions to periodontal pathogenesis are not yet fully understood, their presence suggests their potential as key biomarkers for disease diagnosis.

Currently, chemical-detection technologies such as selected ion flow tube mass spectrometry (SIFT-MS) have been used to measure these VOCs due to its ability to provide rapid and reliable quantification of the VOCs and produce a pattern unique to a specific organism (Wilson, 2018). Moreover, SIFT MS, exploits a fast flow tube reactor combined with chemical ionization in order to analyse trace compounds in air samples real time; the reactions between the precursor ions and the trace compounds in the sample result in characteristic product ions that identify the compounds, while their count rates allow quantification (Casas-Ferreira *et al.,* 2019). The SIFT-MS technique has been extensively discussed in various reviews (Smith and Spanel, 2011; Spanel and Smith, 2011; Spanel and Smith, 2007).

In summary, the SIFT-MS relies on chemical ionization by specific reagent ions (H_3O^+, NO^+, and O_2^+) generated in a microwave discharge through humid air, which ionize trace amounts of gaseous analytes in air or breath samples. Recently, the range of neutral analytes accessible through SIFT-MS has been expanded by utilizing five additional negative reagent ions (O-, OH-, O_2^-, NO_2^-, and NO_3^-) (Smith *et al.,* 2023). When the sample enters the flow tube at a known flow rate, reactions occur between the chosen reagent ion and the neutral analyte molecules, generating characteristic analyte ions (Hera *et al.,* 2017). These ions' signal intensities are recorded by an analytical mass spectrometer located downstream (Fig 1.10). Identification of the neutral trace compounds from the analyte ions produced by different reagent ions is based on understanding the relevant ion-molecule chemistry

(Spanel *et al.,* 2006). Figure 1.10 below displays a simplified illustration of the instrument and how it works. Concentrations of the trace analyte molecules in the sample are calculated by considering several measured physical parameters, including flow tube gas pressure, temperature, carrier gas (typically helium) flow rate, and sample gas (air/breath) flow rate (Spanel and Smith, 2013). Consequently, an ion kinetics library has been developed through extensive studies of the reactions between individual reagent ions and a wide range of VOCs found at trace concentrations in exhaled breath and other gaseous media, such as the headspace of blood serum, urine, and cell cultures (Zhu *et al.,* 2017).

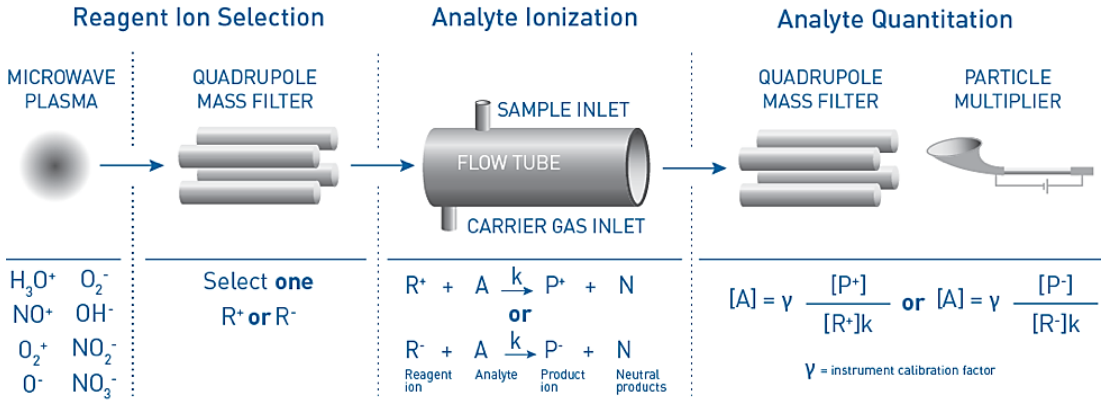


Figure 1.10 Simplified diagram of SIFT-MS (Grira, 2021).

Typically, the SIFT-MS instruments currently available achieve limits of detection below one part per billion by volume (ppbv) in the sample (Zhu *et al.,* 2017). The raw data obtained from SIFT-MS analysis of a breath/air sample is usually presented as a mass spectrum acquired by the downstream analytical mass spectrometer, typically a quadrupole, focusing on a chosen mass-to-charge ratio (m/z) as described by Smith and Spanel (2015). The spectrum exhibits peaks corresponding to the reagent ions and analyte ions. By utilizing the kinetics library, the analyte ions can be correlated with the trace compounds (VOCs) present in the breath sample (Smith and Spanel, 2015). The SIFT-MS instrument may be operated in two modes: (i) the scan mode where a whole spectrum is captured over a desired mass-to-charge (m/z) ratio range, ideal for identifying compounds of interest (Ioannidis *et al.,* 2020). For more precise analysis of specific neutral compounds, the count rates of their respective analyte ions are measured, allowing longer ion integration times and improved precision. This mode is called (ii) multiple ion monitoring (MIM) or selected ion monitoring (SIM) and is suitable for analyzing single breath exhalations to detect chosen metabolites (Smith *et al.,* 2023). Another valuable feature of SIFT-MS is the ability to perform online and real-time analysis of both mouth-exhaled and nose-exhaled breath using the MIM mode (Wondimu, Wang and Ross, 2014; Drummer *et al*., 2013; Smith *et al.,* 2013).

While the SIFT-MS can be useful, limitations including high cost, equipment size, need for technical expertise, lack of portability and ability to only detect VOCs that can be ionized by preselected precursor ions, makes it impractical for widespread use as a point of care device (Fitzgerald and Fenniri, 2017). Consequently, the logical progression in SIFT-MS development involved designing a portable instrument that could be transported to different locations, enabling real-time analysis of air and breath without the need for sample collection. This led to the creation of transportable SIFT-MS Instruments, Mk.1 and Mk.2, which brought significant improvements but still remained cumbersome and expensive to operate (Spanel and Smith, 2009). Nevertheless, despite the success of the Mk.1 and Mk.2 instruments, there was a clear demand for a smaller, more manageable, cost-effective, quieter, and simpler SIFT-MS instrument that could be utilized by non-specialists in various healthcare settings. Consequently, the *Profile3* SIFT-MS instrument was developed, incorporating the many desired features. Moreover, researchers have obtained promising results using *Profile3* instruments for breath and urine headspace analysis, as well as other areas of trace gas analysis, including enhanced sensitivity and improved quality of the obtained mass spectra (Smith *et al.,* 2022). These advancements in sensitivity, coupled with the combination of SIFT-MS instruments with GC-MS and sample collection procedures, are opening up new possibilities for trace gas analysis and potential diagnosis of various diseases.

1.10 Current oral care products available and their role in controlling periodontitis.

1.10.1 Antimicrobial agents used in toothpaste and oral mouthwashes.

Studies have demonstrated a strong link between oral health (OH) and periodontitis, suggesting that OH presents as a stronger risk factor for periodontitis than smoking or obesity, increasing risk by approximately two to five-fold (Lertpimonchai *et al*., 2017). Therefore, it is logical to state that proper oral hygiene could potentially reduce the risks of periodontitis in the oral cavity by reducing the accumulation of dental plaque which causes gingivitis, a pre-requisite for the development of periodontitis. Various studies and meta-analysis (Saad *et al.*, 2018; Villa *et al.,* 2018; Riley and Lamont, 2013; Moharamzadeh, 2017; Araujo *et a*l., 2015) have illustrated the antiplaque and anti-gingivitic effects of various antimicrobial agents available in oral care products currently such as chlorhexidine (CHX),

Fluoride, and Zinc. In this study (Chapter 3), chlorhexidine (CHX) is used as a reference toothpaste since it is considered the most potent chemotherapeutic agent and gold standard in reducing oral biofilms (Shrimathi *et al.,* 2019; Hugar *et al.,* 2016).

Chlorhexidine (CHX) is a bis-biguanide compound known for its ability to inhibit the growth of bacteria and effectively kill them (Rajendiran *et al.,* 2021). It has been extensively studied and recognized as the most effective agent for preventing plaque formation and treating gingivitis, making it the standard choice in this regard (Varoni *et al.,* 2012). The antimicrobial properties of CHX are broad-spectrum, targeting both Gram-positive and Gram-negative bacteria (Rajendiran *et al.,* 2021). This effectiveness is attributed to its cationic nature, which allows it to bind indiscriminately to negatively charged phospholipids in the bacterial membrane (Deus *et al.,* 2022). The mechanism of action begins with the rapid attraction of a positively charged chlorhexidine molecule to the surface of a negatively charged bacterial cell, which contains phosphates and sulphate groups (Solderer *et al.,* 2019). Due to its positive charge, CHX forms bonds with negatively charged sites within the biofilm, including bacteria, extracellular polysaccharides, and glycoproteins (Solderer *et al.,* 2019). This specific and strong adsorption primarily occurs on phosphate-containing components that make up the surface of the bacterial cell (Deus *et al.,* 2022).

As a result of passive diffusion, CHX is able to penetrate through the bacterial cell wall, attracting towards the cytoplasmic membrane and causing damage that compromises its integrity (Łukomska-Szymańska et al., 2017). This event enables CHX to infiltrate the inner cell membrane, leading to increased permeability (Solderer *et al.,* 2019). Consequently, low-molecular-weight molecules and cytoplasmic components, such as potassium ions, are released from the microorganism (Deus *et al.,* 2022; Łukomska-Szymańska *et al.,* 2017). This release inhibits the activity of certain enzymes associated with the cytoplasmic membrane (Deus *et al.,* 2022; Solderer *et al.,* 2019; Łukomska-Szymańska *et al.,* 2017). The mode of action of CHX varies depending on its concentration: at low concentrations (0.02–0.06%), it inhibits bacterial growth, while at higher concentrations (0.12–0.20%), it actively kills bacteria (Rajendiran *et al.,* 2021). Furthermore, CHX exhibits a sustained antibacterial effect by binding to the oral mucosa after use (Loe *et al.,* 1970). CHX finds extensive use in the field of dentistry and is available in various formulations, such as oral rinses (0.02–0.3%), gels (0.12–1%), sprays (0.12–0.2%), dental varnishes (1%, 10%, 40%), as well as toothpastes and mouthwashes (Rajendiran *et al.,* 2021).

Despite its widespread use, long-term application of CHX can lead to localized adverse effects. Some of the most commonly observed adverse reactions are dry mouth (xerostomia), altered taste perceptions (hypogeusia), specifically related to salt and bitter flavours, and the development of a discoloured or coated tongue (Rajendiran *et al.,* 2021). Furthermore, the utilization of 0.12% CHX mouthwash has been associated with increased formation of dental calculus, despite its anti-plaque properties (Deus *et al.,* 2022). Less frequently encountered side effects include sensations of burning (glossodynia), shedding of the oral mucosa, enlargement of the parotid gland, and oral paraesthesia (Deus *et al.,* 2022). However, the primary drawback that discourages patients from employing CHX mouthwash is the occurrence of tooth staining (Teles *et al.,* 2009). This staining is prevalent when the usage extends beyond a few weeks, attributable to non-enzymatic browning (known as the Maillard reaction) and the production of pigmented metal sulfide compounds within the pellicle (Brookes *et al.,* 2020). Consequently, this process can facilitate reactions between tin and iron with dietary aldehydes and ketones, leading to the increased deposition of food components onto the teeth (Varoni *et al.,* 2012).

The more potentially serious side effects associated with the oral use of CHX are the possible type IV and type I hypersensitivity reactions accompanied by severe anaphylaxis (Brookes *et al.,* 2020). The reported incidence of these reactions for CHX stands at 0.78 per 100,000 exposures (Brookes *et al.,* 2020). In certain cases, CHX mouthwash has been implicated in respiratory arrest and even death due to severe anaphylactic responses (Deus *et al.,* 2022). Therefore, although these allergic reactions are rare and documented in limited numbers, they have influenced the decision-making process among clinicians regarding the use of CHX in recent years. Consequently, the risk-benefit analysis must be carefully considered when determining the appropriate use of CHX for managing various oral conditions.

Table 1-1 below presents a comprehensive overview of the active ingredients or compounds utilized in toothpastes and mouth rinses for the purpose of plaque control, as well as the prevention of gingivitis and periodontitis. Extensive research has demonstrated the significant efficacy of these substances in achieving these desired outcomes (Kwon *et al.,* 2021). However, there are many limitations associated with these products such as side effects to patients (Azimi *et al*., 2016), thus the need to develop novel alternatives with the potential to reduce these unwanted side effects.

Table 1-1 Summary of active ingredients in mouthwashes and toothpastes for periodontal diseases, including their mode of action, benefits and limitations (Rajendiran *et al.,* 2021).

Active Ingredient	Available Forms	Antiplaque Effect	Anti-gingivitis Effect	Anti-periodontitis Effect	Antimicrobial Effect	Effect on Bacteria	Side Effects
Chlorhexidine	Toothpastes Mouthwashes Oral rinses Gels Sprays Varnishes	√	√	√	Effective against gram-positive and gram-negative bacteria, yeast, and viruses like *Porphyromonas gingivalis, Aggregatebacter actinomycetemcomitans*, Bacteriodetes, Fusobacteria, phyla SR1, TM7	Bacteriostatic and bactericidal	Temporary alteration of taste, pigmentation of teeth, tongue and prosthetic crowns
Cetylpyridinium chloride	Mouthwashes Toothpastes Chewing subgingival plaques	√	√	√	Effective against gram-positive bacteria, yeast, and viruses like *Streptococcus salivarius*, viridans streptococci, *Candida albicans*	Bacteriostatic and bactericidal	Gingival irritation, mild teeth staining
Fluorides	Toothpastes Mouthwashes Gels Foams Varnishes	√	√	√	Effective against *Streptococcus mutans*, lactobacilli, *Streptococcus oralis, Streptococcus mitis, Streptococcus sanguinis*	Bacteriostatic and bactericidal	Dental/enamel fluorosis
Stannous chloride	Toothpastes Mouthwashes	√	√	×	No specific species reported	Unknown	Tooth and tongue discoloration

Zinc	Toothpastes Mouthwashes	**√**	**√**	**√**	Effective against *Streptococcus mutans, Streptococcus aureus, Streptococcus salivarius, Streptococcus sobrinus, Lactobacillus casei, Porphyromonas gingivalis, Prevotella intermedia, Fusobactereum nucleatum, Treponema denticola, Tanerella forsythia*	Bacteriostatic and bactericidal	Neurological effects like numbness or tingling sensation and nerve damage
Licorice	Candies Lollipops Capsules Tablets Liquid extracts	**√**	**√**	**√**	Effective against *Streptococcus mutans, Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*	Bacteriostatic	Hypokalemia, hypertension, metabolic alkalosis, edema

√—Positive effect, ×—No effect, NA—Not Available.

1.10.2 Novel strategies for controlling oral biofilms.

According to current research (Horev *et al.,* 2015), the effectiveness of topically applied antibacterial agents in toothpastes and oral rinses is limited due to the complex nature of the oral cavity and the rapid clearance of saliva. Traditional antimicrobial agents such as chlorhexidine (CHX) have been widely used, but novel treatment strategies have emerged to target specific characteristics of oral biofilm and address drug resistance issues (Kuang, Cheng, & Xu, 2018). These strategies involve the utilization of nanomaterials such as silver, copper oxide, titanium oxide, and graphene, which have gained significant attention in recent years (Allaker *et al.,* 2014). Additionally, quaternary ammonium salts (QAS) have shown promise in controlling oral biofilms due to their broad-spectrum antimicrobial activity, low toxicity, and chemical stability (Liang *et al.,* 2017). Similarly, small molecules and arginine have demonstrated inhibitory effects on periodontal pathogens such as *P. gingivalis*, *F. nucleatum*, and *P. intermedia*, suggesting their potential in biofilm control (Kuang, Cheng, & Xu, 2018). However, it is important to acknowledge that the current

available data primarily stem from *in vitro* or animal studies that focus on single-species biofilms. Further investigation is necessary to evaluate the antimicrobial activities of these novel agents in human subjects, ensuring a balance between their bioactivity and biocompatibility.

1.11 Overall aim and objectives

1.11.1 Aim

This thesis focused on a comprehensive exploration of the oral microbiome underscoring its significance for both oral and systemic health among diverse ethnic cohorts, particularly focusing on Black African and White European populations. The primary aim was to explore the complex interplay between oral microbiota composition, periodontal disease, and ethnic variations, thereby contributing to the body of knowledge necessary for developing targeted oral health interventions and policies.

1.11.1 Objectives

- To assess the effectiveness of oral care products by evaluating the efficacy of specific oral care formulations on controlling periodontal pathogens and their impact on oral microbial biofilms *in-vitro.*
- To compare the oral microbiota profiles of healthy individuals from Black African and White European backgrounds, uncovering the underlying similarities and differences that may exist.
- To explore ethnic differences in oral health through a detailed analysis of microbial composition, diversity, and functional capabilities which may influence oral health and potentially predispose individuals to systemic conditions.

Chapter 2 Effectiveness of the human oral microbe identification microarray in identifying periodontal pathogens: A systematic review.

2.1 Introduction

Periodontal disease is a multifactorial, chronic inflammatory disease of the mouth involving the gingivae, teeth and alveolar bone, initiated and sustained by an aberrant host immune response against resident bacterial biofilm on the teeth (Persson, 2011). It is usually characterized by loss of connective tissue attached to the teeth and alveolar bone loss thus if left untreated can cause exfoliation of the tooth (Preshaw *et al.,* 2012). According to Casanova, Hughes and Preshaw (2014), gingivitis and periodontitis are the most frequent types of periodontal diseases; the former is characterized by inflammation confined to the gingivae and is reversible with good oral hygiene, while the latter is mostly irreversible and is usually characterized by extended inflammation, resulting in tissue destruction and alveolar bone resorption. Furthermore, in periodontitis, periodontal pockets are formed between the gingivae and the tooth due to the breakdown of collagen fibres of the periodontal ligament as a result of the tissue destruction mentioned above (Preshaw *et al.,* 2012). Research shows that periodontitis is a highly prevalent disease globally, with its mildest form having a prevalence of 45–50% in adults and its most severe form estimated to affect 10–15% of adults in most populations (Sanz *et al.,* 2018). Current research unequivocally confirms diabetes as a major risk factor for periodontitis, with the prevalence of periodontitis estimated to be two to three times higher in diabetics than in an otherwise healthy population (Teeuw *et al.,* 2017).

The terms “oral microbiome”, “oral microflora” or “oral microbiota” are commonly used to describe the microbial community within the human oral cavity (He *et al.,* 2015). Research has identified over 700 bacterial species in the human oral cavity, with 400 identified from the periodontal pockets and 300 from other oral sites such as the tongue, oral mucous membranes and carious lesions (Mougeot *et al.,* 2016). Teeuw *et al.,* (2017) classifies these bacteria into different categories based on their Gram stain results (Gram-positive or Gram-negative bacteria), their shape (coccus, bacillus or spirochetes) and their tolerance to oxygen (aerobic, facultative anaerobes, microaerobic or obligate anaerobes). Some known oral pathogens are believed to contribute to the development of oral diseases such as

dental caries and periodontal diseases (Gao *et al.,* 2018) as well as systemic diseases such as diabetes mellitus, cardiovascular diseases and the development of tumours (Mougeot *et al.,* 2016). In healthy populations, organisms such as *Streptococcus salivarius* and *Rothia mucilaginosa* are usually predominant in the oral microbiome (Zhang *et al.,* 2018). However, with the development of periodontitis, organisms such as *Porphyromonas gingivalis, Tannerella forsythia, Treponema denticola* and *Aggregatibacter actinomycetemcomitans* become more dominant within the oral community (Seerangaiyan *et al*., 2018).

The isolation and identification of oral organisms can be difficult because oral microorganisms are numerous and composed of diverse species and new genera and species are constantly being discovered, while the classification of some previously discovered species changes with time. Earlier methods such as Denaturing Gradient Gel Electrophoresis (DGGE) have been used to identify oral bacteria, and DGGE was first introduced to microbial ecology by a study in 1993 (Muyzer, De Waal and Uitterlinden, 1993). This technique separates short-to-medium-length Polymerase Chain Reaction (PCR)-amplified DNA fragments according to their melting point (Gafan and Spratt, 2005). The technique works on the basis that DNA fragments of the same size but with differing base pair sequences can be separated, and this separation by DGGE relies on the electrophoretic mobility of partially denatured DNA molecules in a polyacrylamide gel (Gafan *et al.,* 2005). Studies have, however, shown that current 16S rRNA sequence hybridization methods such as Human Oral Microbe Identification Microarray (HOMIM) are more effective in providing a comprehensive representation of the oral bacterial community (Lourenço *et al.,* 2014; Ledder *et al.,* 2007; Ahn *et al.,* 2011; Mougeot *et al.,* 2016). As stated by Mougeot *et al.* (2016), HOMIM, first introduced in 2008, is a custom array-based approach that utilizes specially designed probes to detect over 300 of the most prevalent bacterial species. Briefly, 16S rRNA-based oligonucleotide probes are covalently attached to aldehyde-coated slides (Lourenço *et al.,* 2014). The 16S rRNA genes are PCR amplified from bacterial DNA extracts and are labelled with fluorescent dye, producing a fluorescent signal when the bacterial DNA hybridizes to a specific spot on the slide (Ledder *et al.,* 2007). The output data from the HOMIM assay are usually merged onto the Human Oral Microbiome Database (HOMD), which is based on a curated 16S rRNA gene-based provisional naming scheme that provides comprehensive information on the prokaryote species present in the human oral cavity (Mougeot *et al.,* 2016).

This review will focus on investigating the effectiveness of the Human Oral Microbe Identification Microarray (HOMIM), a current molecular profiling technology used for identifying and quantifying bacterial species of the oral microbiome in periodontal disease. The aim of this study is to compare this current profiling technology to earlier identification methods such as Denaturing Gradient Gel Electrophoresis (DGGE).

2.2 Materials and Methods

The following databases were searched by two independent researchers (Stephanie Jay, Eloise Bovill) from their January 1990 records through March 2021: MEDLINE via PubMed, EMBASE, The Cochrane Central Register of Controlled Trials (CENTRAL) and CINAHL. The strategy was developed for MEDLINE using controlled vocabulary, with words derived from "Medical Subject Headings" (MeSH) associated with free terms relevant to the topic in question (Table 2-1). The search strategy and the flow of information through the different phases of the systematic review were established according to the PRISMA statement for systematic reviews and metanalysis. In total, 18 articles were selected, evaluated and classified by two independent readers (Stephanie Jay., Emmanuel Adukwu.); the full-text article was obtained whenever a study seemed to meet the inclusion criteria, but complete information was lacking. By using a pre-defined data-extraction form, the reviewers independently extracted the data on characteristics of the study population, length of follow-up, interventions and outcomes. The results of both readers were compared, and eventual differences were resolved by discussion.

Table 2-1 Summary of keywords used for database search.

Periodontitis OR periodontal disease OR periodontal infection OR periodontal diseases OR adult periodontitis OR chronic periodontitis OR aggressive periodontitis OR juvenile periodontitis OR localized periodontitis.
AND
Oral microbiota OR oral microbiology OR oral microorganism OR oral microbiome OR oral pathogen OR oral pathogens OR oral biofilm OR red complex OR orange complex OR oral bacteria OR oral bacterial species OR oral subgingival microbiome OR gingivitis OR treponema OR forsythia OR denticola OR actinomycetemcomitans OR buccal mucosa microbiome OR hard palate microbiome OR keratinized gingivae microbiome OR saliva microbiome.

AND

molecular profiling OR molecular profiling technology OR NGS OR DGGE OR DNA probes OR sequencing OR next generation sequencing OR metagenomics OR denaturing gradient gel electrophoresis OR culture-based approach OR genetic fingerprinting OR Human Oral Microbe Identification Microarray OR HOMIM OR HOMINGS OR 16S rRNA profiling OR HOMD.

Initially, all articles were selected by title and abstract; articles with duplicate records were considered only once. The publications selected were essentially observational clinical studies conducted in humans. They were required to quantify and characterize the bacterial species and periodontal pathogens in the oral microbiome using HOMIM or DGGE. Afterwards, inclusion and exclusion criteria were applied. The following inclusion criteria were applied: observational clinical studies in humans aged between 18 and 65 years; presence of periodontal disease; DNA extraction of samples collected from the subgingival plaque in the mouth. Conversely, the exclusion criteria were inclusion of humans under 18 years or over 70 years; inclusion of patients with systemic diseases or under any condition that could influence oral microbiota or periodontal support tissues; antibiotic therapy three months before and during the study; absence of periodontal disease; studies written in any other language than English. At this stage, if articles did not meet any exclusion criteria but met part of the inclusion criteria, they were included.

Subsequently, full text articles were read and those that met the inclusion and exclusion criteria were carefully analysed and qualified according to their methodological aspects, as described in Table 2-2. A detailed checklist for quality assessment was adapted for this review, based on the study design, sample, follow-up period, collection and DNA extraction methods, statistical treatment, results and discussion (Freitas *et al.,* 2014). The selected articles were finally classified according to the total score after qualification. Their methodological quality was classified as high (score 7 to 8), moderate (score from 5 to 6.9) or low (score from 0 to 4.9) (Table 2-3). Those classified as low were excluded. A hand search was performed to complement the previous searches, by which the references of the selected articles were analysed. The Cochrane Collaboration tool for assessing risk of bias was used in the included studies (Higgins, J. and Green, S., 2011). The following domains were evaluated as having low, high or unclear risk of bias: random sequence generation, allocation concealment, blinding, incomplete outcome data, selective outcome reporting and other potential bias.

Table 2-2 Methodological quality score (Freitas *et al.,* 2014).

Score Protocol	Maximum Score (8 points)
1. Study design**: description of the study design**	0.2
2. Participants	1.2
• **Sample standards: participant's inclusion and exclusion criteria**	0.2
• **Sample characterization: number and characteristic of participants**	0.2
• **Calculation of sample size**	0.6
• **Ethics: evidence of ethical factors**	0.2
3. Collection methods	3.0
• **Control of factors influencing collection: removal of supragingival plaque and debris.**	-
4. DNA extraction**: adequate description of extraction method**	3.0
5. Statistical analysis: **adequate indication of test applied and significance level**	0.2
6. Results: **adequate presentation of results (presentation of all results; comparison between results considering microorganism, participant dropout with justification)**	0.2
7. Discussion**: consideration and possible explanation for the findings presented; comparison with previous published results**	0.2

Table 2-3 Quality assessment of studies.

Author/Year	Study design	Participants				Collection methods	DNA extraction	Stats Analysis	Results	Discussion	Total points/ quality
		Sample Standards	Sample Characterization	Calculation of sample size	Ethics	Control of factors influencing collection					
Fujimoto et al *2003*	0	0	0.2	0	0	3	3	0	0.2	0.2	6.6 Moderate

Zijnge et al *2006*	0.2	0	0.2	0	0	0	3	0	0.2	0.2	3.8 Low
Zijnge et al *2003*	0	0.2	0.2	0	0	3	3	0	0.2	0.2	6.8 Moderate
Liu et al 2010	0	0	0.2	0	0	3	3	0	0.2	0.2	6.6 Moderate
Mougeot et al 2016	0	0.2	0.2	0	0.2	0	3	0.2	0.2	0.2	4.9 Low
Colombo et al 2009	0.2	0.2	0.2	0	0.2	3	3	0.2	0.2	0.2	7.4 High
Colombo et al 2012	0.2	0.2	0.2	0	0.2	3	3	0.2	0.2	0.2	7.4 High
Aspiras et al 2013	0.2	0.2	0.2	0	0.2	0	3	0.2	0.2	0.2	4.4 Low

A narrative synthesis was used to systematically explore the similarities and differences between results of different studies, identifying data patterns in associations between study characteristics and outcomes. Furthermore, different interventions, outcomes, study designs and the rationale of their effects were explored and analysed to integrate and synthesize data.

2.3 Results

The electronic search retrieved a total of 2931 records from the following databases: CENTRAL (224), CINAHL (0), MEDLINE (2704) and EMBASE (3). After excluding duplicates and records marked as ineligible by automation tools, there were 261 records, 230 of which did not meet the inclusion criteria; 31 were selected for full reading. A manual search of the references from the 31 articles was performed, retrieving an additional 20 new titles. Titles and abstracts not related to the topic were initially excluded. Having selected articles based

on the inclusion and exclusion criteria, a total of 51 full text articles have been assessed for eligibility according to all selection criteria; 43 studies were excluded at this stage. After careful reading and quality assessment, 8 articles were categorized according to methodological quality as follows: high (2), moderate (3), low (3). Low-quality articles were excluded from this systematic review. Consequently, 5 studies were included (Figure 2.1). The general characteristics of the included studies are presented in Table 2-4. Furthermore, risk of bias for the included articles appeared to be low.

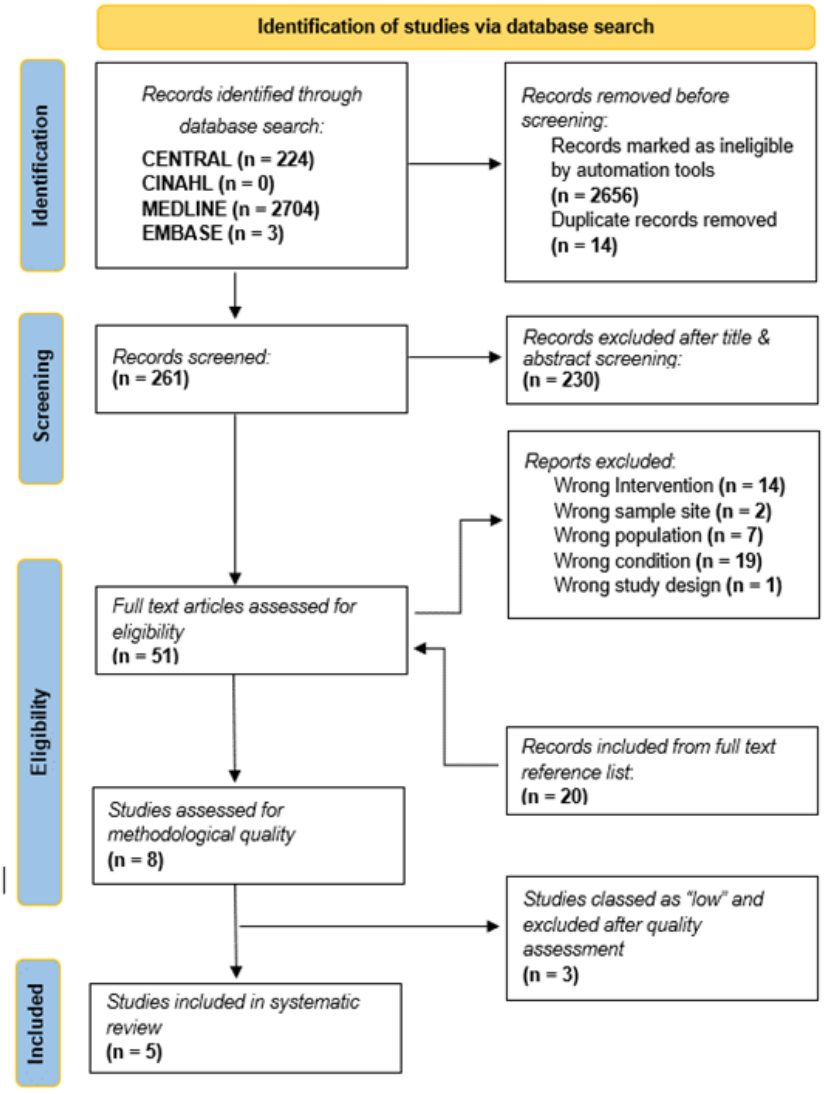


Figure 2.1 PRISMA flow chart.

Table 2-4 Study results of individual studies.

Author/ Year	Study design	Participants		Collection methods	DNA extraction	Stats Analysis	Conclusion
		Sample Standards	General Sample Description				
Colombo et al 2012	Longitudinal study	No history of systemic disease, periodontal therapy or use of antibiotic therapy within the preceding 6 months. Absence of pregnancy.	47 subjects over 20 years of age.	After removal of supragingival plaque, subgingival plaque was collected with sterile periodontal curettes from the mesio-buccal aspect of up to 14 teeth in different quadrants.	Universal (Proteinase K &Tween 20).	Mann-Whitney and Chi-square tests to compare differences in demographic and clinical parameters between groups. Wilcoxon signed rank test to compare pre and post therapy.	HOMIM results indicated reduced prevalence of majority of subgingival species in combined and antimicrobial therapy. Results also identified novel species or phylotypes of periodontal pathogens in subjects with refractory periodontitis.
Colombo et al 2009	Longitudinal study	No history of systemic disease, periodontal therapy or use of antibiotic therapy within the preceding 6 months. Absence of pregnancy.	67 subjects over 20 years of age.	After removal of supragingival plaque, subgingival plaque was collected with sterile periodontal curettes from the mesio-buccal aspect of up to 14 teeth in different quadrants.	Proteinase K &Tween 20	Kruskal-Wallis, Mann-Whitney and Chi-square tests to compare differences in clinical parameters among groups.	HOMIM results indicated a greater diversity in subgingival microbiota of subjects with periodontitis at baseline compared to healthy subjects. Results also showed a

							high frequency of several species not commonly associated with periodontitis at baseline in subjects with refractory periodontitis.
Fujimoto et al 2003	Not mentioned	Not mentioned	4 subjects; 2 males and 2 females aged between 18 and 65 years.	Subgingival plaque was collected by insertion of 3 sterile paper points into subgingival lesion pockets, after removal of supragingival plaque with sterile cotton swabs.	Instagene Matrix	n/a	DGGE results were able to reveal dominant microflora. However, due to low sensitivity, DGGE was unable to efficiently detect pathogens with a low abundance.
Zijnge et al 2003	Not mentioned	No history of systemic disease, periodontal therapy or use of antibiotic therapy within the preceding 6 months. Absence of pregnancy.	15 adult subjects with minimum of 20 teeth.	After supragingival plaque was removed, subgingival plaque was collected by inserting sterile endodontic absorbing points to the bottom of periodontal pockets.	Phenol/ Chloroform	n/a	DGGE results indicated a change in band profiles before and after treatment of subjects with periodontitis. However, DGGE presented difficulties indicating species based

							on band positioning.
Liu et al 2010	Not mentioned	Not mentioned	6 subjects: four women and two men aged between 29-52 years.	After removal of supragingival plaque, subgingival samples were collected from periodontal pockets using sterile curettes.	Tiangen Kit	Cs=2j/(a+b) x 100	DGGE results indicated no change in band pattern before and after treatment for the V3-V5 and V6-V8 region, suggestive of bacterial recolonizatio n. However, recolonizatio n by small numbers of periodontal pathogens would not be identified by this method.

2.4 Discussion

The oral cavity is colonized by a large and diverse group of bacteria, which form biofilm communities in several habitats within the mouth, including the tooth, subgingival sulcus, tongue, buccal mucosa and tonsils (Zijnge *et al.*, 2003; Colombo *et al.,* 2012). More than half of these species have been detected in the subgingival habitat, many of which have not yet been formally named, or are novel species that cannot yet be grown or are difficult to grow in culture (Colombo *et al.,* 2009; Colombo *et al.,* 2012; Zhou *et al.,* 2011). Although most of these organisms are commensal, numerous oral bacterial species have been associated with oral disease and oral health, including those that cannot be cultivated *in-vitro* (Colombo *et al.,* 2009; Zhou *et al.,* 2011).

Various microbiological studies presented in the retrieved articles revealed different aspects of the supragingival plaque and important changes in the subgingival plaque of patients with periodontal disease (Colombo *et al.,* 2012). Although the articles included in

this review reported that HOMIM was an effective method for quantifying the oral community in periodontal disease, it is relevant to indicate that insufficient methodological information was provided. For instance, only two publications outlined the design and ethical information of the study (Colombo *et al.,* 2009; Colombo *et al.,* 2012). However, in all included studies, collection of the biological material was carefully monitored to avoid interference from other sites in the mouth (Fujimoto *et al.,* 2003; Zijnge *et al.*, 2003; Colombo *et al.,* 2009; Colombo *et al.,* 2012). Methodologically poor articles (Zijnge *et al.*, 2006; Mougeot *et al.,* 2016; Aspiras *et al.,* 2013) were characterized by similarities that contributed to their low score and then exclusion from the review. These studies did not describe the control of factors that might influence the collection of biological material, which is an essential aspect of the study. As diverse organisms associated with periodontal disease and periodontal health are characteristic of different oral habitats, isolation of the area and previous removal of other biological material surrounding the collection site is essential (Gafan and Spratt*,* 2005; Fujimoto *et al.,* 2003; Colombo *et al.,* 2009; Colombo *et al.,* 2012).

Periodontal health is usually associated with supragingival Gram-positive microbiota that consists mainly of diverse species of *Streptococci* and *Actinomyces* (Colombo *et al.,* 2009; Colombo *et al.,* 2012). Moreover, they are also predominant in gingivitis; however, the number of Gram-negative bacteria, such as *Fusobacterium* and *Bacteroides*, increases (Colombo *et al.,* 2009). On the other hand, in periodontitis, the microflora is dominated by Gram-negative anaerobes, with increased spirochetes (Colombo *et al.,* 2009; Colombo *et al.,* 2012). The Human Oral Microbe Identification Microarray (HOMIM) provides a semi-quantitative identification of oral microbiome bacterial species (Mougeot *et al.,* 2016; Colombo *et al.,* 2009; Colombo *et al.,* 2012). Earlier methods such as Denaturing Gradient Gel Electrophoresis (DGGE) have been used to study microbial population dynamics in periodontal disease (Fujimoto *et al.,* 2003; Zijnge *et al.,* 2003; Zhou *et al.,* 2011; Ireland *et al.,* 2014; Ledder *et al.,* 2007). This molecular technique, while useful for analyzing bacterial communities and studying shifts in microbial composition at a population level, is often limited by numerous factors, particularly low detection limit and difficulties associated with species identification based on gel positioning (Fujimoto *et al.,* 2003; Zijnge *et al.,* 2003; Zhou *et al.,* 2011; Ireland *et al.,* 2014; Ledder *et al.,* 2007).

For this review, included articles were classified as having high to moderate methodological quality (Fujimoto *et al.,* 2003; Zijnge *et al.,* 2003; Liu *et al.,* 2010; Colombo *et al.,* 2009;

Colombo *et al.,* 2012). The studies classed as having moderate methodological quality reported on the use of Denaturing Gradient Gel Electrophoresis to provide a visual representation of bacterial diversity in periodontal disease. The first study (Fujimoto *et al.,* 2003) aimed to examine the bacterial community of the subgingival plaque using DGGE. Samples were collected from the subgingival plaque of four patients using sterile paper points, and DNA was extracted using the InstaGene Matrix kit. Furthermore, Polymerase Chain Reaction (PCR) was performed using specifically designed primers and genomic DNAs of typical periodontal bacteria. The generated 16S rDNA fragments were separated by denaturing gel. Results indicated that DGGE was able to show distinct banding patterns observed among several samples from identical subjects, but the bands were not always observed at the species-specific positions of periodontal bacteria. The authors in this study highlight the difficulty of the DGGE method to detect bacteria with a low abundance in various samples. This method of bacterial identification assumes that DNA is extracted equally from all bacterial species. Moreover, its reliability depends on the quality and reproducibility of bacteria sample processing and DNA extraction. Thus, any organisms forming < 1% of the microbiota may not be represented. This limitation is widely reported in other studies that have used this method to analyse subgingival microbiota in health and disease (Zhou *et al.,* 2011; Ireland *et al.,* 2014; Ledder *et al.,* 2007).

Similarly, in the second study (Zijnge *et al.,* 2003), the authors highlight the limitations of DGGE due to its low sensitivity and difficulties in identifying species based on their position in the gel. Their research aimed to study the microbial population dynamics in the subgingival pocket of 15 subjects with untreated advanced periodontitis (n=9) or periodontal health (n=6). After the supragingival plaque was removed, subgingival plaque was collected by inserting sterile endodontic absorbing points. DNA extraction was carried out using the phenol/chloroform method and part of the bacterial 16S rRNA was PCR amplified and separated by DGGE. Samples were evaluated at baseline, 1 day after and 3 months after treatment; DGGE banding profile showed that treatment resulted in a decrease in the diversity of the population. DGGE results also indicated that after 3 months, a microbial population 33–47% different from the population before treatment had re-established, highlighting shifts in composition and diversity in the microbial population. Despite DGGE's effectiveness in providing a fingerprint representative of the microbial flora, the culture-independent, PCR-based method can only detect up to 30–40 bands, thus presenting with low resolution and sensitivity issues as reported in another study evaluating

changes in oral bacterial composition (Zhou *et al.,* 2011). Due to the limitations of this method, most studies simply report the relative position of amplicons, while others provide an estimation of denaturing percentage for comparisons due to the limited number of computer programs capable of acquiring DGGE gel images, transferring them to specially designed analytical software and recording the banding patterns (Zhou *et al.,* 2011).

The third study (Liu *et al.,* 2010), similar to the previous two studies, confirms the limitations of DGGE, particularly with its inability to detect bacteria below a certain threshold due to its low sensitivity. The authors of this study aimed to investigate the microbial population in the subgingival community, using DGGE. Specifically, the study examined whether primer choice affected DGGE results and assessed the most appropriate primer pairs for DGGE analysis. Firstly, the authors analyzed the DGGE profiles of different 16S rDNA regions of three periodontal strains (P. gingivalis ATCC 33277, F. nucleatum ATCC 25586 and *P. nigrescens* ATCC 33563) using the target regions (V3, V3–V5 and V6–V8). These regions were cloned into plasmid vectors and the constructed plasmids was used as templates for PCR-DGGE analysis templates in the study. Moreover, the study included non-smoking adults with chronic periodontitis (n = 6), between the ages of 29 and 52 years. Subgingival samples were collected from periodontal pockets using sterile curettes with a probing depth and clinical attachment loss of more than 5 mm at baseline after removal of supragingival plaque. Following mechanical debridement, patients were examined six weeks later and their periodontium was found to have improved significantly. Again, subgingival plaque was sampled from the same pockets (the probing depth was decreased by 2 or 3 mm). Using a bacterial genomic DNA extraction kit (TIANGEN), microbial DNA was extracted and used for PCR amplification of the target fragments. The results suggested that V3–V5 and V6–V8 fragments may be suitable for community analysis of subgingival bacteria; however, it was concluded that 16S rDNA of the V3 region may cause over-estimation of subgingival bacterial populations in DGGE analysis due to multiple banding patterns. Further analysis with the V3–V5 and V6–V8 fragments suggested that, in chronic periodontitis, periodontal bacteria may recolonize within 6 weeks after mechanical debridement with a population very similar to the baseline as there were no significant differences in banding patterns between the two groups. However, these changes would not be identified using DGGE, possibly due to its low sensitivity; thus, there is a need for further analysis with quantitative methods (Liu *et al.,* 2010). The authors were successful in identifying the potential of some targeted regions of 16S rDNA for DGGE analysis, but they highlight the necessity for careful

consideration of the regions used in the analysis as it is currently impossible to predict which regions would yield different results for species identification in DGGE analysis of the same sample. This poses another limitation for DGGE as there is potential for different primers to affect the results generated, as well as certain regions making it difficult to estimate bacterial population due to multiple-band appearance for a single pathogenic bacterium.

Only two studies which described the study design and ethics were classed as having high methodological quality. These studies (Colombo *et al.,* 2009; Colombo *et al.,* 2012) were conducted by the same research group; however, they presented different objectives and distinct samples. Both studies showed evidence of controlled microbial collection by prior removal of the supragingival plaque with a sterile gauze to preserve sample quality. Meanwhile, subgingival biofilm samples were collected using sterile periodontal curettes. For DNA extraction, samples were placed in separate 1.5 mL tubes containing 50 µL of TE (50 mM Tris-HCl, 1 mM EDTA, pH 7.6), 44 µL of each sample was then taken and mixed with 0.5% Tween 20 and 1 µL of Proteinase K (10 mg/mL).

Colombo *et al.* carried out a study in 2009, which aimed to analyse and compare the baseline subgingival microbiota of subjects with refractory periodontitis (RP) to those in subjects with treatable periodontitis (GR) or periodontal health (PH) using the Human Oral Microbe Identification Microarray (HOMIM). A total of 67 subjects were measured at baseline (17 RP individuals, 30 GR individuals and 20 PH individuals), and analysed for the presence of 300 bacterial species using HOMIM. Results indicated a distinct microbial profile in RP patients compared to patients in GR and PH groups. In addition, more species were detected in diseased patients (GR or RP) than those without disease (PH). The authors report that the HOMIM technique allowed for the detection of about 300 species, including cultivable and not-yet cultivable species. Moreover, in periodontal sites losing attachment, HOMIM effectively identified a high prevalence of periodontal pathogens and novel species in low abundance, particularly *S. intermedius*/*constellatus*, *S. anginosus*, *P. micra*, *Selenomonas* spp., *S. parasanguinis*, *Streptococcus* sp. OT 070/071, *F. alocis*, *D. invisus*, *D. pneumosintes*, *C. rectus/concisus*, TM7 spp. OT 346/356/437, *Treponema socranskii*, *Treponema maltophilum*, *Bacteroidetes* sp. OT 274/272, *Prevotella tannerae*, *Tannerella forsythia*, *Eubacterium spp*., *G. sanguinis*, *Porphyromonas endodontalis*, *Peptostreptococcus* sp. OT 113, *Desulfobulbus* sp. OT 041, *P. stomatis*, *S. moorei*, *Sphaerocytophaga* sp. OT 337, *P. gingivalis*, *Megashaera micronuciformis*, *S. satelles*, *Prevotella oralis*, *Mogibacterium*

timidum, *Anaerococcus geminatus*, *Atopobium rimae*, *Atopobium parvulum* and *P. alactolyticus*.

In the last study included in this review (Colombo *et al.,* 2012), the authors compared the changes in the subgingival microbiota of 47 subjects with refractory periodontitis (RP) (n=17) or treatable periodontitis (GR) (n=30) before and after periodontal therapy using HOMIM. Subgingival plaque samples were taken at baseline and 15 months, the HOMIM technique was used to analyse the samples for the presence of over 300 species. HOMIM results indicated that the majority of the evaluated species decreased in prevalence in both groups after treatment. However, only a small subset of organisms was affected significantly. Furthermore, HOMIM data identified several species that increased or persisted in high frequency in RP but reduced significantly in GR, including *Bacteroidetes* sp., *Porphyromonas endodontalis, Porphyromonas gingivalis*, *Prevotella* spp., *Tannerella forsythia*, *Dialister* spp., *Selenomonas* spp., *Catonella morbi*, *Eubacterium* spp., *Filifactor alocis*, *Parvimonas micra*, *Peptostreptococcus* sp. OT113, *Fusobacterium* sp. OT203, *Pseudoramibacter alactolyticus*, *Streptococcus intermedius* or *Streptococcus constellatus*, and *Shuttlesworthia satelle*s. Furthermore, HOMIM analysis was effective in identifying novel species in subjects with RP, suggesting that different microbial profiles, including not only combinations of known species but also novel species, and consequently, significant differences in treatment response.

The results presented in this systematic review indicate that there is moderate scientific evidence that the HOMIM is significantly effective in identifying periodontal pathogens of the oral microbiome. For the HOMIM analysis in both studies (Colombo *et al.,* 2009; Colombo *et al.,* 2012), a total of 400 16S rRNA-based, reverse capture oligonucleotide probes printed on aldehyde-coated slides were used to target over 300 bacterial taxa, suggesting a potential limitation of the technology, as it is possible that HOMIM is only able to recognize bacterial taxa/clusters that have a target probe present on the microarray slides. However, refinement of the HOMIM technology will provide a better identification tool for the oral microbiome and will be beneficial to the understanding of periodontal pathogens in the oral cavity. Moreover, the evidence presented in this review has identified the main limitation for DGGE as its inability to detect species when abundance is below a certain level. Due to the importance of these low-abundance species, a more comprehensive technology such as HOMIM is best to understand the complexity of the periodontal disease process and probable multifactorial aetiology.

While HOMIM has presented as a valuable tool for studying the oral microbiome, other non 16S rRNA sequencing based methods including metagenomics (MTG) and metatranscriptomics (MTT) have emerged as useful tools for bacteria identification, enabling researchers to accelerate the study of genetic information from uncultured microbes and complex microbial communities (Kumar et al., 2023). Metagenomics involves the direct sequencing of DNA extracted from environmental samples, enabling the comprehensive analysis of the entire microbial community present in a given sample (Kumar et al., 2023). By using high-throughput sequencing technologies, researchers can obtain a wealth of genetic information, allowing the identification of individual bacterial species and providing insights into their functional capabilities and potential roles in disease development (Carda-Diéguez *et al*., 2022; Meyer *et al*., 2023). Metatranscriptomics, on the other hand, focuses on the analysis of the transcriptome, which represents the entire set of RNA molecules present in a microbial community (Sun *et al*., 2022). This approach allows the study of gene expression patterns of different bacterial species within the community, providing information on their activity and metabolic functions (Kumar *et al.,* 2023).

In the context of periodontal diseases, MTG and MTT have proven particularly valuable. By analyzing the oral microbiome of individuals with periodontitis, specific bacterial species associated with disease progression and severity have been identified (Carda-Diéguez *et al*., 2022). Moreover, MTT has enabled the investigation of gene expression patterns in these bacterial communities, revealing the activation of virulence factors and inflammatory response-related genes (Guo *et al.,* 2023). The integration of MTG and MTT data has also allowed for the construction of microbial interaction networks within the oral microbiome, providing insights into the complex dynamics of microbial communities in periodontal diseases.

In the exploration of the oral microbiome, various sequencing methodologies offer distinct insights and challenges, notably when comparing the HOMIM and DGGE techniques discussed in this chapter with the HOMINGS approach outlined in Chapter 4. HOMIM is known for its ability to rapidly identify a broad array of oral bacteria through hybridization to specific probes, providing a high-throughput, semi-quantitative overview of the microbial community. DGGE on the other hand, separates nucleic acid sequences by their electrophoretic mobility in a gradient gel, offering a visual representation of microbial diversity that, while informative, lacks the specificity and depth of molecular identification.

In Chapter 4 the study employs the High-throughput Oral Microbial Identification using Next-Generation Sequencing (HOMINGS) method. HOMINGS leverages the power of next-generation sequencing (NGS) technologies to provide a comprehensive, in-depth analysis of the oral microbiome with high specificity and sensitivity. This method does not rely on predefined bacterial targets; instead, it allows for the identification and quantification of a wide array of microorganisms, including those not previously catalogued in existing databases. Compared to HOMIM and DGGE, HOMINGS offers enhanced resolution in microbial identification, enabling the detection of low-abundance species and providing a more detailed picture of microbial diversity and community structure.

Moreover, the transition from HOMIM and DGGE to HOMINGS represents a significant advancement in oral microbiome research, moving from broad-spectrum, semi-quantitative analyses to a highly detailed, quantitative understanding of microbial presence and abundance. While HOMIM provides a valuable tool for identifying known microbes efficiently, and DGGE offers insights into community diversity, HOMINGS surpasses these methods in its ability to uncover the full complexity of the oral microbiome, including the detection of rare or novel organisms. This progression underscores the evolution of microbiome research from focusing on the presence and relative abundance of microbes to a nuanced understanding of microbial ecosystems, their dynamics, and their implications for health and disease. While HOMIM and DGGE have played crucial roles in the foundational understanding of the oral microbiome, the adoption of HOMINGS in Chapter 4 signifies a step forward in the precision, depth, and comprehensiveness of microbial analysis.

2.5 Conclusions

This is the first systematic review regarding the significance and effectiveness of HOMIM in quantifying the human oral microbiome. A search of the literature found moderate evidence that the Human Oral Microbe Identification Microarray (HOMIM) is significantly effective in identifying and quantifying bacterial species of the oral microbiome in periodontal disease. Despite the limitations associated with this current molecular profiling technology, HOMIM expanded oral bacterial species identification compared to earlier

methods such as DGGE. Furthermore, the species probes utilized in HOMIM provided a more comprehensive representation of the oral bacterial community, critical for future characterization of oral microbes in periodontal disease. It is important for future work to explore the effectiveness of Human Oral Microbe Identification using Next-Generation Sequencing (HOMINGS), a successor technology of HOMIM, as well as MTG and MTT methods for identifying and quantifying the human oral microbiome.

Chapter 3 Effect of oral formulations on oral bacteria in tongue derived microbial biofilms: An *in-vitro* study.

3.1 Introduction

3.1.1 Oral microbial biofilms and their role in the development of periodontal disease.

The oral cavity is an important organ that plays a huge role in social interactions, and as such, maintaining its health is crucial for an individual's personality, confidence, and communication (Wu *et al.,* 2022). Moreover, the oral microbiome within the oral cavity is composed of a unique and diverse ecosystem of microbial organisms that interact metabolically and physically, creating complex biofilm communities (Sedghi *et al.,* 2021). These communities have distinct niches for microorganisms of differing metabolic needs, which can be disrupted, leading to microbial dysbiosis (Hajishengallis and Lamont, 2012). This disruption in microbial community dynamics plays a major role in the aetiology of gingivitis and the development of periodontal disease such as periodontitis (Sedghi *et al.,* 2021).

Periodontal disease is a condition that is characterized by a progressive destruction of both soft and hard tissues in the periodontal complex. This destruction is primarily mediated by the interplay between dysbiotic microbial communities and aberrant immune responses in gingival and periodontal tissues (Sanz *et al*., 2020). In addition, periodontitis is also characterized by immune dysregulation, inflammation, and an increased representation of periodontal pathogens, which bi-directionally promote each other and ultimately contribute to the destruction of tooth-supporting structures such as the periodontal ligament and alveolar bone (Sedghi *et al.,* 2021). The development of periodontitis is associated with profound shifts in the composition of subgingival communities (Curtis *et al.,* 2020). Specifically, Gram-negative species that differ from those enriched during gingivitis outgrow health-associated taxa (Musić *et al*., 2021). Furthermore, periodontitis has been identified as a risk factor for several systemic diseases, including diabetes (Curtis *et al.,* 2020). The pathological changes associated with diabetes can exacerbate and accelerate the onset and progression of periodontal inflammation, and effective management of periodontitis plays a crucial role in regulating blood sugar levels (Peng *et al.,* 2022). The oral microbiome can influence systemic immune homeostasis and contribute to the development of diabetes. For example, oral microorganisms can induce insulin resistance

by stimulating local periodontal tissue immune responses, resulting in the production of high levels of inflammatory cytokines and promoting oxidative stress (Peng *et al.,* 2022). These events can have a significant impact on the pathogenesis of diabetes.

Research has established that the biofilm environment is the preferred mode of bacterial growth. It is widely accepted that most bacteria prefer to grow in matrix-enclosed biofilms that are adherent to surfaces in nutrient-rich aquatic ecosystems (Mgomi *et al.,* 2023). A biofilm is a microbial community characterized by cells that are irreversibly attached to a substrate, interface, or to each other and are embedded in a matrix of extracellular polymeric substances (EPS) that they have produced (Mgomi *et al.,* 2023). Furthermore, Mamdoh *et al*., (2023) suggest that these sessile bacteria differ significantly from their planktonic counterparts as biofilms often exhibit an altered phenotype with respect to growth rate and gene transcription. During biofilm development, bacteria first attach to a surface, then certain adherent cells proliferate and produce exopolysaccharides until they generate a microcolony in which morphologically similar "sister cells" are embedded in a thick polysaccharide matrix (Roilides *et al*., 2015). Moreover, as the biofilm thickens and matures, individual microcolonies may lose their association with the colonized surface, and, in multispecies populations, cells of several species may collaboratively produce a functional consortium that carries out physiologically cooperative processes, which can generate altered patterns of bacterial growth and metabolic efficiency (Mariselvam *et al*., 2023).

In the oral cavity, distinct habitats exist, including the lips, tongue, cheeks, palate, subgingival plaques and teeth that can support the growth of a characteristic microbial community (Saad, 2006). These habitats containing soft and hard tissue surfaces are potential substrates for the development and maintenance of oral biofilms, as they are kept well lubricated by salivary glands, providing a suitable nutritious aqueous environment (Saad, 2006). The dorsum of the tongue is a highly populated surface, supporting a higher bacterial density than other oral mucosal surfaces due to its large surface area (Saad *et al.,* 2013). Studies have confirmed that this site can harbour a significant population of Gram-negative anaerobic rods such as *Bacteriodes* spp., *Fusobacterium* sp., as well as other anaerobes such as *P. intermedia*, and *Veillonella* spp., which have been implicated in periodontal disease (Curtis *et al.,* 2020; Hampelska *et al.,* 2020; Patil *et al*., 2013; Sanz *et al.,* 2017; Saad *et al.,* 2013;). Additionally, the tongue biofilm is believed to be a major

contributor to oral malodour, a condition with linked to periodontitis and accounts for 60 - 70% of volatile compounds (VOC's) and volatile sulphur compounds (VSC's) (Khalid *et al.,* 2013).

Oral malodour (Halitosis) is a common complaint amongst the adult population, with research worldwide indicating a high prevalence of moderate halitosis, whereas severe cases are restricted to around 5% of the populations (Rösing and Loesche, 2011). Musić *et al*. (2021) defines halitosis as an unpleasant smell emanating from the oral cavity, with 80-90% of halitosis cases arising from intra-oral origin. Halitosis can be classified as intra-oral or extra-oral depending on the origin of the compounds (Wu *et al.,* 2022). The general population is still poorly informed about the causes and treatment of halitosis, although, oral malodour can still affect the lives of many individuals who genuinely suffer from it. According to Musić *et al*. (2021), the dorso–posterior portion of the tongue where microbial populations are present in the form of biofilms, was the most common cause of intra oral halitosis, followed by gingivitis/periodontitis and a combination of the two, accounting, respectively, for 43%, 11% and 18% of the case. Research suggests that the gases emanating from the mouth that cause oral malodour are generated by anaerobic bacteria; particularly anaerobic Gram-negative bacteria of the red complex, (*Porphyromonas gingivalis*, *Tanerella forsythia* and *Treponema denticola)*, which have shown strong relation to clinical measures of periodontal disease and are also associated with higher production of VSCs (Musić *et al*., 2021). These anaerobes decompose proteinaceous components of saliva, desquamated epithelial cells, and trapped foodstuff, transforming them into foul-smelling volatile sulphur compounds (VSCs) and volatile compounds (VOCs) (Saad *et al.,* 2018).

The pathogenesis of intraoral halitosis is associated with the microbial degradation of both sulphur-containing (cysteine, cystine, and methionine) and non-sulphur-containing amino acids (i.e. tryptophan, lysine, and ornithine) (Saad *et al.,* 2012). Furthermore, these amino acids are mainly derived from proteins present in bacterial plaque, saliva, blood and tongue coating, and usually undergo decarboxylation, which generates malodorous amines (putrescine and cadaverine) and other volatile aromatic compounds such as indole and skatole (Tungare *et al.,* 2018). Studies (Hampelska *et al.,* 2020; Wu *et al.,* 2022; Tangerman and Winkel, 2013; Musić *et al*., 2021; Saad *et al.,* 2012) report that VSC's such as Hydrogen sulphide (H_2S), Methyl mercaptan (CH_3SH) and Dimethyl sulphide [$(CH_3)_2S$], are the major contributors to halitosis. Although, non-sulphur-containing volatile compounds such as

indole, skatole, amines and ammonia, are known to contribute to halitosis to a lesser extent (Saad *et al.,* 2012). Furthermore, research suggests that these volatiles themselves can be harmful and are deleterious to oral soft tissues even at low concentrations as they contain thiols (-SH groups) that interact with other proteins and support the negative interaction of bacterial antigens and enzymes (Caroline *et al.,* 2020). For instance, Hydrogen sulphide and Methyl mercaptan act as potent gaseous neuromodulators involved in the regulation of crucial cellular processes such as inflammation, oxidative stress, autophagy, and apoptosis.

Saad *et al.* (2018) reported two fundamental means of evaluating oral malodour: organoleptic or instrumental. In the organoleptic method, oral malodour is evaluated at various distances from the oral cavity by an examiner's sense of smell, or assigned a severity grade given a constant distance (Wu *et al.,* 2019). In breath malodor research, a frequently employed scale is the 0-5 intensity scale developed by Rosenberg *et al*. (1991) and modified by Greenman *et al*., (2004). In this scale, a rating of 0 corresponds to odorant concentrations below the detection threshold, while a rating of 5 signifies odorant concentrations that are exceptionally potent and presumed to be near saturation of the smell receptors. The advantages of the organoleptic method are ease of performance and low costs. However, studies have shown that the results of organoleptic measurement are often not reproducible, because they depend on the subjective assessment of the examiner and other influencing factors including age, gender and time of day (Brunner *et al*., 2010). Additionally, the recent COVID-19 pandemic introduced some additional challenges and limitations of using this method as it proved unsafe for both researchers and participants. For instrumental measurement, various devices are used including gas chromatographs (e.g. Oral Chroma), electronic noses, and sulphide monitors (e.g. Halimeter). However, these instruments are often limited by what volatiles can be measured or indirect sampling techniques (Tangerman and Winkel, 2012). While the Halimeter offers direct measurement of VSCs in breath, it cannot discriminate between specific VSCs, and though the OralChroma can discriminate between VSCs, it cannot sample breath in real time (Saad *et al*., 2018). Additionally, neither instrument can detect other volatile compounds such as cadaverine, putrescine, indole, and skatole which may also play an important role in periodontal disease (Zurcher *et al.,* 2014). Meanwhile, selected ion flow tube–mass spectrometry (SIFT-MS) offers a solution to these limitations as it is a real-time, quantitative method for analyzing a wide range of volatile compounds. Recent applications of SIFT-MS include the analysis of

volatiles in the headspace of microbial cultures and in exhaled human breath, and other gas samples for medical diagnosis and monitoring of treatments (Saad *et al*., 2018).

3.1.2 Oral formulations and their Impact on oral microbial biofilms

Halitosis treatment often involves meticulous oral hygiene, including the use of dental floss and brushes to remove bacteria from oral surfaces, as well as the use of oral care products containing antimicrobial and odour-neutralizing agents (Zurcher *et al.,* 2014). Chlorhexidine (CHX) is recognised as a benchmark treatment for oral malodour and periodontal disease due to its broad-spectrum activity against both Gram-negative and Gram-positive bacteria and its dual antimicrobial and odour-neutralizing properties. (Sharma *et al.,* 2019). Furthermore, the cationic nature of CHX enables it bind to tooth surfaces and the oral mucosa, increasing its substantivity through slow-release Saad *et al.,* (2012). Various studies have demonstrated the efficacy of CHX in reducing oral bacterial viability, strongly preventing gingivitis, reducing oral malodour *in-vitro* and *in-vivo* (Sharma *et al.,* 2019; Erovic *et al.,* 2017; Herrera, 2013; *Saad et al.,* 2012; Van Strydonck *et al.,* 2012; Greenman *et al*., 2008). However, Van Strydonck *et al.* (2012) highlights various CHX side effects including teeth staining, taste alteration, allergic reactions and potential impacts on fibroblast formation in periodontal pocket. Hence, it is imperative to explore alternative compounds that exhibit similar or superior efficacy in managing periodontal disease and oral malodour.

3.1.3 Study aim

The study aimed to investigate the effects of a toothpaste containing 5% potassium nitrate and 0.24% sodium fluoride, compared to 1% chlorhexidine, on a malodour flat-bed perfusion biofilm model derived from a real tongue-scrape inoculum. The study used SIFT-MS to quantify the levels of volatile compounds (VOC) and volatile sulphur compounds (VSC) in headspace gas samples from the biofilm matrix before and after treatment with the oral formulations. The study also investigated the antimicrobial activities of these oral formulations by assessing the final bacterial load after treatment compared to controls, assessing antibacterial susceptibility and evaluating the effects of the treatments on cell membrane permeability of oral tongue scrape bacteria.

3.2 Methods

3.2.1 The flat bed perfusion matrix model

3.2.1.1 Biofilm enclosure

In principle, the flat bed perfusion matrix model was used for this project as it allows bacteria to be grown in a biofilm formation thus, more accurately reproducing the environment of the bacteria as they would be found in the mouth. Moreover, this model allows the studying of oral malodour effectively since it can maintain bacterial population as a culture for long periods (appx. 7 days in this case). The methodology has been adapted from Saad *et al.,* (2013), with some modifications as described below. Multiple systems (n = 4) were set up in parallel as in fig 3.1, drawing medium continuously from a single reservoir. One biofilm sample box was filled with the test toothpaste, another with the reference toothpaste, while the remaining two boxes were used as controls. The biofilm enclosure consisted of a modified polypropylene freezer box (Lock & Lock, Amazon, UK) within which a previously inoculated loose fibred 1 cm^2 cellulose matrix was held in place on a specially constructed slope. Media was fed drip-wise by a peristaltic pump (Watson-Marlow, Falmouth, UK) via a 23G hypodermic syringe (Terumo, Japan). The feed line for this syringe was connected to the pump via a grow-back inhibitor. One fifth strength Brain Heart Infusion (BHI) (7.4 g/L w/v) (Neogen, UK) supplemented with haemin (0.001 g/L w/v), dithiothreitol (0.05 g/L w/v) and cysteine (0.1 g/L w/v) (Sigma-Aldrich, UK) media was used for the oral bacterial strains. The pH was adjusted to 7.0 prior to autoclaving by addition of 10 M NaOH. Two peristaltic pumps were used, (one for media and the other for gas) which needed to be calibrated to control the flow rate. To calibrate the pumps, water was pumped through the system and collected in a graduated cylinder for half an hour, after which the flow rates were calculated using the volume collected and time elapsed.

The box was continuously filled with anaerobic gas (90 % nitrogen, 10 % carbon dioxide) from a cylinder of compressed gas through a peristaltic pump set at 10 RPM to enable an anaerobic atmosphere inside each box. The flow rate of media through the peristaltic pump was set at 6 RPM, closely mimicking the environment of the oral cavity, which has an average salivary flow rate approximately 0.3-0.4 mL/minute for unstimulated salivary production and up to 7 mL/minute when stimulated (Iorgulescu, 2009). Positive pressure of media was maintained by peristaltic pump to prevent backward flow of media and waste flowed out of the box via silicone tubing attached to a PTFE elbow connector under the box.

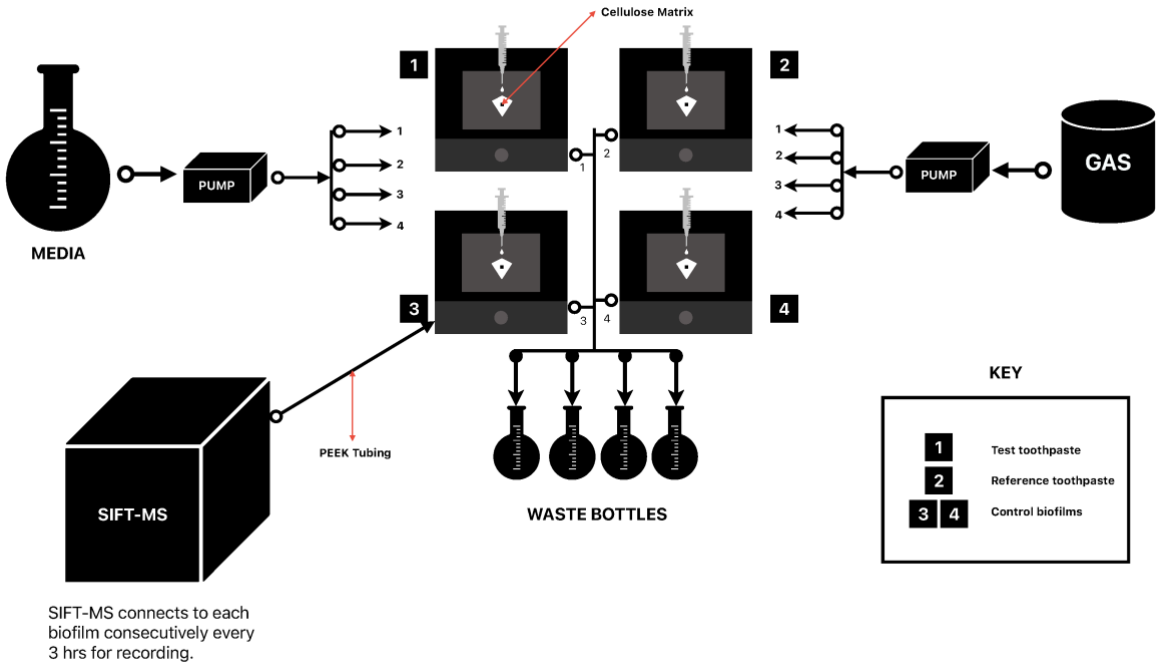


Figure 3.1 Schematic representation of biofilm setup.

3.2.1.2 Protocol for anaerobic biofilm development for VOC analysis.

Using a sterile toothbrush, tongue scrape samples were collected from a healthy participant (Ethics ref: HAS.20.03.148) at the University of the West of England, then resuspended in 10 ml of BHI broth (Neogen, UK) (pH 7) and incubated anaerobically at 35 ˚C for 24 hrs. A 300 µl aliquot was then transferred aseptically onto the surface of a 1 cm^2 cellulose matrix, layered on Fastidious Anaerobe Agar (FAA) (Neogen, UK) supplemented with 5% defibrinated horse blood (TCS Biologicals, Bucks, UK). The matrices were left to stand for 4 hrs in the anaerobic cabinet (Don Whitley, Anaerobic Workstation) to allow attachment of bacterial cells to matrix and then transferred to the support slope within the airtight autoclavable boxes serving as growth chambers (Saad *et al.,* 2013). The biofilm enclosures were mounted into a modified shelf, and each placed inside a separate mini-incubator (VWR International Ltd., Lutterworth, UK) set to 37 °C. The front panel of these incubators was replaced with a laser-cut polycarbonate panel allowing inlet ports for gas and media, and outlet ports for waste and VOC's analysis. Once set up, the system was left to run uninterrupted for 48hrs before testing commenced, allowing each biofilm reach quasi-steady state.

3.2.1.3 Treatment protocol.

A modular approach was adopted, which enabled the split biofilm system to be fed from a single reservoir, allowing for statistical stability to be assessed. By employing control groups

and comparing them with treatments, interventions displaying clear inhibitory effects on both volatile organic compounds (VOCs) and volatile sulphur compounds (VSCs) output levels, as well as on viable counts, were identified in contrast to those with minimal or no inhibitory activity. Two oral formulations, the test toothpaste and the reference toothpaste, each containing different active ingredients (Table 3.1), were selected for testing. To prepare the toothpastes for testing, 2g of each toothpaste was measured out and left to dry anaerobically in desiccators for a week on sterile microporous membrane discs. During the intervention, the resulting thick, dried toothpaste film was then placed on the surface of inoculated cellulose matrices using sterile forceps. Toothpastes were introduced twice a day with an 8-hour gap, and old discs were replaced at each time point, to simulate multiple treatments over a 3-day period. Chamber openings were standardized across experimental and control groups to address potential concerns regarding changes in headspace volatile concentrations. The 'no treatment' controls underwent a procedure identical to that of the treatment groups, with the addition of a blank disc mirroring the intervention without introducing active substances. This standardized approach ensured any temporary fluctuations in volatile levels due to chamber opening were equally experienced across all test conditions. Although this procedure may have led to transient losses of volatiles, these levels were rapidly restored within minutes, ensuring that any such losses did not significantly affect the overall volatile profiles and the integrity of the recorded values. At the end of the run, on day 4, (precisely 12 hours after the completion of the final treatment on day 3), all four biofilms were sacrificed and harvested by disruption, then bacterial viable counts were conducted for microbiology analysis. The composition of each treatment used is detailed in Table 3-1. Experiments were repeated three times for each treatment).

Table 3-1 Active ingredients in selected oral formulations.

	Key or active ingredients	Other Ingredients
Test Toothpaste	5% potassium nitrate (KNO_3), 0.24% sodium fluoride (0.14% w/v fluoride ion)	Water, glycerine, hydrated silica, sorbitol, PEG-12, PVM/MA copolymer, sodium lauryl sulphate, flavour, trisodium phosphate, poloxamer 405, sodium hydroxide, sodium saccharin, cellulose subgingival plaque, xanthan subgingival plaque and titanium dioxide.
Reference Toothpaste	1% chlorhexidine digluconate (CHX)	Hydroxypropylcellulose, macrogolglycerol hydroxystearate, sodium acetate, levomenthol, peppermint oil, isopropyl alcohol and water.

3.2.1.4 SIFT-MS Sampling of Biofilm Enclosures

Volatile organic compounds produced by the biofilms were monitored in real time with the use of a Voice200 SIFT-MS (Syft Technologies, Canterbury, New Zealand). Gas sampling was through a protected port in the biofilm enclosure described above, through a Poly-Ether Ether Ketone (PEEK) tubing of 1/16 in external diameter and 0.030 in internal diameter (Supelco, Penns, USA). This is introduced through the front of the enclosure and protected from contamination by a 1 ml pipette tip. Prior to sampling, the instrument had both capped and uncapped pressure, as well as calibrant (Helium) pressure, checked and recorded each time. Validation with standard gases (Nitrogen, Oxygen and Hydrogen) was also performed before testing each time, in line with the manufacturer (Anatune) recommendation. For biofilm sampling, a full scan was run before each test to ensure that (i) the instrument was functioning as normal and (ii) the gas being sampled was from the headspace of the biofilm rather than ambient air retained in the tubing. Furthermore, the SIFT-MS was used in selected ion mode, which allows for quantification of the concentration of specific compounds in a given sample. A method was then created on the instrument to measure specific compounds known to be associated with oral malodour, including [Hydrogen sulphide (H_2S), Methyl mercaptan (CH_3SH), Dimethyl-disulphide (DMDS) (CH_3SSCH_3), Indole (C_8H_7N), Skatole (C_9H_9N), 1-Pentanol ($C_5H_{12}O$), Methanol (CH_3OH), Trimethylamine (C_3H_9N), Propionic acid ($CH_3CH_2CO_2H$), Butanoic acid ($CH_3CH_2CH_2CO_2H$), Cadaverine ($C_5H_{14}N_2$) and Putrescine ($C_4H_{12}N_2$)] selected from the drop-down library list. The SIFT-MS device sequentially measured each biofilm sample through a single port, continuously gathering data for about 3 hours each day. It performed scans at intervals of every 5 milliseconds. Approximately 5 minutes of anaerobic gas (10% CO_2/10% Hydrogen/Nitrogen) scan was used to flush the machine between each biofilm sampling to remove any residual compounds from the tubing. Data was saved and exported using the SIFT-MS profile viewer software (Saad *et al.,* 2018).

The experimental design dictated the timing and feasibility of volatile concentration measurements, particularly impacting the data collection after the 5pm treatments. Due to the structured schedule of sequentially recording volatile levels from each of the four biofilms for three hours, measurements immediately following the 5pm treatments were not conducted. This approach resulted in the absence of immediate post-treatment data at 5pm. However, the experimental protocol ensured that volatile levels were assessed at the start of each day, before any treatments were administered, thereby incorporating the

cumulative effects of the 5pm treatments from the preceding day into the overall analysis. Additionally, data from only one control biofilm was used for analysis as both controls provided identical data, thus n=3 as displayed on graph.

3.2.1.5 Microbiological Analysis

3.2.1.5.1 Sampling of biofilm perfusate

To accurately assess the bacterial population in the biofilm and ensure the absence of contamination, approximately 5ml of perfusate samples were collected pre-treatment and daily during treatment from the biofilm by attaching a sterile universal container to the waste tube. These samples were subjected to serial dilutions (1 in 10) in phosphate buffered saline (PBS), and 50 µl from each dilution was spiral plated (WASP, Don Whitley Scientific, Shipley, UK) onto a range of selective and non-selective media. To isolate strict anaerobic species, plates of Fastidious Anaerobe Agar (Neogen, UK) enriched with 5% defibrinated horse blood (TCS Biologicals, UK) and containing vancomycin (0.0025% w/v) were used. The same medium without vancomycin was employed to isolate both strict and facultative anaerobes. All plates were incubated at 35°C in an anaerobic cabinet (Don Whitley, Anaerobic Workstation) for up to 10 days, and the resulting colonies were counted and recorded as colony-forming units per mL (CFU/mL).

3.2.1.5.2 Destructive sampling of biofilm matrices

After a 4-day period (12 hrs after last treatment on day 3), biofilm matrices were aseptically removed from their enclosures using sterile forceps, then placed into 10 ml of PBS and vortexed for 2 minutes at full speed, to give “neat” suspension. A series of serial dilutions (1-in-10) were performed in PBS (down to 10^{-5}) and plated using a spiral plater (WASP, Don Whitley Scientific, Shipley, UK.) Plates were incubated at 35 °C in an anaerobic cabinet (Don Whitley, Anaerobic Workstation) for up to 10 days. Plates of Fastidious Anaerobe Agar (Neogen, UK), enriched with 5% defibrinated horse blood (TCS Biologicals, UK) with vancomycin (0.0025% w/v) were used for the isolation of strict anaerobic species. The same medium without vancomycin was used to isolate both strict and facultative anaerobes and the resulting colonies were counted and recorded as colony-forming units per millilitre (CFU/mL).

3.2.2 Agar well diffusion assay

Varying concentrations of two oral formulations with different active ingredients were tested on oral bacteria in tongue scrape samples in an agar well diffusion assay to assess their antimicrobial effect. FAA plates (Neogen, UK) were inoculated with 100 µl of a 10^8 CFU/ml tongue scrape culture, spread round with a sterile cotton swab and allowed to dry in the anaerobic cabinet (Don Whitley, Anaerobic Workstation). Furthermore, 9 mm wells were punched on media and 500 µl of oral formulations (varying concentrations) were dispensed into the respective wells. Oral formulation concentrations used ranged from 2.5%, to 0.63% for the test toothpaste (5% KNO_3 and 0.24% NaF) and 0.5%, to 0.13% for the reference toothpaste (1% CHX). A negative control without the oral formulation was included as well as a control with distilled water. Additionally, a 500 µl aliquot of pure 5% KNO_3 solution included in one of the wells used for this assay. Plates were incubated for 48 hrs at 35°C anaerobically and inhibition zones (mm) were measured using a Vernier calliper. Experiment was carried out independently on three separate occasions in triplicates.

3.3.3 Determination of the minimum inhibitory concentration (MIC) minimum bactericidal concentration (MBC)

The standard broth microdilution method was used to study the antimicrobial efficacy of two commercial oral formulations (Table 3-1) by evaluating the visible growth of oral bacteria in tongue scrape samples. Due to opacity interference from oral formulations used, an MIC assay could not be performed effectively; however, an MBC assay was performed. Serial dilutions (1 in 2) of the test toothpaste and the reference toothpaste were prepared in sterile 12 well plates using BHI (Neogen, UK) to create a concentration range from 2.5% (w/v) to 0.02% (w/v) (test toothpaste) and 0.5% (w/v) to 0.002% (w/v) (reference toothpaste). A pipette was used to transfer 1 ml of each sample into correspondingly labelled wells containing 100 µl of 10^8 CFU/ml of a tongue scrape sample. Wells containing BHI (Neogen, UK) were used as a negative control while inoculated BHI and inoculated dH_2O wells were used as a positive control. Moreover, 50 µl samples from each well were then inoculated onto FAA plates and incubated at 35 °C for 48hrs. MBC was determined as lowest concentration which no visible growth was observed, resulting thus in 99.9% bactericidal activity. Experiments were performed in triplicates on three independent occasions.

3.3.4 Cell leakage assay

Adapted from Huang *et al*. (2017) & Adukwu *et al.* (2020) with some modifications, the cellular content leakage assay method was used to assess the integrity of the cell membranes of bacterial cells in tongue scrape samples following exposure to the test and reference toothpastes (Table 3.1.). Briefly, tongue scrape samples were collected from six healthy participants (n=6) at the University of the West of England (Ethics ref: HAS.20.03.148). Each was then transferred into 10 ml of BHI broth (pH 7) and incubated anaerobically at 35°C for 24hrs. Following incubation, 200 µL of cell suspensions were transferred to wells of the 24-well plates containing 1800 µL of the different oral formulations at different concentrations (0.3% and 0.1% (w/v)). Oral formulations in BHI (Neogen, UK) and untreated cells in uninoculated broth acted as controls. Plates were incubated anaerobically at 35 °C for 24 h. After the incubation period, the samples were transferred aseptically into Eppendorf tubes and centrifuged (Allegra X-30R) at 12,000 g for 10 min. Release of DNA and RNA was determined by using spectrophotometric measurement of cell supernatant at 260 nm. The protein concentration was measured according to Bradford (1976). A linear calibration curve (Fig. 3.9) was made with Bovine Serum Albumin (BSA) (Sigma-Aldrich, UK). Using a 2 mg/ml BSA solution in PBS buffer, a calibration standard curve of serial dilutions from 0, 125, 250, 500, 1000 to 1400 µg/ml was made. Each standard dilution (50 µl) was mixed in a sterile cuvette with 1.5 ml of Bradford reagent (Sigma-Aldrich, UK). After incubation at room temperature for 15 minutes, standard dilutions were measured at 595 nm with a Jenway 7205 spectrophotometer (manufacturer), and protein concentrations were determined by comparing the absorbance values of unknown samples with the calibration standard curve of BSA.

3.3.5 Scanning electron microscopy

The anti-bacterial activity of the toothpastes was examined through Scanning Electron Microscopy (SEM). Tongue scrape samples were collected from a healthy participant (Ethics ref: HAS.20.03.148) at the University of the West of England and treated with two oral formulations with different active ingredients, test toothpaste (5% KNO_3 and 0.24% NaF) and reference toothpaste (1% CHX) in an *in-vitro* biofilm system. Treatment occurred twice daily at 8:00 am and 17:00 pm for a period of 5 days before matrices were sacrificed. Biofilm matrices were then removed and washed three times with phosphate buffer saline (PBS). Following, matrices were fixed with 4% glutaraldehyde for 2hrs before dehydration in a 20%,

30%, 50%, 70%, 80%, and 90% dilution series of ethanol for 5 minutes each, respectively. The samples were then dehydrated in 100% ethanol for an additional 15 minutes before being transferred into a mixture of 100% ethanol and hexamethyldisilazane (HMDS) in a 2:1, 2:2 and 1:2 ratio for 5 minutes each. Samples were then transferred to a 100% HMDS solution for 15 minutes and left to air dry overnight before attachment to metallic stubs coated, under vacuum, with a film of gold. Once coated with gold, the specimens were ready for examination under scanning electron microscope (FEI Quanta FEG 650, Thermo Fisher Scientific, USA). From each treatment group, representative SEM images were selected for further analysis. A total of 50 images were analysed, with 2-3 images each taken from 7 randomly selected sections. The SEM images included in this thesis were chosen from three biological replicates and are representative of the overall findings.

3.3.5 Statistical analysis

Descriptive statistical analyses were employed to generate summary statistics, including means, standard deviations, and ranges, for continuous variables. Inferential statistics were subsequently applied to evaluate the hypotheses formulated for each study. To assess statistical differences within the in-vitro biofilm systems, an unpaired t-test was utilized to compare the two treatment groups. For the examination of significant differences among various treatment concentrations, a one-way ANOVA followed by Tukey's post hoc test was employed. Additionally, to ensure the robustness and consistency of the analyses, the data were analysed using two distinct software packages, namely Excel and SPSS.

3.3 Results

3.3.1 In-vitro effects of oral formulations on volatile organic compounds and microbial populations

3.3.1.1 Effect of multiple dose treatment of toothpastes on VOC and VSC

Headspace analysis using SIFT-MS showed a consistent decrease in total VSC and VOC levels in tongue scrape biofilms treated with both oral formulations. Control (untreated) biofilms showed an increase in both VOC and VSC levels over the 3-day treatment period, while biofilms treated with the test and reference toothpaste showed a general decrease in all levels except for DMDS. The test toothpaste exhibited the most significant decrease in total volatile organic compound levels with an average 1 log ppb drop compared to the control

(p<0.001) and an average 0.5 log ppb drop compared to the reference toothpaste (p<0.01). The reference toothpaste also showed significant drop in total VSC's compared to the control with an average 0.5 log ppb drop (p<0.05). All volatile organic compounds measured during the study are listed in the methodology.

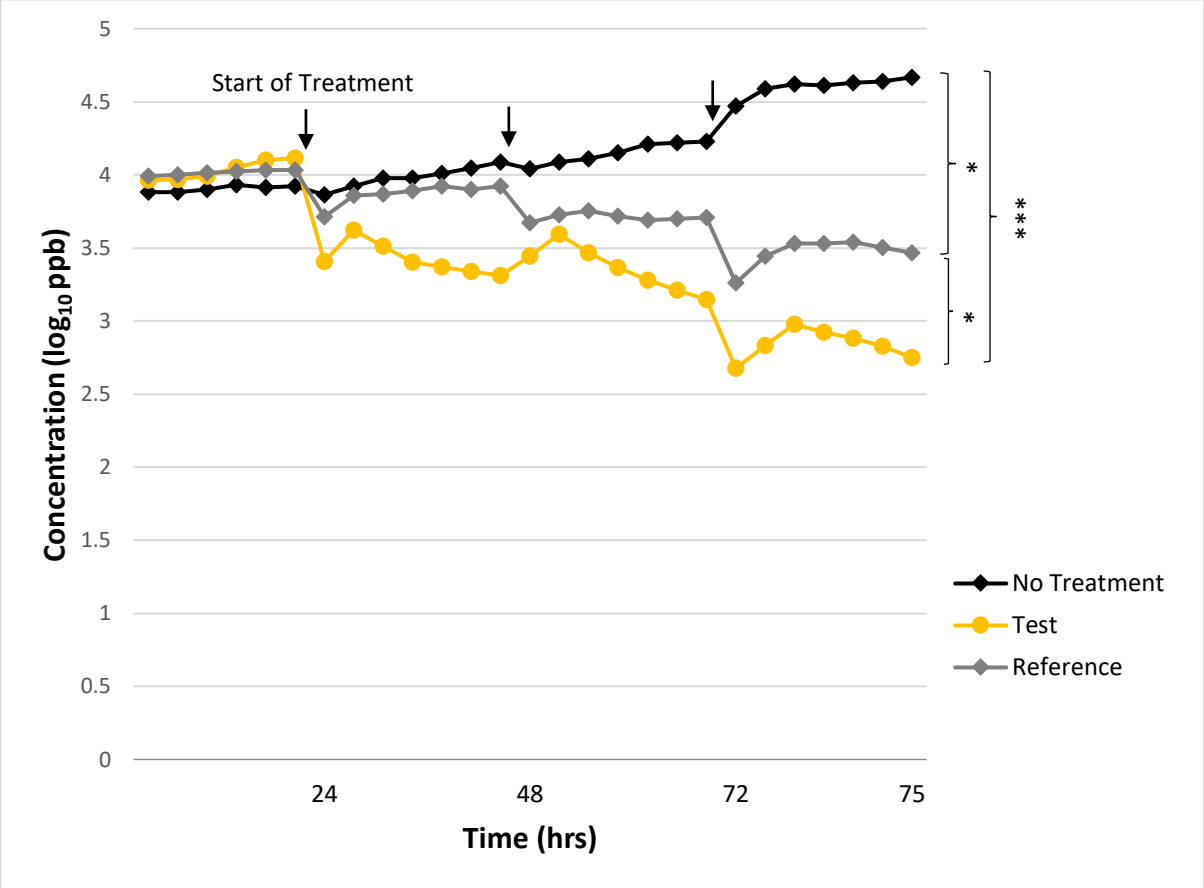


Figure 3.2 Test toothpaste significantly decreases levels of total volatile organic compounds detected from biofilm headspace after 3-day treatment. Tongue scrape samples were grown in an *in-vitro* biofilm system and exposed to treatment over a 3-day period, biofilm was treated twice a day, in the morning and evening with 2g of the test toothpaste (5% KNO_3, 0.24% NaF) and 2g of the reference toothpaste (1% CHX). Experiment was repeated thrice independently with triplicates (n=3). Arrows indicate daily treatment points at 8am. Unpaired t-test statistical significance is indicated as (*) $p \leq 0.05$ and (***) $p \leq 0.001$.

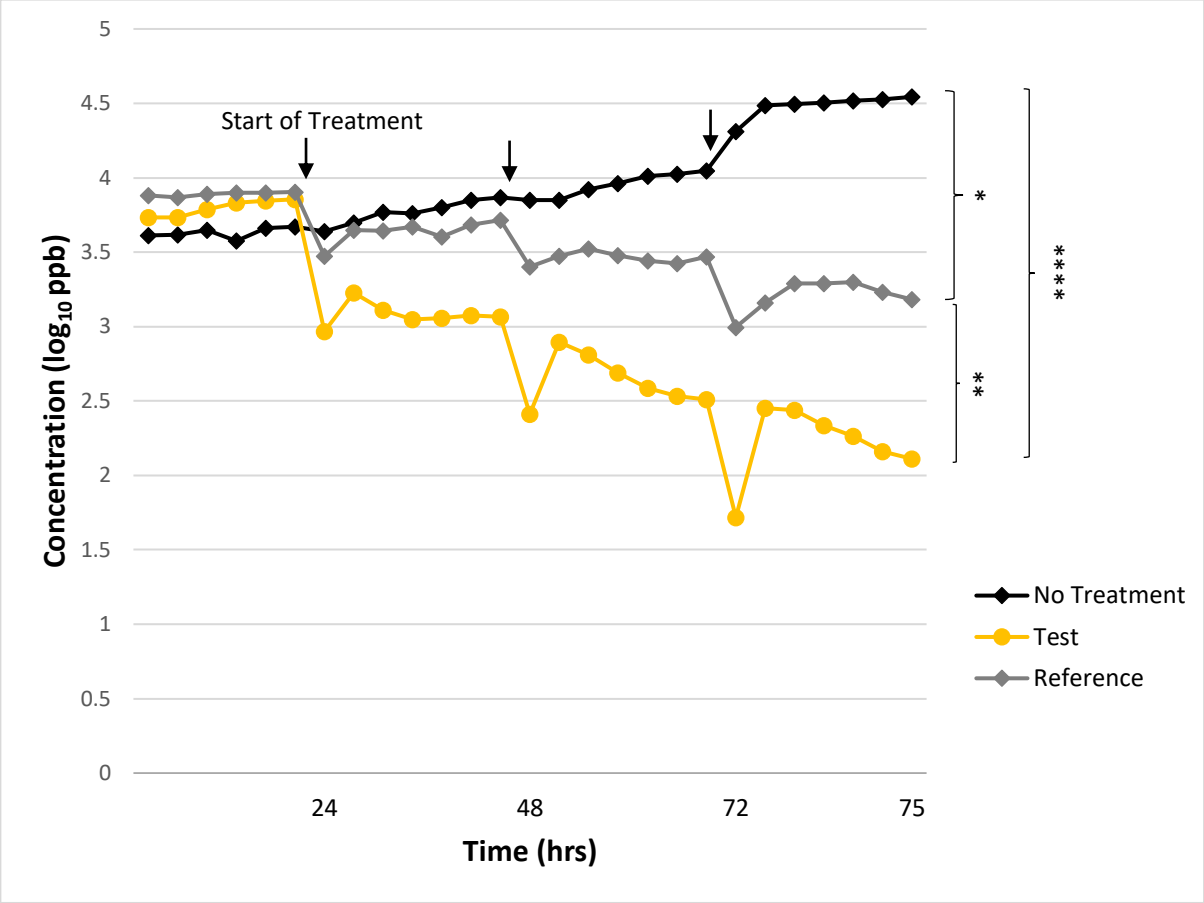


Figure 3.3 Test toothpaste significantly decreases levels of Hydrogen sulphide (H_2S) detected from biofilm headspace after 3-day treatment. Tongue scrape samples were grown in an *in-vitro* biofilm system and exposed to treatment over a 3-day period, biofilm was treated twice a day, in the morning and evening over a 3-day period with 2g of the test toothpaste (5% KNO_3, 0.24% NaF) and 2g of the reference toothpaste (1% CHX). Experiment was repeated thrice independently with triplicates (n=3). Arrows indicate daily treatment points at 8am. Unpaired t-test Statistical significance is indicated as (*) $p \leq 0.05$, (**) $p \leq 0.01$ and (****) $p \leq 0.0001$.

The results presented in Figs. 3.3 and 3.4 demonstrate the effects of the test toothpaste (5% KNO_3 and 0.24% NaF) and the reference toothpaste (1% CHX) on the levels Hydrogen sulphide and Methyl mercaptan respectively in tongue scrape biofilm headspace analysis. Both treatments resulted in a significant reduction in Hydrogen sulphide levels; however, the test toothpaste exhibited the stronger effect with an average decrease of 0.8 log ppb compared to the reference toothpaste ($p<0.01$) and a 1.4 log ppb drop compared to the control ($p<0.0001$). The reference toothpaste also showed a significant reduction in Hydrogen sulphide levels compared to the control with an average decrease of 0.6 log ppb ($p<0.05$). Similarly, both toothpastes caused a significant decrease in Methyl mercaptan levels, with the test toothpaste being the more effective with an average reduction of 0.7 log ppb compared to the reference toothpaste ($p<0.01$). The reference toothpaste also

exhibited a significant reduction in Methyl mercaptan levels with an average decrease of 0.5 log ppb ($p<0.05$). These results demonstrate that both toothpastes have an effect on both gas levels, with the test toothpaste exhibiting a stronger effect than the reference toothpaste on both Hydrogen sulphide and Methyl mercaptan.

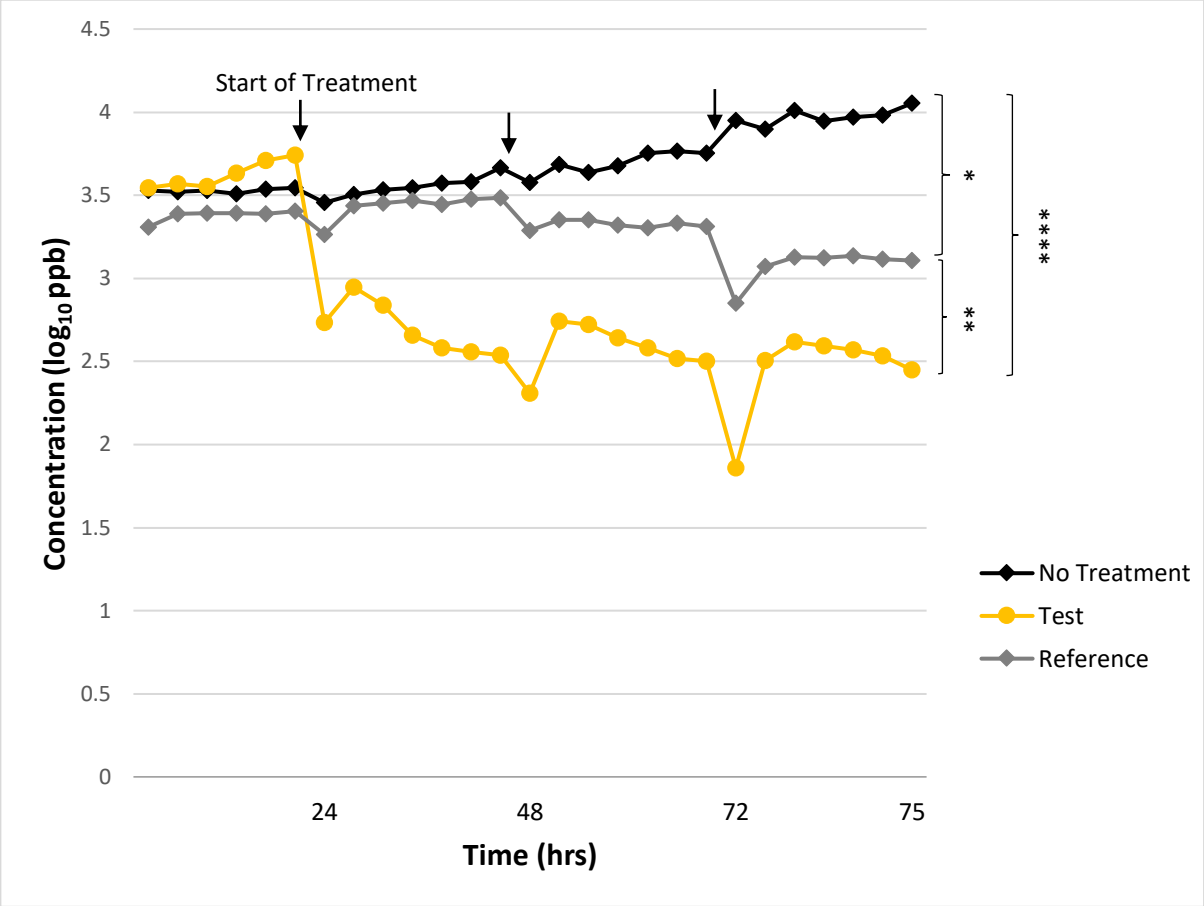


Figure 3.4 The test toothpaste significantly decreases levels of Methyl mercaptan (CH_3SH) detected from biofilm headspace after 3-day treatment. Tongue scrape samples were grown in an *in-vitro* biofilm system and exposed to treatment over a 3-day period, biofilm was treated twice a day, in the morning and evening over a 3-day period with 2g of the test toothpaste (5% KNO_3, 0.24% NaF) and 2g of the reference toothpaste (1% CHX). Experiment was repeated thrice independently with triplicates (n=3). Arrows indicate daily treatment points at 8am. Unpaired t-test Statistical significance is indicated as (*) $p \leq 0.05$, (**) $p \leq 0.01$ and (****) $p \leq 0.0001$.

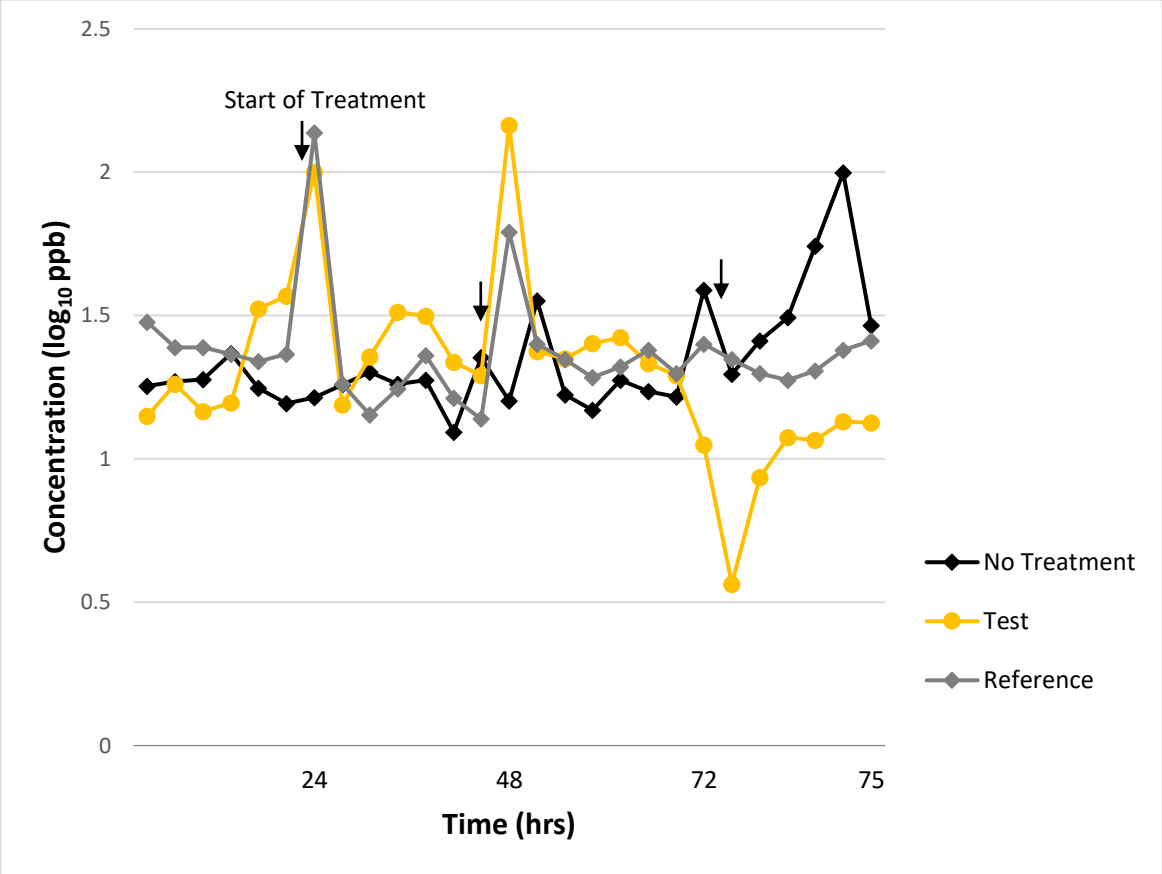


Figure 3.5 No effect from the test toothpaste and the reference toothpaste on levels of Dimethyl disulphide detected from biofilm headspace after 3-day treatment. Tongue scrape samples were grown in an *in-vitro* biofilm system and exposed to treatment over a 3-day period, biofilm was treated twice a day, in the morning and evening over a 3-day period with 2g of the test toothpaste (5% KNO_3, 0.24% NaF) and 2g of the reference toothpaste (1% CHX). Experiment was repeated thrice independently with triplicates (n=3). Arrows indicate daily treatment points at 8am. No statistical significance between all groups as indicated by unpaired t-test.

The results of the study indicated that both the test toothpaste and the reference toothpaste had similar effects on DMDS levels (Figure 3.5) with no significant difference observed between the two treatments ($p=0.258$) or compared to the control (untreated) biofilm ($p=0.367$, $p=0.364$) as determined by unpaired t-test analysis. The readings for the treated biofilm exhibited a degree of volatility, with the DMDS levels fluctuating over the 3-day period. However, when the effect of the treatments on total VSC levels (Hydrogen sulphide + Methyl mercaptan + Dimethyl disulphide) (Figure 3.6) was assessed, the test toothpaste recorded the largest average reduction (0.7 log ppb) compared to the reference toothpaste ($p<0.01$). Although the decrease in total VSC levels observed for the reference toothpaste was lower than that of the test toothpaste, it was still statistically significant, with an average 0.5 log ppb reduction compared to the control ($p<0.05$). These findings suggest that both the test toothpaste and the reference toothpaste have a significant

impact on reducing VSC levels in the headspace of the tongue scrape biofilm, with the test toothpaste demonstrating a greater effect than the reference toothpaste.

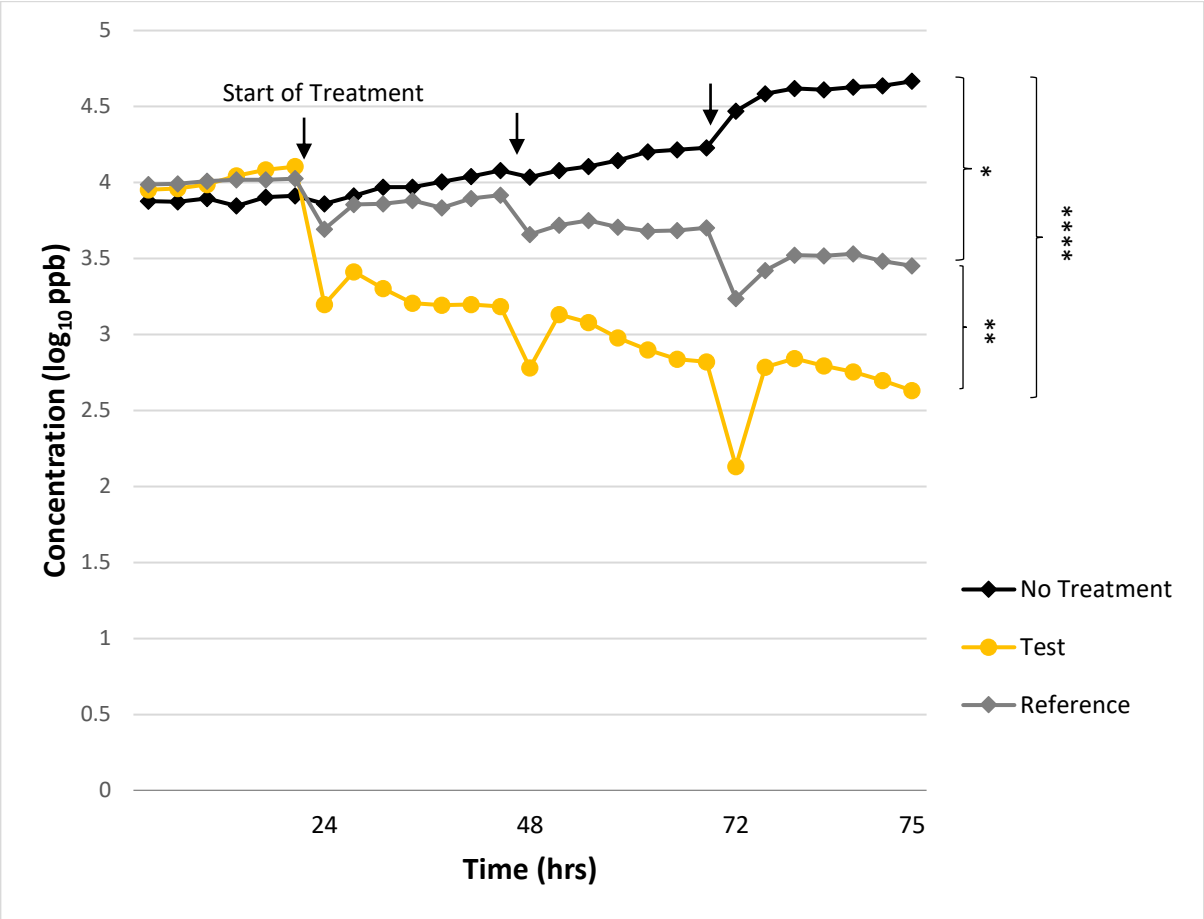


Figure 3.6 Test toothpaste significantly decreases levels of total levels of volatile sulphur compounds (VSC's) (Hydrogen sulphide + Methyl mercaptan + Dimethyl disulphide) detected from biofilm headspace after 3-day treatment. Tongue scrape samples were grown in an *in-vitro* biofilm system and exposed to treatment over a 3-day period, biofilm was treated twice a day, in the morning and evening over a 3-day period with 2g of the test toothpaste (5% KNO_3, 0.24% NaF) and 2g of the reference toothpaste (1% CHX). Experiment was repeated thrice independently with triplicates (n=3). Arrows indicate daily treatment points at 8am. Unpaired t-test Statistical significance is indicated as (*) $p \leq 0.05$, (**) $p \leq 0.01$ and (****) $p \leq 0.0001$.

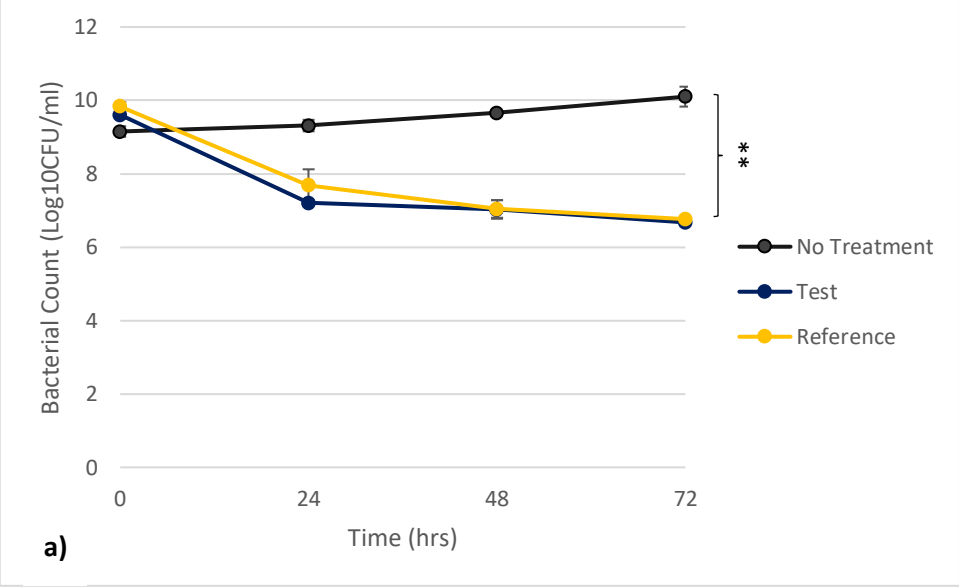
3.3.1.2 Effect of multiple dose treatment on microbiology

Results of the study showed that twice-a-day treatment with the test toothpaste and the reference toothpaste resulted in significant reductions in both facultative anaerobes and strict anaerobes in the biofilm perfusate and sacrificed matrices. The reductions are illustrated in Figures 3.7 and 3.8. Treatment with the test toothpaste resulted in a 2.72 log drop ($p<0.05$) for facultative anaerobes and a 2.76 log drop ($p<0.05$) for strict anaerobes in the perfusate. The reference toothpaste also showed a significant reduction in the perfusate with a 2.53 log drop ($p<0.05$) for facultative anaerobes and a 2.50 log drop ($p<0.05$) for strict

anaerobes. There was no significant difference between the two treatments in reducing either facultative or strict anaerobes (p=0.64 and 0.58 respectively), as determined by unpaired t-test.

On day 4, 12 hours after the final treatment, destructive sampling of biofilms showed high bacterial populations in the control group in the order of 10^{10}. However, the test toothpaste demonstrated a significant reduction in bacterial yield with a 4.02 log drop for facultative anaerobes and a 3.72 log drop for strict anaerobes ($p<0.05$) compared to the control. Similarly, the reference toothpaste was also observed to reduce bacterial yield, with a 3.74 log drop ($p<0.05$) for strict anaerobes but was not as effective as the test toothpaste in reducing facultative anaerobe yield, with a 3.63 log drop ($p<0.05$) compared to the control. No statistical difference was observed between the two treatments on either the facultative anaerobes (p=0.14) or strict anaerobes (p=0.48).

These results suggest that twice-a-day treatment with the test toothpaste and the reference toothpaste is effective in reducing both facultative anaerobes and strict anaerobes in the biofilm perfusate and sacrificed matrices. The test toothpaste demonstrated superior efficacy in reducing bacterial yield compared to the reference toothpaste, especially for facultative anaerobes, while both toothpastes showed similar efficacy in reducing strict anaerobes.



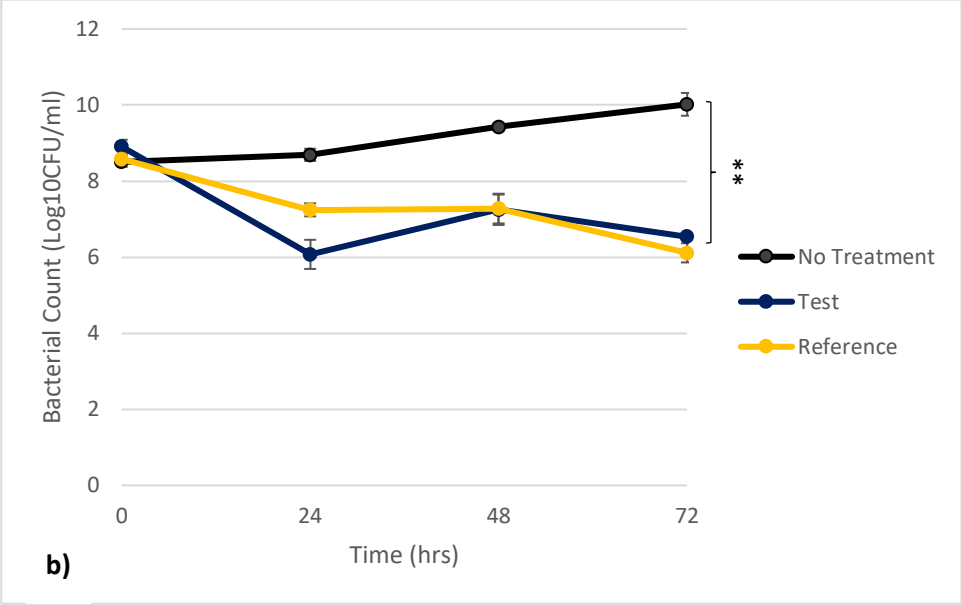


Figure 3.7 Showing average a) total anaerobes and b) strict anaerobes in biofilm perfusate after treatment with oral formulations for 3 days. Tongue scrape samples were grown in an *in-vitro* biofilm system and exposed to treatment over a 3-day period, biofilm was treated twice a day, in the morning and evening with 2g the test toothpaste (5% KNO_3, 0.24% NaF) and 2g the reference toothpaste (1% CHX). Experiment was repeated thrice independently with triplicates (n=3). Results are presented as mean ± 2SE, unpaired t-test statistical significance is indicated as (**) p≤ 0.01.

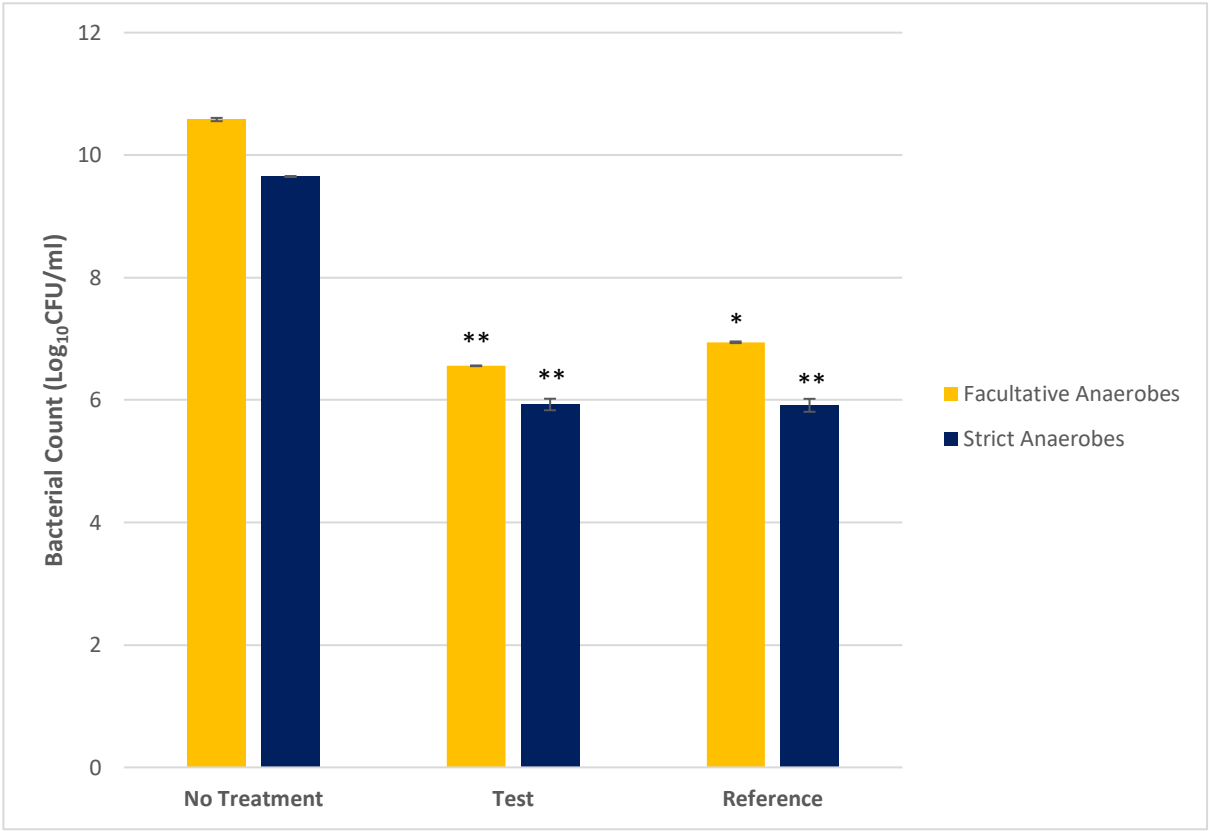


Figure 3.8 Showing facultative anaerobes and strict anaerobes in sacrificed biofilm matrix after exposure to oral formulations for 4 days. Tongue scrape samples were grown in an *in-vitro* biofilm system and exposed to treatment over a 3-day period, biofilm was treated twice a day, in the morning

and evening with 2g the test toothpaste (5% KNO_3, 0.24% NaF) and 2g the reference toothpaste (1% CHX). Biofilm matrices were sacrificed on day 4, 12 hrs after final treatment. Experiment was repeated thrice independently with triplicates (n=3). Results are presented as mean ± 2SE, unpaired t-test statistical significance is indicated as (*) $p \leq 0.05$ and (**) $p \leq 0.01$.

3.3.2 Agar well diffusion assay

Figure 3.9 displays the mean zone of inhibition of two oral formulations, the test toothpaste (5% $KN0_3$, 0.24% NaF) and the reference toothpaste (1% CHX), at different concentrations were compared to a control group with no toothpaste. This experiment was conducted on three different occasions in triplicates.

The results showed that the mean zone of inhibition for the control group was 0 mm ± 0 mm for all concentrations, indicating no antibacterial activity. Moreover, results indicated that the mean zone of inhibition increased with increasing concentration for both oral formulations. For the test toothpaste, the highest mean zone of inhibition was observed at a concentration of 0.05 g/ml (19.4 mm ± 2.2 mm), followed by 0.03 g/ml (11.6 mm ± 2.3 mm) and 0.01 g/ml (9.9 mm ± 1.8 mm). Similarly, for the reference toothpaste, the highest mean zone of inhibition was observed at a concentration of 0.01 g/ml (12.4 mm ± 1.2 mm), followed by 0.005 g/ml (10.9 mm ± 1.3 mm) and 0.003 g/ml (10.0 mm ± 0.7 mm). Furthermore, results from a one-way ANOVA suggested statistically significant differences between both treatments at all concentrations compared to the control ($p < 0.001$). A post-hoc Tukey test was conducted to compare the mean zone of inhibition between the oral formulations at each concentration. Due to the varying starting concentrations between both oral formulations, graphs indicate percentage strength of each oral formulation for clarity; corresponding concentrations to percentage strengths have been listed in table 3-2. The results showed that at 50% strength the mean zone of inhibition for the test toothpaste was significantly higher than that of the reference toothpaste at all concentrations ($p < 0.001$). However, at 25% and 12.5% strengths, there were no significant differences between the two oral formulations.

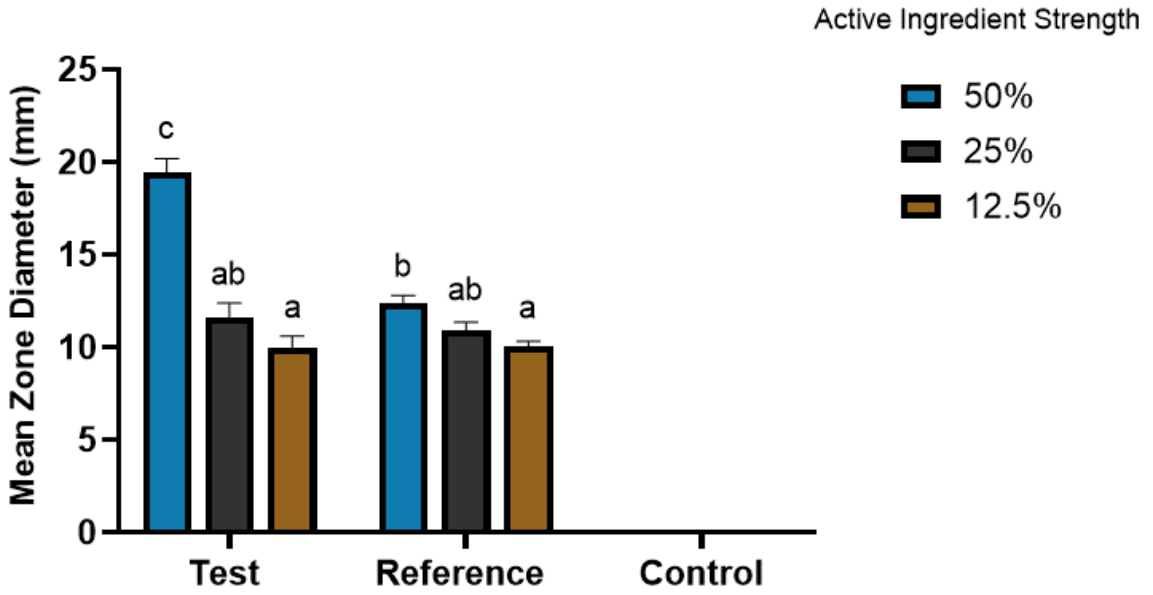


Figure 3.9 Mean zone of inhibition against bacterial cell growth from tongue scrape culture treated with two oral formulations (The test toothpaste - 5% KN03, 0.24% NaF and the reference toothpaste – 1% CHX) on FAA plates. One-way ANOVA and Tukey's post hoc analysis suggest significance between both oral formulations ($p < 0.001$). Groups with the same letter are not significantly different from each other, but all other groups are significantly different from each other at the specified level of significance ($p<0.05$). All groups are significantly different from the control group at $p < 0.001$. Error bars showing ±SE, (n=6). (a vs. b, $p< 0.05$; a vs. c, $p<0.001$; b vs. c $p<0.001$).

3.3.3 Inhibitory and bactericidal activity

As mentioned earlier, the MIC for both toothpastes could not be investigated due to interference with turbidity caused by the toothpaste colour. However, the broth microdilution assay results (Table 3.2) showed that the MBC of the test toothpaste was 0.075%, while the MBC of the reference toothpaste was 0.015%.

Table 3-2 Minimum bactericidal concentrations for test and reference toothpastes. Various concentration tested for MBC of oral formulations on oral bacteria in tongue scrape samples MBC defined as 0.075% for the test toothpaste with active ingredient (5% KNO3, 0.24% NaF) and 0.015% for the reference toothpaste (1% CHX), n=3. *+ visible growth observed *- no visible growth observed.

Oral Formulation	% Strength	Active ingredient Concentration (g/ml)	Observation
Test toothpaste (5% KNO_3 and 0.24% NaF)	50	0.05	-
	25	0.025	-
	12.5	0.0126	-
	6.3	0.0062	-
	3.1	0.003	-
	1.6	0.0016	+

Reference toothpaste (1% CHX)	50	0.01	-
	25	0.005	-
	12.5	0.0026	-
	6.3	0.0012	-
	3.1	0.0006	-
	1.6	0.0004	+
Pure KNO_3 solution	100	5	+
Control	n/a	n/a	+
dH_2O	n/a	n/a	+

3.3.4 Cell leakage assay

3.3.4.1 Action of oral formulation on protein release from tongue scrape bacterial cells

To determine the effect of both oral formulations on leakage of cytoplasmic proteins, the amount of protein released into the supernatant was estimated using the Bradford method. It was found that cells treated with the test toothpaste at 0.3% concentration leaked significantly more protein than controls and cells treated with 0.1% and the reference toothpaste at both concentrations 0.3% and 0.1% ($p < 0.001$) (Fig. 3.11). The large amounts of protein released into the supernatant after 24 hrs incubation of cells in the presence of oral formulations indicates that cell membranes had been disrupted. However, no significant difference in protein leakage were observed for cells treated at 0.1% with the reference toothpaste ($p > 0.05$). Moreover, protein leakage was observed to be significantly greater at the higher concentration of 0.3% than 0.1% for the test toothpaste ($p<0.001$).

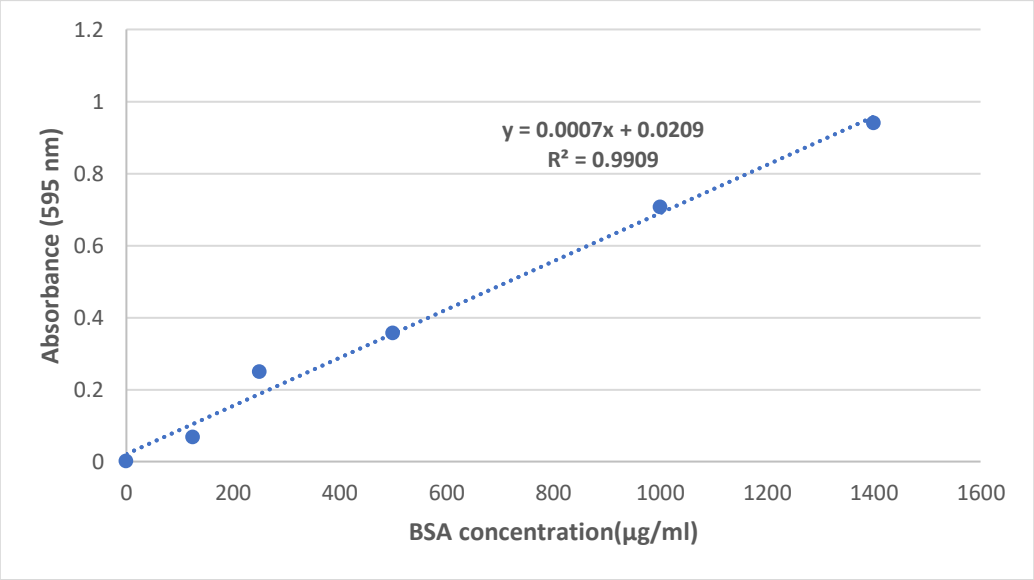


Figure 3.10 Bovine Serum Albumin standard curve for the Bradford Protein Assay. Bovine serum albumin (BSA) was used as a standard and diluted to the following concentrations: 0, 125, 250, 500, 1000

to 1400 µg/ml. Absorbance was measured at 595 nm. Each data point represents an average of three absorbance readings per BSA concentration. The concentration of protein (in µg/ml) was determined using the equation y=0.0007x + 0.0209 with an R^2 value of 0.9909 where y is absorbance and x is concentration.

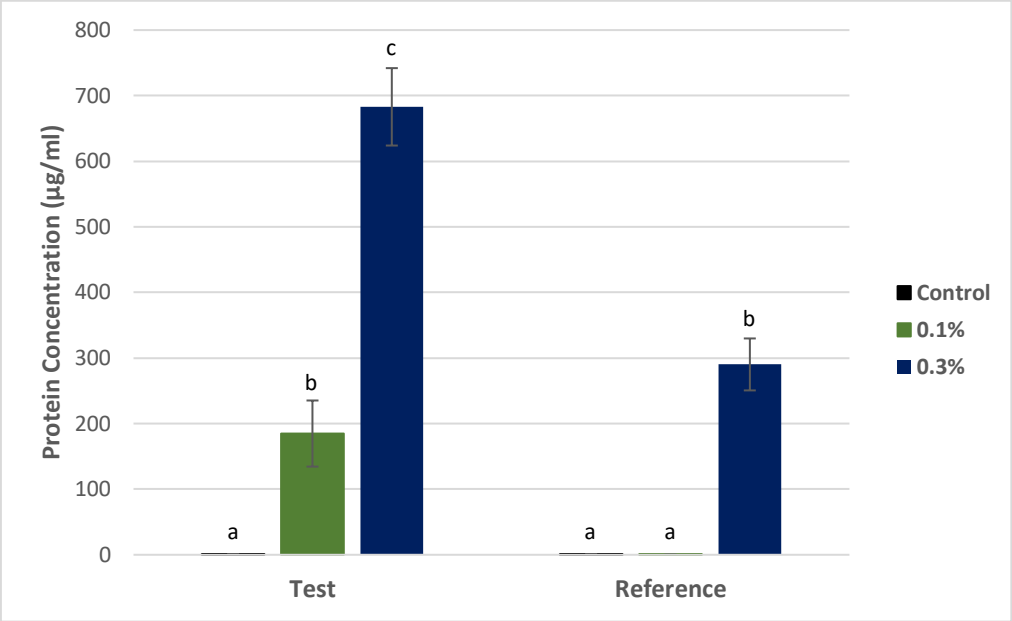


Figure 3.11 Assessment of protein release from bacterial cells in tongue scrape culture following treatment with (i) The test toothpaste at 0.3 and 0.1% (w/v) and (ii) The reference toothpaste at 0.3 and 0.1% (w/v), results are representative of pooled participant data. One-way ANOVA results showed statistical difference between control and treatment groups at p<0.001. Tukey's post hoc analysis indicates that groups with the same letters are not significantly different from each other, but all other groups are significantly different from each other at the specified level of significance. Error bars showing ± 2SE, (n=6). (a vs. b, p< 0.01; a vs. c, p<0.001; b vs. c p<0.001).

3.3.4.2 Action of oral formulation on nucleic acid release from tongue scrape bacterial cells

This study investigated the effect of the test and reference toothpaste on the release of nucleic acid from bacterial cells, which serves as a crucial indicator of bacterial cell lysis and has significant implications for the effectiveness of antimicrobial treatments. Figure 3.12 shows the pooled concentration of nucleic acid released from bacterial cells for all participants after exposure to the test and reference toothpastes at two different concentrations. The study findings revealed that both treatments resulted in nucleic acid leakage at both concentrations. However, at 0.3%, both treatments showed a more significant increase in nucleic acid release (p<0.001), compared to 0.1% (p<0.05). A more detailed effect of the treatments across individual participants is shown in figure 3.13. Study

findings suggest that both toothpastes have a dose-dependent effect on nucleic acid leakage, with higher concentrations resulting in greater leakage.

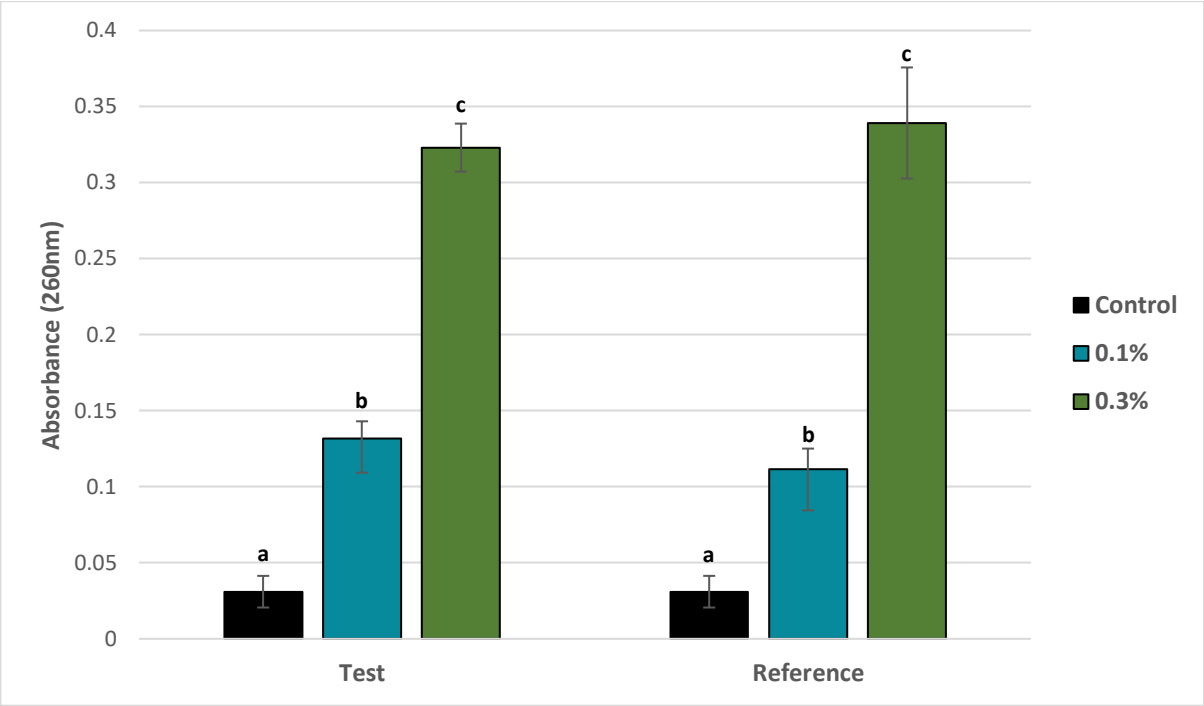


Figure 3.12 Assessment of nucleic acid release from bacterial cells in tongue scrape culture following treatment with (i) the test toothpaste at 0.3 and 0.1% (w/v) and (ii) The reference toothpaste at 0.3 and 0.1% (w/v), results are representative of pooled participant data. One-way ANOVA results showed statistical difference between control and treatment groups at $p<0.001$. Tukey's post hoc analysis indicates that groups with the same letters are not significantly different from each other, but all other groups are significantly different from each other at the specified level of significance ($p<0.05$). Error bars showing ± 2SE, (n=6). (a vs. b, $p< 0.05$; a vs. c, $p<0.001$; b vs. c $p<0.001$).

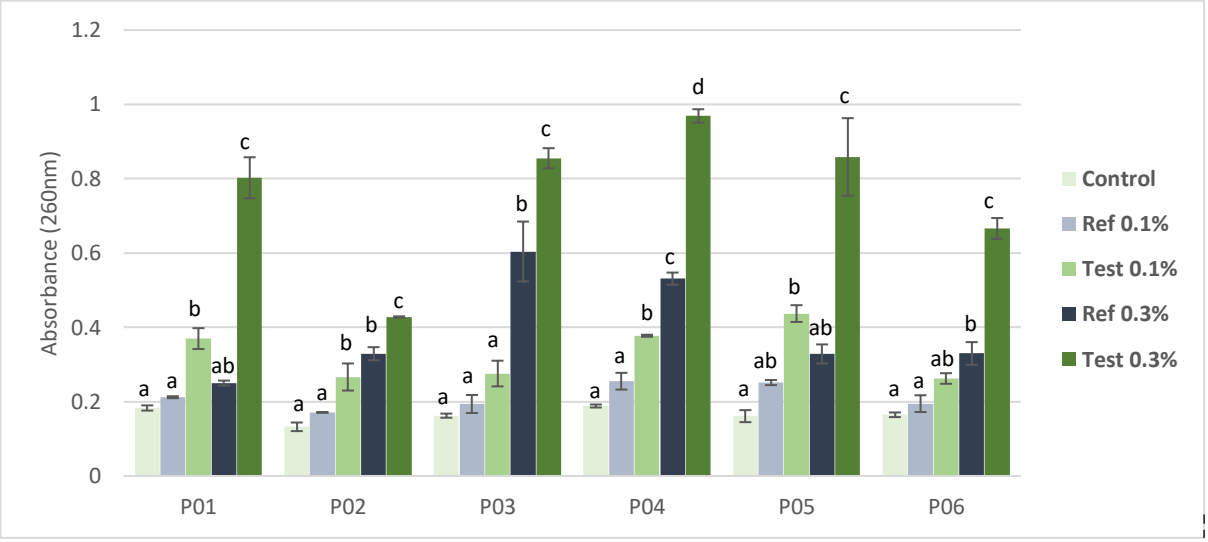


Figure 3.13 Assessment of nucleic acid release from bacterial cells in tongue scrape culture following treatment with (i) the test toothpaste at 0.3 and 0.1% (w/v) and (ii) the reference (ref) toothpaste at 0.3 and 0.1% (w/v), results are representative of individual participant data. Tukey's post hoc analysis

indicates that groups with the same letters are not significantly different from each other, but all other groups are significantly different from each other at the specified level of significance ($p<0.05$). Error bars showing ± 2SE, (n=6).

3.3.5 Scanning electron microscopy

SEM images in figures 3.14, 3.15 and 3.16 were obtained from three biological replicates for each treatment group. Analysis of the SEM images showed that there were significant morphological changes in the cells treated with the test toothpaste and the reference toothpaste compared to the control cells. A larger proportion of the control cells appeared plump and whole with smooth and intact surfaces. Furthermore, cells in the control group were tightly aggregated and clustered together to form intact mature biofilm structure. Moreover, some cells appeared shrivelled, although this was a lower proportion. In contrast, cells treated with the test toothpaste showed disrupted integrity of the biofilm architecture and an increased proportion of shrivelled, wrinkled, and deformed cells with less granular surfaces, which may indicate cell damage and lysis in some cases. Some cells in the test toothpaste group appeared smooth and whole, but the number of shrivelled and wrinkled cells appeared higher compared to the control group. On the other hand, cells treated with the reference toothpaste showed a greater proportion of plump and smooth cells with intact surfaces compared to the control group. Some cells in the reference toothpaste group appeared shrivelled, indicating cell damage, but these cells seemed significantly lower in number compared to test toothpaste group.

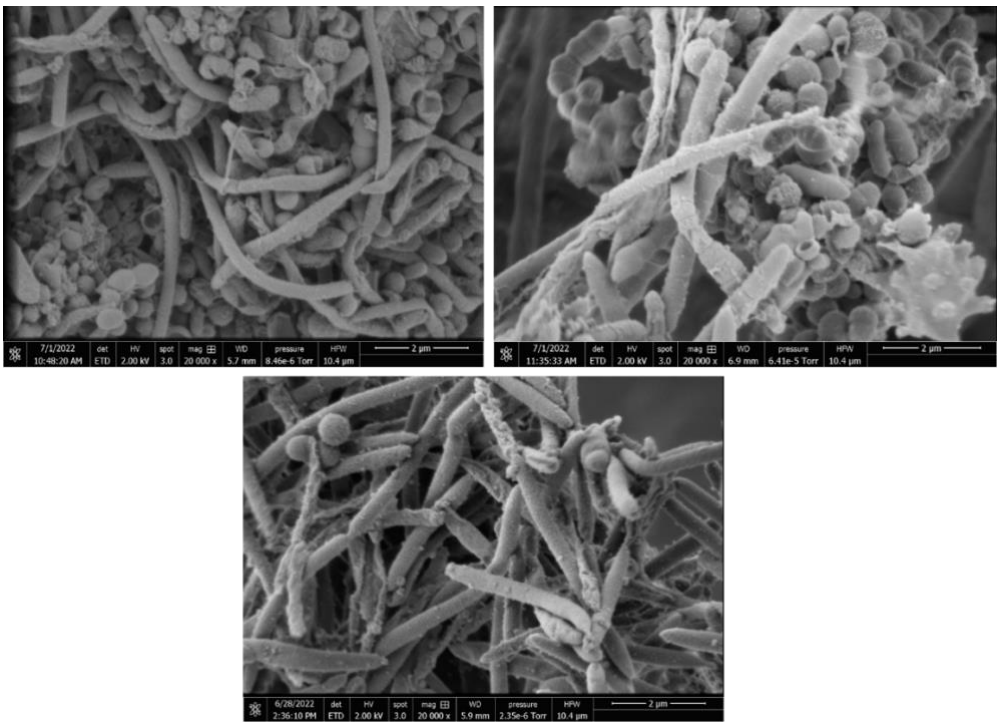


Figure 3.14 Scanning Electron Micrograph (SEM) of 1-week old mixed biofilm from tongue scrapes grown in-vitro, untreated. Most cells look plump and whole with smooth and intact surfaces. Some cells appeared shrivelled but most cells looked plump and smooth (n=3).

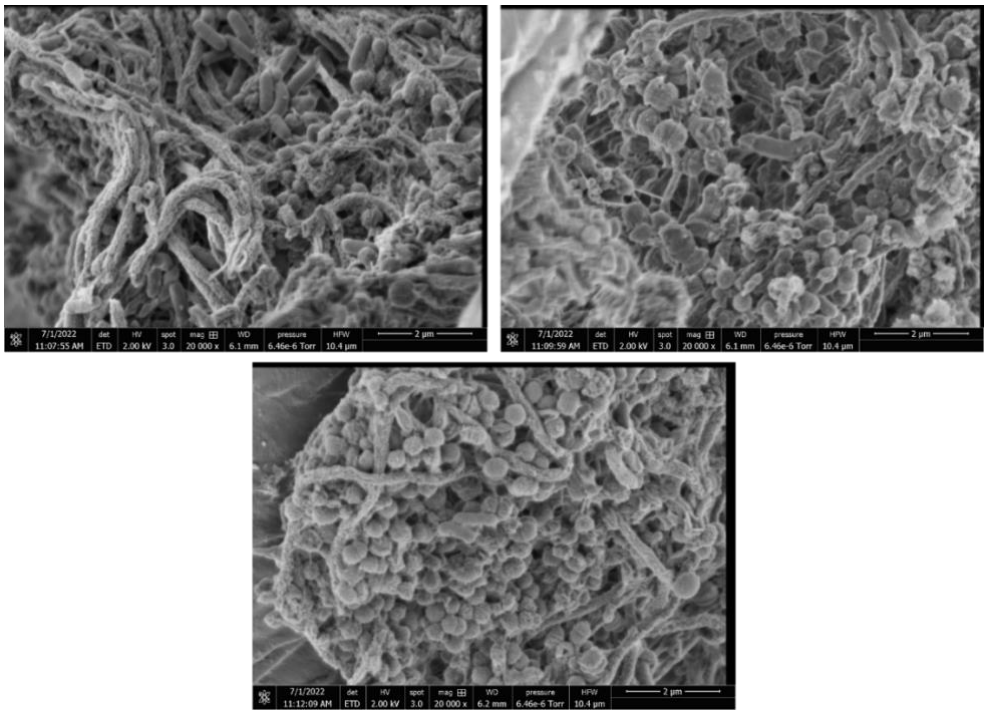


Figure 3.15 Scanning Electron Micrograph (SEM) of 1-week old mixed biofilm from tongue scrapes grown in-vitro, treated with the test toothpaste (5% KN03, 0.24% NaF) for 5 days. Most cells look shrivelled, wrinkled and deformed with less granular surface, indicating cell damage and rupture in some

cases. Some cells appear smooth and whole but there was a greater proportion of shrivelled and wrinkled cells (n=3).

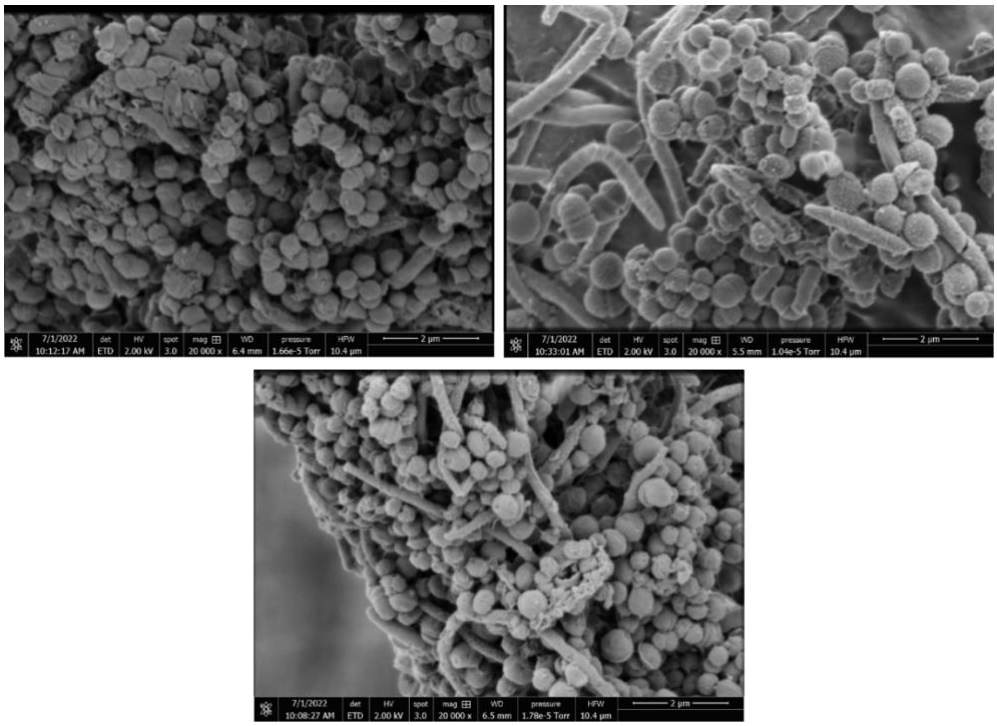


Figure 3.16 Scanning Electron Micrograph (SEM) of 1-week old mixed biofilm from tongue scrapes grown in-vitro, treated with the reference toothpaste (1% CHX) for 5 days. Most cells look plump and whole with smooth and intact surfaces. Some cells appeared shrivelled indicating cell damage but there was a greater proportion of plump and smooth cells (n=3).

3.4 Discussion

Bacterial cell growth and removal of unattached daughter cells in the oral cavity are facilitated by constant nutrient availability and saliva flow (Saad *et al.,* 2013). The balance between cell growth and removal by hydrodynamic shear forces determines the steady state, which is reflected in the growth rate. Previous studies on malodour processes *in-vitro* have used saliva and saliva sediment in closed batch "accumulative" systems, which do not replicate the open system of the mouth, where cells and agent molecules are continually being removed (McNamara *et al*., 1972; Kleinberg and Codipilly, 1997; Goldberg *et al.,* 1997). To address this issue, this study utilized a flat-bed perfusion model to compare the efficacy of toothpaste formulations on mixed flora biofilms derived from human tongue scrapes. This model offers a dynamic state that mimics what happens in the real mouth, where the media continuously perfuses the matrix, feeding the cells and allowing daughter

cells to be eluted from the matrix. To ensure strict replication of biofilm units (n=4), they were constructed to be as similar as possible to each other. However, there were slight variations in the starting levels of VSCs and VOCs, and studies have shown that after 72 hours, biofilms are able to reach a quasi-steady state. This steady state allows for the study of the efficacy of oral formulations designed to combat biofilm activities through bacteriostatic or bactericidal action (Saad *et al.,* 2013; Greenman *et al.,* 2008; Spencer *et al.,* 2007). Although the starting positions of the biofilms in this study varied slightly, the observed differences were not significant enough to interfere with the interpretation of the results.

This study aimed to investigate the efficacy of different oral formulations in inhibiting VSC production and biofilm growth. Multiple interventions were employed to observe the effect of these formulations. It is believed that certain agents may provide rapid inhibition of volatile sulphur compounds (VSCs), while others may inhibit biofilm growth, with a greater degree of inhibition observed after repeated interventions (Saad *et al.,* 2013). Results from the multiple treatment study showed a larger reduction in viable count (4 log drop) for the test toothpaste compared to the reference toothpaste. The test toothpaste also displayed a preferential reduction of strict anaerobes in sacrificed biofilm matrices, and a cumulative reduction in VSC and total volatile organic compound (VOC) production levels over the treatment period. Furthermore, the test toothpaste displayed a marked reduction on total VOC's, total VSC's, Hydrogen sulphide and Methyl mercaptan, compared to the reference toothpaste. Overall, these results suggest that the mode of action for the test toothpaste could be through bactericidal effects on biofilm populations, therefore reducing the enzymes available for biotransformation of substrates to produce the volatile compounds. The control biofilms showed a significant increase in total VOC and VSC concentrations following treatment with a blank disc containing no oral formulation ($p<0.0001$), which supports the theory that control treatments are not significantly effective on biofilms as they may continue to grow and mature (Saad *et al.,* 2012). This constant growth gives an increase in population numbers with concurrent increase in VSC and VOC production levels. For the reference toothpaste, there was also a decrease in VOC and VSC levels although not as significant as the test toothpaste.

Microbiology analysis of treated and untreated biofilms showed that both the test and reference toothpastes gave reduced final cell populations compared to the control biofilm in the order the test toothpaste > the reference toothpaste > control (Fig. 3.7). Results for

the reference toothpaste in this study are consistent with other studies (Srikumar *et al.,* 2022; Saad *et al.,* 2013; Greenman *et al.,* 2008; Carvalho *et al.,* 2004; Ademvoski *et al.,* 2013; Mednes *et al.,* 2015) with a general reduction in VSC levels and bacterial population. Although, a study by Saad *et al.* (2012) demonstrates that CHX has no reduction effect on Methyl mercaptan, it should be noted that a lower concentration (0.2% CHX) was used in that study. While various studies have screened the effect of CHX *in-vitro* using biofilm systems, there are no reported studies using the same systems, particularly the flat-bed matrix model with oral formulations containing 5% KNO_3 and 0.24% NaF. In this study, the density of the tongue biofilm inocula deposited onto the cellulose matrices was $10^7 ml^{-1}$. After perfusion with media, the biofilm density reached a steady state of 10^{10} to 10^{11} prior to treatment. The aerial density of tongue biofilm in previous studies (Saad *et al.,* 2016; Saad *et al.,* 2013; Saad and Greenman, 2008; Washio *et al.,* 2005) has been reported to vary between 10^6 and 10^9 cfu per cm^2 and was shown to correlate with the level of malodour. In essence, a thicker biofilm contains more bio-transforming enzymes that can convert existing substrates into malodourous compounds, indicating the presence of oral malodour caused by microbial activity. Therefore, if a treatment displays antimicrobial effects on a high-density biofilm matrix *in-vitro*, it is reasonable to predict similar results in-*vivo* where the bacterial load on the tongue surface may be 1 log-fold lower.

The study also evaluated the susceptibility of oral bacteria in a tongue-scrape-derived inoculum using agar well diffusion. In this investigation, both treatments were used as well as controls and a pure solution of 5% potassium nitrate. The test toothpaste exhibited a concentration-dependent antibacterial effect, with the strongest effect (evident by the largest zone of inhibition) observed at 50% strength (active ingredient concentration 2.5%) ($p<0.001$). Notably, even at lower concentrations, the test toothpaste demonstrated an antibacterial effect (Fig. 3.8). Previous studies (Andini *et al.,* 2018; Seong *et al.,* 2021; Majou and Christieans, 2017; Hong *et al*., 2016; Pradeep and Sharma, 2010; Salin *et al.,* 2010; Orsini *et al.,* 2010) have primarily investigated the effect of potassium nitrate containing formulations on dentine hypersensitivity as that is what the active ingredient is well known for, with no studies reporting specifically on its antimicrobial properties on oral bacteria in comparison to a positive control such as CHX. Potassium nitrate is a commonly used in toothpaste formulations to reduce tooth sensitivity by blocking pain signals (Hong *et al*., 2016). The result from this study suggests that the antimicrobial effect from the test toothpaste may be due to a combined effect of both potassium nitrate and sodium fluoride

or complexes formed between the potassium nitrate and other compounds in the toothpaste formulation.

In SEM studies, the test toothpaste was found to damage the bacterial envelope, which was evident by the shrinkage of cell surfaces and receding of cytoplasm, ultimately leading to cell lysis (Fig. 3.14). This finding was supported by the significant increase in the level of extracellular nucleic acid and protein material ($p<0.001$) (Fig. 3.10-3.12). Moreover, the different mode of action between the test toothpaste and the reference toothpaste is indicated by these results. Potassium nitrate has been shown to create a hypertonic environment that disrupts the cell membrane of bacteria, causing dehydration and cell death (Francius *et al.,* 2011). Moreover, it is possible that the excipients used in the toothpaste formulation may interact with KNO_3 and NaF to enhance their antibacterial properties. For example, surfactants such as sodium lauryl sulphate (SLS), often used in toothpaste formulations exhibit antimicrobial properties and can disrupt the cell membrane of bacteria by interacting with the lipids in the membrane (Leoty-Okombi *et al.,* 2021). This interaction may facilitate the penetration of KNO_3 and NaF into the cell membrane, thus increasing their efficacy. Additionally, SLS can interact with other ingredients in the formulation to form complexes, which may further damage the cell membrane. The presence of potassium nitrate and sodium fluoride in the formulation may increase the potential for complex formation, leading to more extensive damage to the cell membrane. However, additional research is necessary to fully comprehend the mechanisms of action of this toothpaste formulation.

In the UK, regulations have been established to guarantee the safety of cosmetic and personal care products. The European Union's Cosmetic Products Regulation (EC) No 1223/2009 outlines the standards for the safety and labelling of cosmetic products marketed in the EU, such as toothpaste (Ferreira *et al.,* 2022). This regulation obliges manufacturers to ensure that their products are safe for human use and do not contain any substances that are harmful or potentially harmful to human health when used as directed (Ferreira et al., 2022). Although the oral formulation (containing 5% KNO_3) used in this study has shown promising antimicrobial effects, it is currently unavailable commercially in the UK. The levels of potassium nitrate used in this formulation may be the reason for its unavailability. Nevertheless, this study provides evidence that even at lower concentrations, this formulation exhibits antimicrobial efficacy.

Exploring the impact of ethnic variations on the efficacy of tested formulations presents an intriguing avenue for extending the study's scope. Considering the documented diversity within the oral microbiome across different ethnic groups, it's plausible that such variations could influence individual responses to oral health treatments (Diaz *et al.,* 2021; Ghannoum *et al.,* 2010). The incorporation of sequencing individual samples could unveil the depth of microbial diversity and sensitivity to active ingredients, potentially uncovering distinct microbial profiles that correlate with varying efficacy levels of the formulations tested. Additionally, RNAseq studies could offer insights into gene expression patterns, further elucidating the molecular mechanisms underlying the microbiome's interaction with therapeutic agents (Diaz & Dongari-Bagtzoglou, 2021). This approach not only promises to enhance our understanding of the microbiome's role in oral health but also paves the way for personalized oral care solutions that account for the unique microbial landscape of diverse populations. Such research could significantly contribute to the field of precision oral health, tailoring interventions to achieve optimal outcomes across different ethnicities.

3.5 Conclusions

In summary, this study provides evidence on the potential efficacy of the test toothpaste, which contains 5% potassium nitrate and 0.24% sodium fluoride, as an alternative to Chlorhexidine for the immediate and long-term reduction of VSC and VOC levels in an *in-vitro* flat-bed biofilm model. The test toothpaste may achieve this reduction by controlling the number of biofilm cells responsible for the biotransformation of substrates into odorous gases and interfering with the biotransformation process. Additionally, the study reveals that the test toothpaste can significantly reduce biofilm density, including both facultative and strict anaerobes by disrupting the bacterial cell membrane. It is worth noting that the *in-vitro* perfusion model used in this study could replicate many of the microbial activities and reactions that are believed to be occurring on a real tongue biofilm, indicating that these findings could be predicted to occur *in-vivo*. Overall, this study highlights the potential of the test toothpaste as an effective oral care product for managing halitosis and reducing the risk of periodontal disease. Further research is needed to validate these findings and to investigate the potential of the test toothpaste in clinical settings. Nonetheless, this study's results demonstrate that the test toothpaste has the potential to be a viable alternative to Chlorhexidine for the control of oral malodour and periodontal disease.

Chapter 4 Oral microbiota in White European and Black African populations and its association with risk for Type 2 diabetes: A clinical study

4.1 Introduction

Type 2 diabetes mellitus is a prevalent disease with significant morbidity and possible mortality if left uncontrolled (Ampofo and Boateng, 2020). It is a chronic health condition that has reached alarming rates across the globe with a global prevalence estimated at 9.3% (463 million people) in 2019, rising to 10.2% (578 million) by 2030 and 10.9% (700 million) by 2045 (Saedi *et al.,* 2019). Moreover, periodontitis, a chronic multifactorial inflammatory disease in the underlying supporting tissues surrounding the teeth has also proved a global health burden, being the sixth most prevalent chronic disease among the general population and affecting nearly 750 million people worldwide (Wu *et al.,* 2020). Research suggests an evident bidirectional relationship between both conditions, possibly through mechanisms detailed in chapter 1.7, with periodontitis being a risk factor for worsening glycaemic control and increasing the risk for diabetic complications (Agarwal and Baid, 2023). Obesity and ethnicity are other risk factors that have been associated with type 2 diabetes (Tinajero and Malik, 2021). Moreover, research suggests that type 2 diabetes and obesity manifest differently across ethnic groups, primarily because of differences in patterns of body fat accumulation, which may be driven in part by gene-environment interactions occurring during early development in conjunction with the adoption of unhealthy lifestyles later in life (Tinajero and Malik, 2021). However, it is unclear whether ethnicity acts as an independent risk factor for poor glycaemic control in individuals diagnosed with type 2 diabetes. A clearer understanding of the impact of ethnicity on type 2 diabetes risk may not only guide interventions at the social policy level, but also inform health system leaders and providers on the potential value of developing services tailored to the specific needs of their patients. Moreover, since substantial variations in prevalence, disease progression, and response to treatments have been observed across diverse populations (Premaraj *et al.,* 2020), understanding the influence of ethnicity on oral health and the composition of the oral microbiota can provide valuable insights into the underlying mechanisms contributing to these disparities.

The human oral cavity hosts a complex and diverse microbial community, comprising of bacteria, viruses, fungi, and archaea. These communities of microbes are responsible for various functions that can both maintain and deplete oral health through mechanisms such as modulating the immune response, aiding in digestion, and protecting against pathogenic invaders (Renson *et al.,* 2019). For example, the presence of biofilms containing bacterial species such as *Streptococcus mutans* and other aciduric bacteria can damage hard dental surfaces and lead to dental caries (Wade, 2013; Takahashi and Nyvad, 2011). Furthermore, the oral microbiome is known to play a role in a myriad of systemic diseases and other oral diseases, including oral cancer (Karpiński, 2019), periodontitis (Socransky *et al.,* 1998; Kumar *et al.,* 2003), and gingivitis (Murray *et al.,* 1992; Kolenbrander *et al.,* 2006). In addition to well-established associations between oral and cardiac health (Shungin *et al.,* 2019), recent work has also begun to show that the oral microbiome may play a role in the health of other distal sites within the human body (Said *et al.,* 2014). For example, the enrichment of both *P. gingivalis* and *A. actinomycetemcomitans* has been associated with a higher risk of pancreatic cancer (Fan *et al.,* 2018). Furthermore, several oral bacteria, including *Streptococcus* and Prevotella species, have been found to be in higher relative abundance among individuals with colorectal cancer (Flemer *et al*., 2018). Other than these two cancers, a number of other distal diseases have been associated with oral microbiome composition, including prostate cancer (Porter *et al.,* 2018) and inflammatory bowel disease (Said *et al.,* 2014).

Due to the associations between these diseases and the oral microbiome, its composition has been proposed as a useful biomarker for human health and disease. With this in mind, various studies have attempted to identify core members of the "healthy" oral microbiome (Nearing *et al.,* 2020; The Human Microbiome Project Consortium, 2012; De Filippis *et al.,* 2014; Nasidze *et al.,* 2009; Takeshita *et al.,* 2016; Zaura *et al*., 2009) to aid in disease detection. These studies have uncovered that, at the genus level, the oral microbiome remains relatively stable between individuals (The Human Microbiome Project Consortium, 2012; Zaura *et al.,* 2009) and across multiple geographic locations (Nasidze *et al.,* 2009; Li *et al*., 2014), but at deeper taxonomic resolutions it can be variable (Nearing *et al.,* 2020). Emerging evidence suggests that ethnicity can influence the structure and diversity of the oral microbiota, with distinct microbial profiles observed in individuals of different racial and ethnic backgrounds (Li *et al.,* 2014; Mason *et al.,* 2013; Yang *et al.,* 2019). However, these studies have largely focused on the bacterial diversity in saliva only, with no studies

focusing on the tongue and subgingival plaque which is the preferred habitat by most periodontal pathogens.

The relationship between ethnicity, oral microbiota composition, and type 2 diabetes risk is an area of growing interest and holds substantial clinical implications. Several studies have indicated that the oral microbiome's dysbiosis, characterized by an overabundance of certain pathogenic bacteria, may play a role in the pathogenesis of insulin resistance and chronic inflammation (Chapter 1.4). Considering the potential ethnic-specific differences in oral microbial composition, it becomes critical to examine whether these variations are associated with disparities in diabetes risk and disease progression among different racial and ethnic groups. Understanding the interplay between ethnicity, oral microbial composition, and diabetes risk has significant clinical relevance. Firstly, identifying ethnic-specific variations in the oral microbiota could contribute to the development of targeted prevention and intervention strategies for diabetes risk reduction (Pereira *et al.,* 2021). Personalized approaches to diabetes management, accounting for the individual's ethnic background and oral microbial profile, may enhance treatment outcomes and reduce health disparities (Premaraj *et al.,* 2020). Secondly, the findings of this study can inform healthcare policies aimed at reducing diabetes-related health disparities among diverse populations. Tailored public health programs that address the unique needs of different ethnic groups concerning oral health and diabetes risk can lead to more effective and equitable healthcare delivery (Pereira *et al.,* 2021).

This study originally aimed to investigate the oral community in healthy and diabetic individuals with a direct comparison between the Black Africans in Nigeria and White Europeans in the UK. However, due to the global limitations imposed by the COVID-19 pandemic, all clinical studies were put on hold particularly for oral research and the study was modified to assess the healthy cohort only. This study focused on investigating the oral microbial profile of healthy individuals with direct comparisons between the Black African and White European ethnic groups within the UK population. Moreover, comparisons were made between individuals with a low risk for diabetes. Results from Black African individuals with a high risk for diabetes were presented as preliminary data for future studies. By addressing the complexities of ethnicity in relation to oral health, this study aims to contribute to a comprehensive understanding of the microbiota at baseline between two ethnic groups.

4.2 Materials and Methods

4.2.1 Ethical approval

The protocol for this clinical trial was reviewed and approved by the University of the West of England's ethics committee (ref: HAS.20.03.148). The study was conducted in a manner consistent with the ethics of the 'Declaration of Helsinki'. All participants read the participant information sheet (appendix I.c) provided and gave written consent (appendix I.a) before taking part in the study. The CONSORT statement checklist was used for reporting the clinical investigation (Fig 4.1 & 4.2).

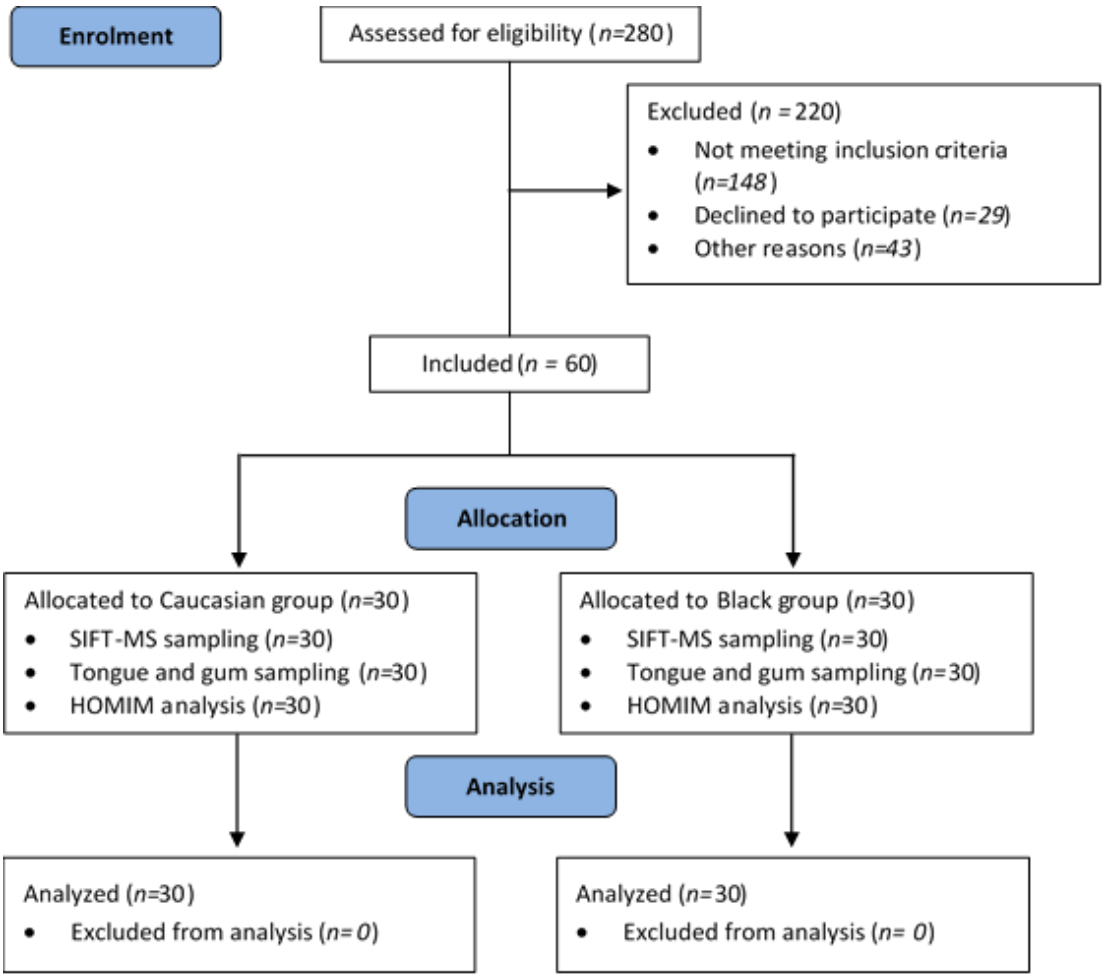


Figure 4.1 CONSORT flow chart of control (low risk) cohort through first phase of the trial.

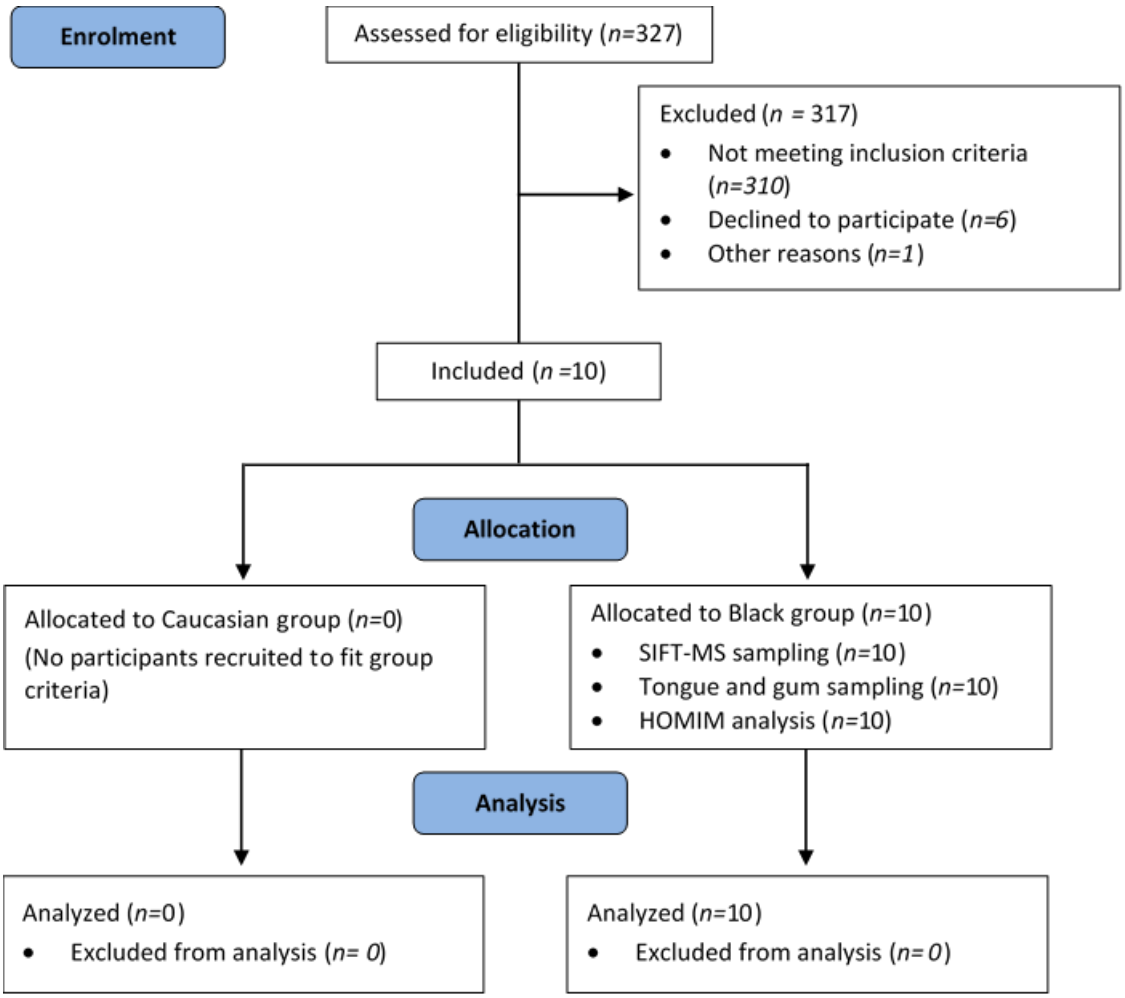


Figure 4.2 CONSORT flow chart of high-risk cohort through second phase of the trial.

4.2.2 Study design and participants

In this case-control study, the term "healthy population" has been defined as individuals without a clinical diagnosis of diabetes, and in good general health. Additionally, prior to the study, all participants were confirmed by their own dentists to have good oral health, with periodontal pocket depths measuring ≤ 3mm. The first phase of the clinical study was carried out on a healthy population of Black African and White European origin at the University of West England (UWE) with all sampling and data collection taking place between November 2022 and February 2023. This involved the recruitment of 60 male and female individuals (30 Black Africans and 30 White Europeans) with mean age 22.1 ± 5.7 who were screened for their risk of diabetes using a tool developed in 2022 by Diabetes UK, the University of Leicester and the University Hospital of Leicester NHS Trust. This tool, designed for individuals without a diabetes diagnosis used factors including gender, age, ethnicity, family history, waist measurement, weight, height, body mass index (BMI) and blood pressure to highlight a person's risk of developing type 2 diabetes in the next 10 years

(Diabetes UK, 2022). On the basis of this screening, a health questionnaire was completed to determine any exclusion criteria (Table 4-1). Additionally, the variable parameters outlined above were carefully measured by the principal investigator in the laboratory before participants were included in the study.

Table 4-1 Eligibility criteria for participants in clinical study.

Inclusion criteria	Exclusion criteria
• Voluntary participation confirmed by consent form. • Availability during specified times to comply with sampling intervals. • Diabetes risk score of 7 or less. • Aged between 18 to 65 years. • At least 20 remaining permanent teeth. • Good oral hygiene and good dental health.	• Recent medical history of infectious disease, severe caries, gingivitis or periodontitis. • Antibiotic medication within 1 month of the trial start date including medicated sweets containing antimicrobial agents. • Pregnant or nursing women. • Substantial false dentition. • Use of highly perfumed cosmetics at time of sampling. • Diagnosis of diseases including diabetes, bronchitis, tonsillitis, sinusitis or other conditions that may contribute to oral malodor.

All subjects were to refrain from smoking 48 hrs before sampling. Participants continued with their normal oral hygiene until the evening before the test, but were asked to abstain from it, and from food intake, in the morning of the test day. Consumption of foods associated with oral malodour (such as garlic, onions or alcohol) on the day prior to, and on the sampling day and using strongly perfumed cosmetics on the sampling day was not allowed. The execution and design of this clinical investigation followed the guidelines proposed by Seemann *et al.,* (2014). The second phase, conducted between March and April 2023, included a healthy population of 10 male and female Black African participants with mean age 28.9. ± 5.8 from the University of the West of England. The same inclusion and exclusion criteria were applied as in the first phase of the study, with the additional inclusion criteria of a diabetes risk score greater than 15 (high risk). Table 4-3 outlines the summary of characteristics for all participants included in the clinical study.

4.2.3 Clinical procedures

The first phase involving the healthy control cohort had 102 participants recruited, with 60 completing the investigation while the second involved the recruitment of 18 participants with 10 completing the investigation. All participants were assigned a participant identification number in chronological order and were provided with their information letter, protocol, diary and appointment dates/times for attending the laboratory through email. On sampling day, SIFT-MS measurement of volatile organic compounds was taken from participants mouths. Tongue scrape and subgingival plaque samples were also collected from participants using a sterile toothbrush and sterile cocktail stick respectively. These methods are detailed below.

4.2.4.1 Instrumental analysis of volatile organic compounds using SIFT-MS

Participants had their oral mouth air measured using a Profile 3 SIFT-MS**,** a mass spectrometry instrument that measures volatile organic compounds (VCs) and permanent gases in air. The SIFT-MS was used in selected ion mode with the compounds investigated listed in Table 4-2. Furthermore, participants were instructed to close their mouth for two minutes and breathe through their nose prior to sampling by the instrument. During this period, approximately one minute of anaerobic gas (10% Carbon Dioxide/10% Hydrogen/Nitrogen mix) was sampled to flush the machine. After two minutes, the participants were instructed to place a clean sampling straw connected to the instrument into their mouth, to place their teeth and lips gently against the straw, without compressing or restricting it, so that the tip of the straw was situated over the back of their tongue without touching the inside of their mouth. The participants were also instructed to keep the muscles around the mouth relaxed and breathe gently through their nose during the sampling. Ninety seconds of data was recorded, and one minute of anaerobic gas (10% Carbon Dioxide/10% Hydrogen/Nitrogen mix) was sampled to flush the machine again. Measurements were taken in triplicates and resultant data were saved and then analysed using the SIFT-MS profile viewer software as well as SPSS software.

Table 4-2 List of compounds measured using SIFT-MS.

Sulphur Compounds	Acids	Amines	Others
Dimethyl disulphide	Butanoic acid	1,4-diaminobutane	3-methylindole
Dimethyl sulphide	Hexanoic acid	1,5-diaminopentane	Indole
Hydrogen sulphide	Propionic acid	Trimethylamine	
Methyl mercaptan			

4.2.4.2 Tongue scrape and subgingival plaque collection

Each participant provided tongue scrape and subgingival plaque samples which were collected under aseptic conditions. Tongue scrape samples were taken using a sterile soft toothbrush with a 1 cm^2 flat bristle field applied with gentle oscillations on the dorsum of the tongue, 7 cm from the tip. Meanwhile, subgingival plaque samples were collected using sterile cocktail sticks from the periodontal pockets of the upper right and left quadrant, an average of 6 teeth (teeth #s 1–3 UL & 1-3UR), were sampled for each participant. The samples were each re-suspended in 10 ml of phosphate buffered saline, vortexed for 2 minutes then serially diluted and plated with a spiral plater (Don Whitley, West Yorkshire, UK). Dilutions were plated on fastidious anaerobe agar (FAA) (LabM, Bury, UK) supplemented with 5% defibrinated horse blood (TCS Biologicals, Bucks, UK) for isolation of anaerobes, and supplemented with vancomycin (2.5 mg L^{-1}) for isolation of strict anaerobes. Plates were incubated anaerobically (Don Whitley, West Yorkshire, UK) with 10% CO_2, 10% H_2 and 80% N_2 at 37°C degrees for 10 days. Furthermore, for both tongue scrape and subgingival plaque samples collected, 2ml of each vortexed sample was transferred to Eppendorf tubes and centrifuged at 3000rpm (Allegra X-30R, SX4400 rotor) for 15 minutes. The supernatant was then removed, and pellets were stored in a -80°C freezer. After completion of the samples by all participants, all frozen samples were shipped to Biomarker Technologies, Germany for Human Oral Microbe Identification Microarray (HOMIM) analysis using 16S rRNA sequencing.

4.2.4 HOMINGS analysis

DNA extraction and 16S rRNA sequencing was carried out by Biomarker Technologies (BMK Gene), Münster, Germany. In summary, for library construction, total DNA was extracted from the samples using the Quick-DNA™ Miniprep Kit (Zymo Research, US), and specific degenerate primers (F: AGRGTTTGATYNTGGCTCAG; R: TASGGHTACCTTGTTASGACTT) targeting full-length amplicons were designed. Degenerative bases (R, Y, N, H and S) used in the primer sequences represent positions in a DNA or RNA sequence where there is more than one possible nucleotide present. PCR was then employed to amplify the target sequences, followed by purification, quantification, and homogenization to create single molecule real time (SMRT) bell libraries. Library quality control (QC) was performed, and only qualified libraries were selected for sequencing on the PacBio Sequel II platform. The

initial bam files generated by PacBio Sequel II were subsequently converted into Circular Consensus Sequencing (CCS) files using SMRT Link (version 8.0) setting a minimum of 5 passes and a minimum predicted accuracy of 99%. CCS sequencing involves circularizing DNA fragments and repeatedly sequencing the same DNA molecule multiple times to generate a consensus sequence (Wenger *et al.,* 2019). This process helps reduce sequencing errors and improve the quality of the resulting sequences.

CCS file processing for the participant samples involved several steps. Firstly, reads from different samples were distinguished by barcode sequences using software Lima v1.7.0 with default setting, resulting in raw-CCS sequences. To confirm the high quality of CCS reads independently, error rates were measured through read-to-read alignments and software cutadapt v1.9.1, was utilized to identify and remove forward and reverse primers with error rate of 20%. CCS reads without primer sequences were also removed and the remaining CCS reads were processed for length-based filtration to produce clean-CCS sequences. The next step involved de-noising and chimeric removal to generate non-chimeric CCS sequences. The high-quality CCS reads obtained after removing chimeras were then utilized for subsequent analyses. Amplicon Sequence Variant (ASV) analysis was generated using dada2 (Callahan *et al.,* 2016) in software QIIME2 (Bolyen *et al.,* 2019) while Operational Taxonomic Unit (OTU) clustering was performed using USEARCH v10.0 with a similarity threshold of over 97% (Edgar, 2013).

Species annotation was conducted using databases including Silva, Unite, Greengenes, NCBI, Fungene, and MaarjAM. For species annotation, two methods were employed (i) The blast-based method for species annotation which utilizes the classify-consensus-blast feature in QIIME2 to compare a query DNA or protein sequence with a reference database, identifying regions of similarity and determining the species by selecting the closest matching sequences. (ii) The Naive Bayes classifier-based approach which uses the classify-sklearn feature in QIIME2 and employs machine learning and Bayesian probability, learning patterns from a labelled dataset to predict species annotations for unlabelled sequences by recognizing species-associated features.

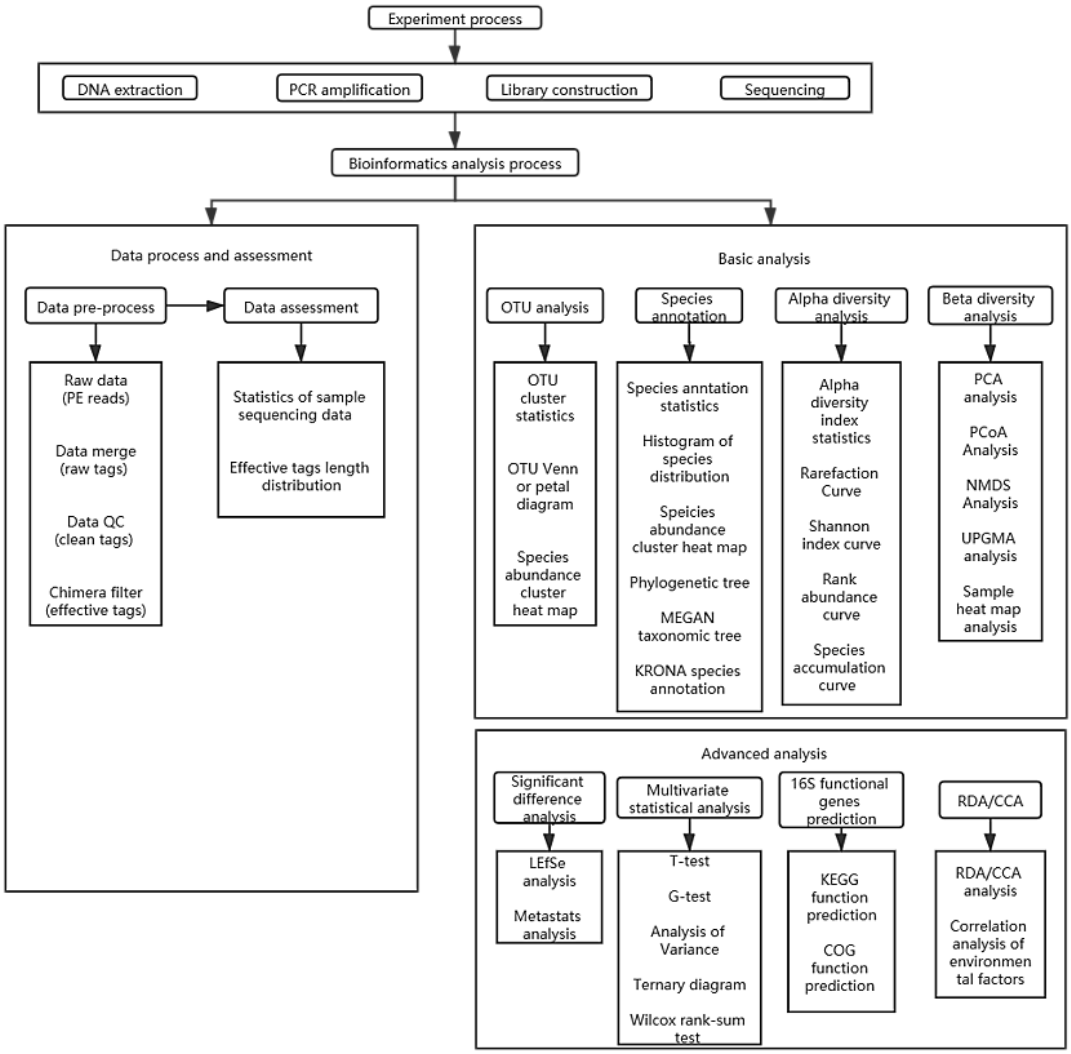


Figure 4.3 Bioinformatics analysis workflow (BMK Gene, 2023).

4.2.5 Sequencing data statistical analysis

Alpha diversity analyses were conducted using software QIIME to assess richness and diversity within and between samples. This involved quantifying species abundance and creating distribution histograms at various taxonomic levels with R packages. Metrics such as Chao1, abundance-based coverage estimator (ACE), Shannon, Simpson, and Phylogenetic diversity (PD) whole tree were employed to measure microbial alpha diversity. Statistical comparisons within and between groups were made using the student's t-test.

Beta diversity analysis was also performed using software QIIME, to explore microbiota variation between the two ethnic groups. Principal Coordinates Analysis (PCoA) and Non-Metric Multi-Dimensional Scaling (NMDS) were used for visualization. A distance matrix of unweighted UniFrac among samples obtained before was transformed to a new set of

orthogonal axes, the maximum variation factor is demonstrated by first principal coordinate, and the second maximum one by the second principal coordinate. Both PCoA and NMDS were generate in the R software. A one-way analysis of similarity (ANOSIM) and Adonis test was performed to determine the differences in bacterial communities among groups.

To assess the significant differences in relative abundance across various taxa between the two groups, the Wilcoxson rank sum test was employed. Additionally, the Linear Discriminant Analysis Effect Size Analysis (LEfSe) utilized the non-parametric factorial Kruskal–Wallis (KW) sum-rank test, in combination with logarithmic linear discriminate analysis using a threshold of 4.0 to identify significantly different features within the groups. Functional capabilities of the oral microbiota were predicted using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) software. By variance analysis of Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathways, the differences and changes of metabolic pathways of functional genes in microbiota between the samples of different groups could be observed. Comparison of the percentages of functional gene content between groups was performed by G-test, adjusted for multiple comparisons.

4.2.6 SIFT-MS and microbiology statistical analysis.

SPSS software was used to log transform, plot and statistically analyse the data using a Mann-Whitney test to compare the level of volatile organic compounds detected in the mouth air between the Black African and White European group. The same test was used to compare the subgingival plaque and tongue bacterial density between the two ethnic groups.

4.3 Results

4.3.1 Characteristics of the study participants

Table 4-3 presents an overview of the participants included in the current study. The low-risk group comprised a total of 60 individuals, with 30 participants from the Black African ethnic group and 30 participants from the White European ethnic group. On the other hand, the high-risk group included 10 participants, all belonging to the Black African ethnic group.

All study participants were classified as "healthy" since they had not been diagnosed with type 2 diabetes and demonstrated overall periodontal and general health. In detail, participants in the low-risk group were further categorized based on their scores on the Diabetes UK risk tool calculator. For the Black African group, individuals scoring below 7 points (6 points for females and 7 points for males) were classified as low risk. Similarly, for the White European group, individuals scoring below 1 point (0 points for females and 1 point for males) were considered low risk.

It is important to note that data from the high-risk group participants were included in this study primarily as preliminary data for future research. Despite the uneven sample sizes between the groups, direct comparisons to the low-risk Black African group were made to identify any unique changes in the microbial community.

Table 4-3 Summary of participant characteristics by group.

Characteristics	Low risk group	High risk group
Ethnicity (n)		
White European	30	-
Black African	30	10
Sex (n)		
Female	35 (12 **B**; 23 **C**)	2 (**B**)
Male	25 (18 **B**; 7 **C**)	8 (**B**)
Age (y) mean ± SD	22.1 ± 5.7	28.9. ± 5.8
Average *BMI (kg/m^2, mean ± SD)	23.1 ± 2.7	32.4 ± 4.8

* BMI: Body Mass Index; B: Black African; C: White European

4.3.2 Headspace analysis of volatile organic compounds using SIFT-MS

Figure 4.4 below shows the concentration levels of total volatile organic compounds (VOCs) and total volatile sulphur compounds (VSCs) between healthy individuals from different ethnic backgrounds. To further dissect the interethnic differences, Figures 4.5 and 4.6 show comprehensive comparisons of various volatile organic compound groups and specific volatile sulphur compounds known to be associated with periodontal disease.

Generally, the level of total volatile organic compounds (Hydrogen sulphide, Methyl mercaptan and Dimethyl disulphide) in participants from both ethnic groups were low with an average of 150 parts per billion (ppb) in the White European group and 190 parts per billion (ppb) in the Black African group. The findings from Mann-Whitney analysis reveal that there were no statistically significant differences in the concentrations of total volatile sulphur compounds between participants belonging to the two distinct ethnic groups. However, a noteworthy distinction ($p < 0.05$) emerged in the concentrations of total volatile organic compounds, particularly in the category of acids, when comparing the two ethnic groups (Figure 4.6). Mann-Whitney results suggest that the difference is attributed to higher acid levels among participants in the Black African ethnic group. No significant differences were identified in the amine and indole groups when comparing participants from both ethnic backgrounds (Figure 4.6).

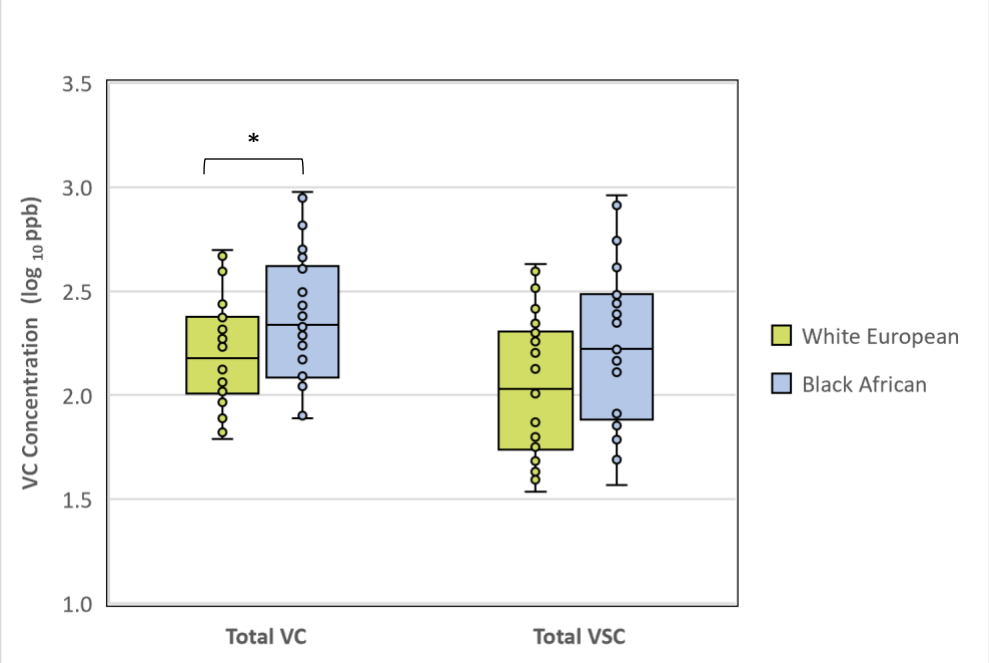


Figure 4.4 Box and Whisker plot of total volatile organic compounds (VOC) and total volatile sulphur compounds (VSC) concentration in healthy participants from White European ethnic group (n=30) and Black African ethnic group (n=30). Mann Whitney test showed significance at * $p < 0.05$.

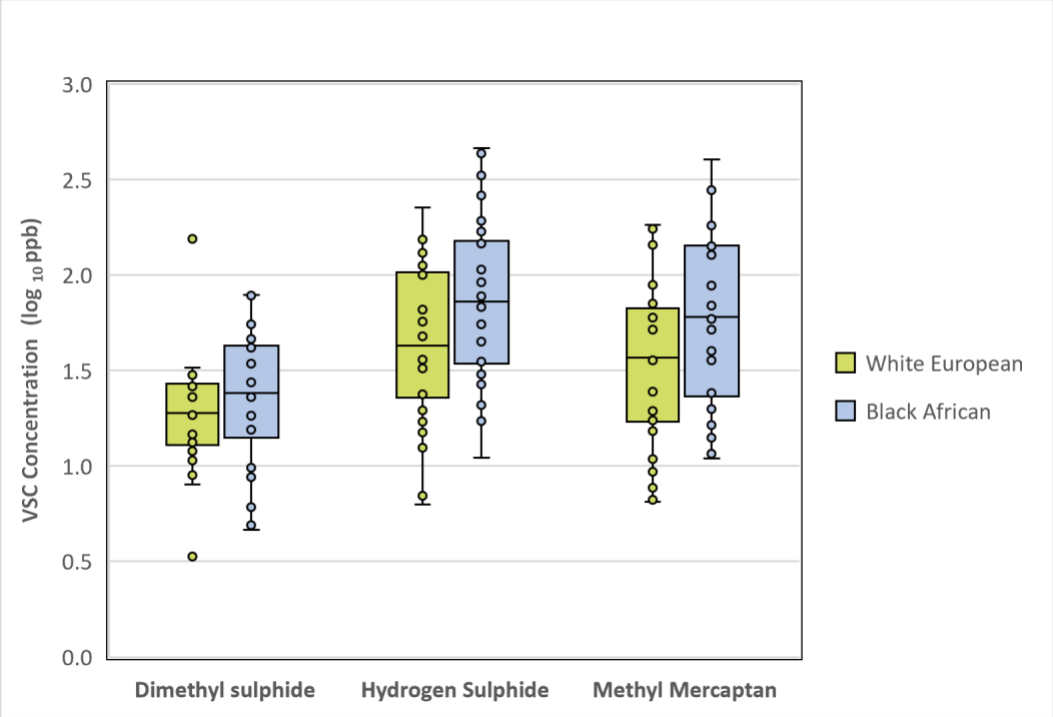


Figure 4.5 Box and Whisker plot of VSC concentration in healthy participants from White European ethnic group (n=30) and Black African ethnic group (n=30). Mann Whitney test showed no significance at $p > 0.05$.

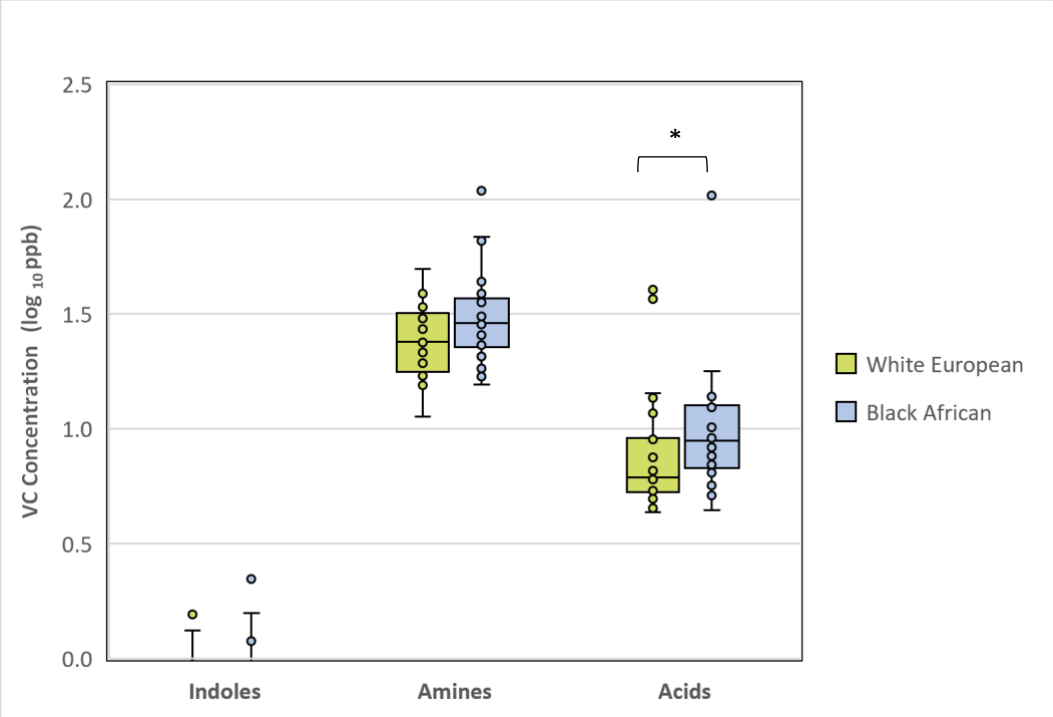


Figure 4.6 Box and Whisker plot of different volatile organic compound groups in healthy participants from White European ethnic group (n=30) and Black African ethnic group (n=30). Mann Whitney test showed significance at * $p < 0.05$.

4.3.3 Microbiological analysis of tongue and subgingival plaque samples in different ethnic groups

Figure 4.7 provides an overview of the tongue and subgingival plaque microbial compositions in healthy individuals from two distinct ethnic backgrounds. The results from Mann-Whitney analysis revealed no significant difference between the ethnic groups concerning the composition of facultative anaerobes in both tongue and subgingival plaque samples. However, a notable difference was observed in the total tongue microbial composition ($p < 0.05$) between the two ethnic cohorts, particularly in the category of strict anaerobes ($p < 0.01$). The White European group exhibited a higher prevalence of strict anaerobes in their tongue microbial composition, compared to the Black African group. In contrast, no significant differences were observed in subgingival plaque bacterial composition between the two ethnic groups.

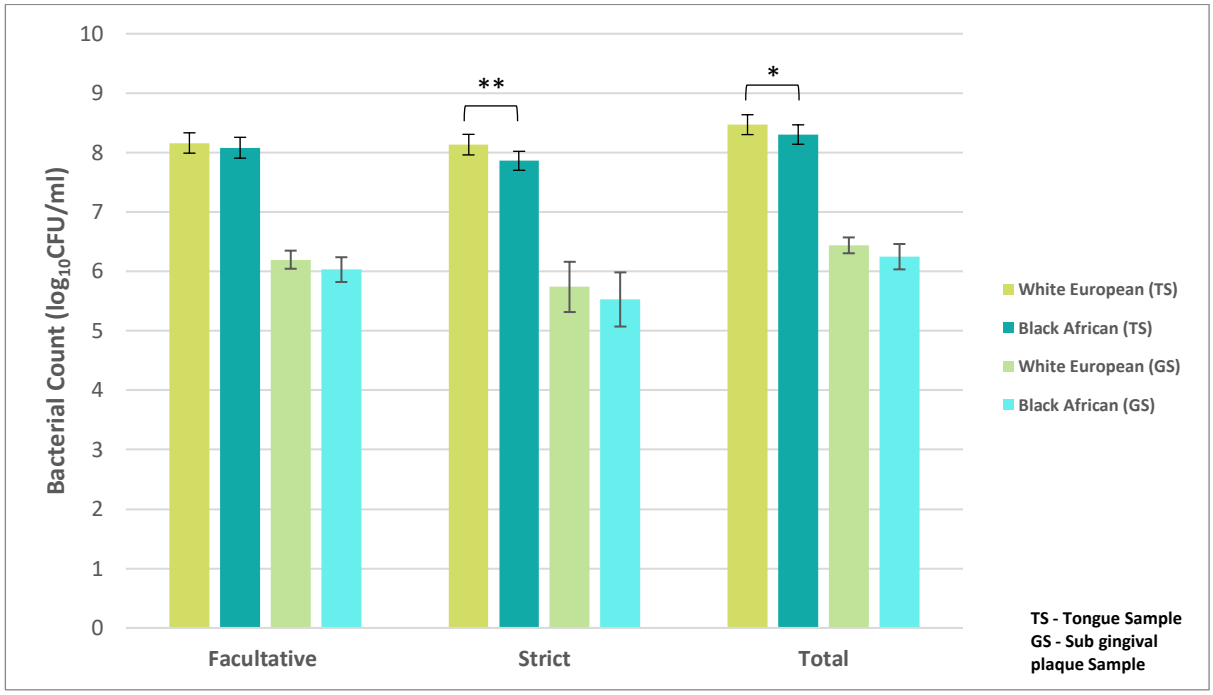


Figure 4.7 Total viable count recovery from tongue and subgingival plaque scrape samples of healthy participants from White European (n=30) and Black African ethnic backgrounds (n=30). Mann Whitney test showed significance at * $P < 0.05$ and ** $P < 0.01$. Error bars show +/- 2SE

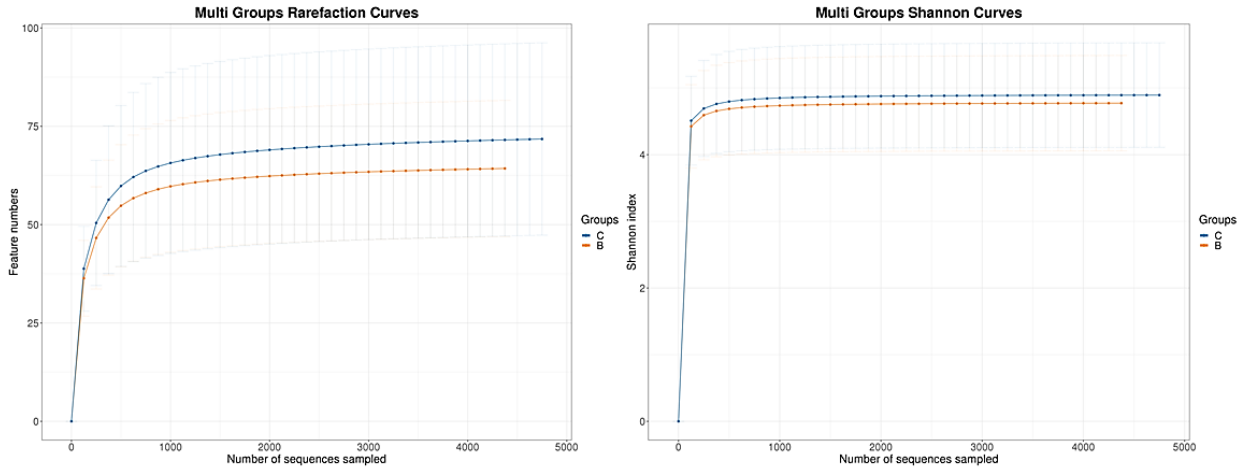
4.3.4 Overall sequencing data and microbial profile

The V1-V9 region of 16S rRNA genes sequencing yielded over 1 million CCS reads in total, with the average number of sequences for each individual sample being 7290 (minimum: 5028; maximum 8,477). All sequences were clustered with representative sequences, and a 97% sequence identity cut-off was used.

4.3.5 Overall oral microbial diversity between the low risk Black African and White European groups.

The adequacy of sequencing data to reflect species diversity in the samples was assessed using rarefaction and Shannon curves. In the rarefaction curve, an abrupt increase indicates the discovery of numerous species as sequence counts rise within a specified range. Conversely, when the curve levels off, it signifies that further sequencing will not significantly augment the species count. Likewise, a higher Shannon index corresponds to a greater number of observed OTU species and increased species richness, implying that most microbial species information is captured in the samples. A plateauing curve suggests that sequencing data suffices, and OTU species do not increase with additional sequencing data. Both the rarefaction and Shannon curves below (Figs. 4.8A and 4.8B) indicate that most of the microbial diversity has been captured in the subgingival plaque samples. Although, rarefaction curves for the tongue samples suggest that deeper sequencing may have revealed additional and rare OTUs.

A



B

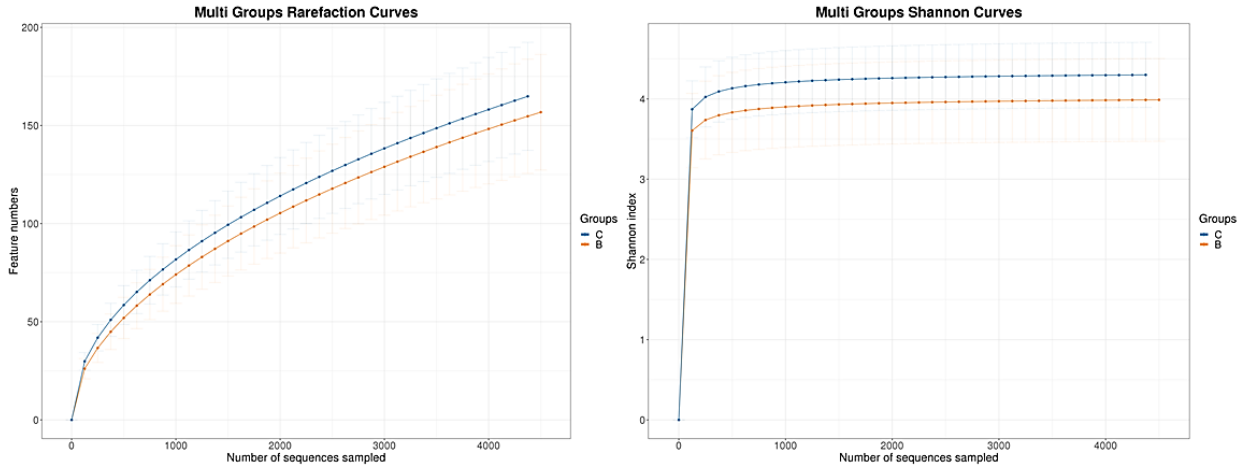


Figure 4.8 Rarefaction and Shannon curves for calculated OTUs in low-risk group. (A) represents subgingival plaque samples and **(B)** represents tongue samples. The curves represent the observed

number of species in the two groups at various sequencing depths. The abscissa represents the number of sequences extracted by resampling, and the ordinate indicates the diversity value or the average number of OTUs per sample in each group (B: Black African group; C: White European group).

These curves depict a slightly higher diversity in samples from the White European group compared to the Black African group. To confirm this, the Chao1 indices were calculated and subjected to a student's t-test (as shown in Figs. 4.10 and 4.11). Interestingly, the analysis found no statistically significant difference in mean microbial abundance between the two groups ($p > 0.05$, student's t-test). Other alpha diversity indices including ACE, PD whole tree, Simpson and Shannon index were used to also describe alpha diversity as illustrated in Figures 4.10 and 4.11. Within the subgingival plaque microbiome, no significant differences were found between the low risk White European group and Black African group ($P > 0.05$; student's t-test) across all indices with the exception of PD whole tree which indicated a higher abundance in the White European group than in the Black African group ($P=0.0025$). Moreover, in the tongue microbiome, the Shannon index revealed a significant difference between the groups, with the White European group exhibiting greater diversity than the Black African group ($P = 0.012$). Nonetheless, no significant differences were observed among the other alpha diversity indices ($P > 0.05$).

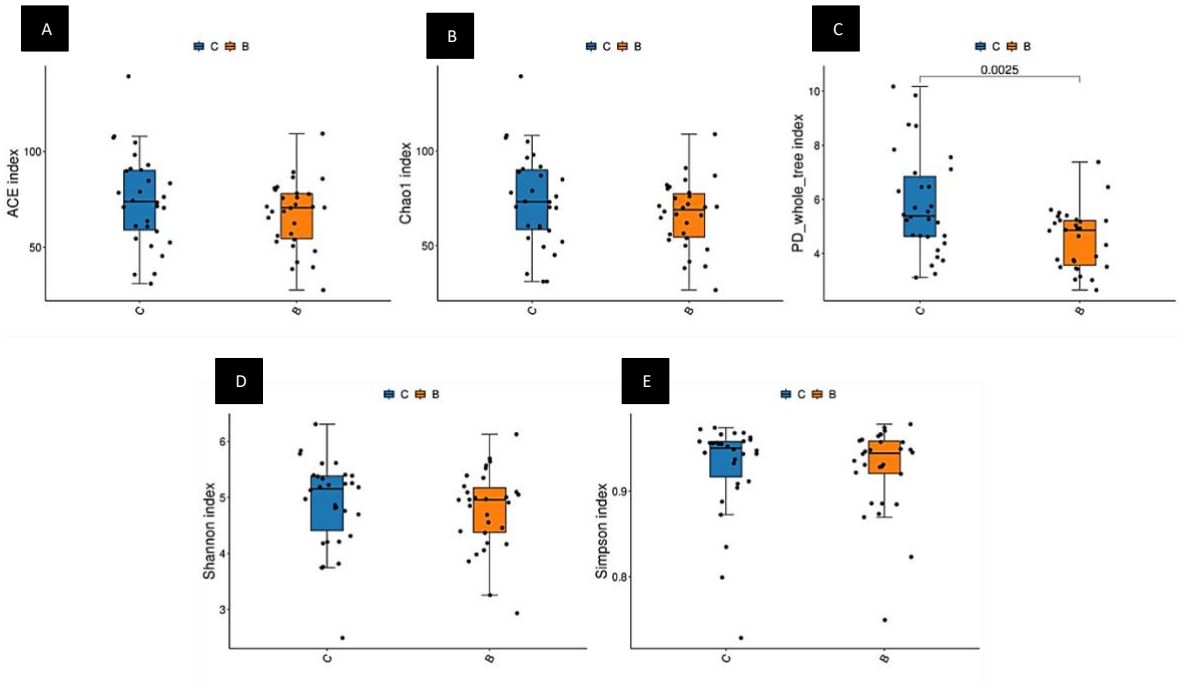


Figure 4.9 Alpha diversity metrics (ACE index, Chao1 index, PD whole tree, Shannon index and Simpson index) of OTU-level subgingival plaque bacterial communities. (A) (B) Box plots for comparison of species richness (ACE and Chao1 index); **(C)** Boxplots for comparison of phylogenetic diversity (PD whole tree); **(D)** Boxplots for comparison of species diversity (Shannon index) **(E)** Boxplots for comparison of species dominance (Simpson index) (B: Black African group; C: White European group). Boxes represent

the interquartile range (IQR) between the first and third quartiles (25th and 75th percentiles, respectively), and the horizontal line inside the box defines the median. Whiskers represent the lowest and highest values within 1.5 times the IQR from the first and third quartiles, respectively.

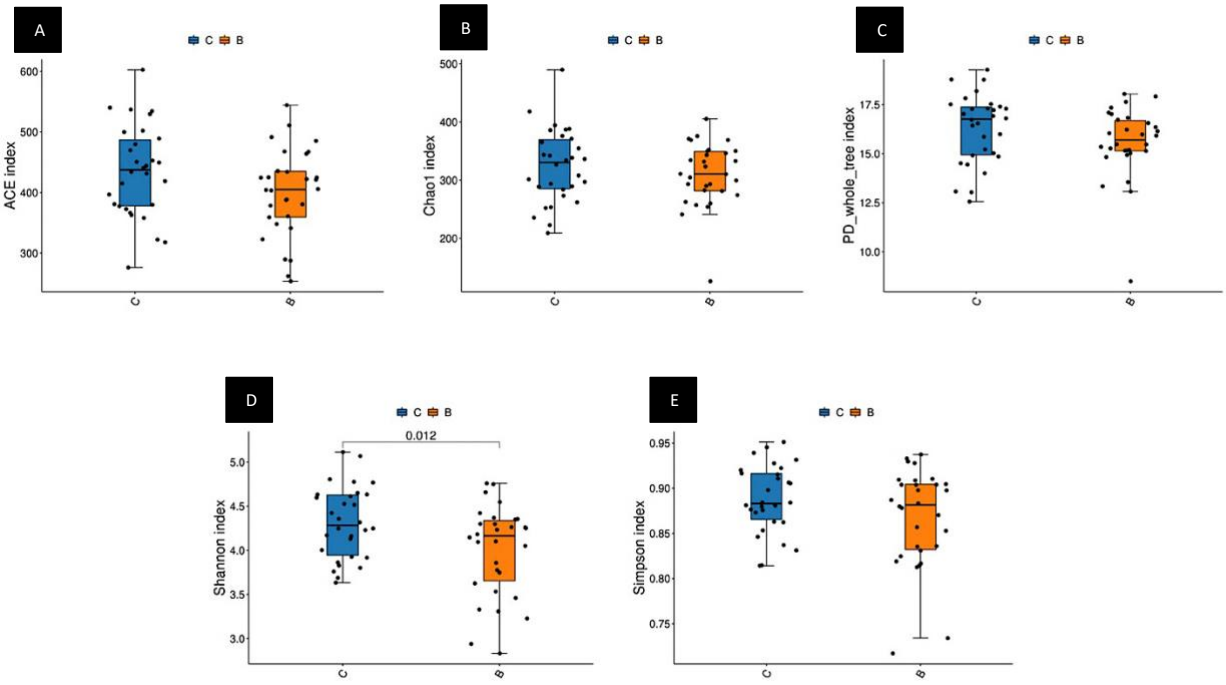


Figure 4.10 Alpha diversity metrics (ACE index, Chao1 index, PD whole tree, Shannon index and Simpson index) of OTU-level tongue bacterial communities. (A) (B) Box plots for comparison of species richness (ACE and Chao1 index); **(C)** Boxplots for comparison of phylogenetic diversity (PD whole tree); **(D)** Boxplots for comparison of species diversity (Shannon index) **(E)** Boxplots for comparison of species dominance (Simpson index) (B: Black African group; C: White European group). Boxes represent the interquartile range (IQR) between the first and third quartiles (25th and 75th percentiles, respectively), and the horizontal line inside the box defines the median. Whiskers represent the lowest and highest values within 1.5 times the IQR from the first and third quartiles, respectively.

In addition to the alpha diversity evaluation, the unweighted UniFrac analysis (beta diversity) was applied to compare similarities among oral microbial communities in the tongue and subgingival plaque between both ethnic groups. Data for this particular metric has been discussed as it is specific to bacteria and considers the presence/absence of species while considering their evolutionary relationships. Looking into the bacterial composition profiles, statistically significant differences between the White European and Black African group were observed in the subgingival plaque with the White European group exhibiting a higher beta diversity (R^2 = 0.045, P = 0.002, adonis; R = 0.07, P = 0.006 anosim). Meanwhile, in the tongue, the Black African group exhibited a higher beta diversity (R^2 = 0.032, P = 0.03, adonis; R = 0.1, P = 0.001 anosim) compared to the White European group.

In both cases, while the p-values are significant, it is important to note that the effect sizes (R and R^2) are relatively small. NMDS and PCoA, relying on OTU abundance, highlighted distinctions and commonalities in microbial composition between the White European and Black African groups (see Figs. 4.11 and 4.12). In these observed variations, two principal component scores accounted for 16.67% and 19.82% of the total variations in the subgingival plaque samples and 6.86% and 9.96% of the total variations in the tongue samples.

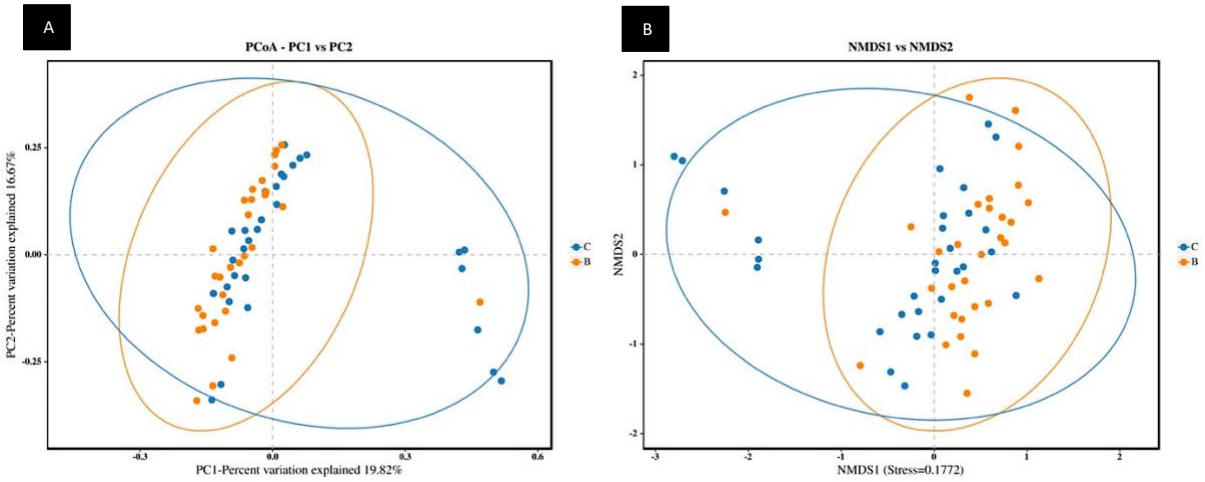


Figure 4.11 (A) Principal coordinate analysis and (B) non-metric multi-dimensional scaling plots showing the similarity relationship among the subgingival plaque microbiota between the two ethnic groups using an unweighted UniFrac distance matrix. The closer the spatial distance of the sample, the more similar the species composition of the sample. (B: Black African group; C: White European group).

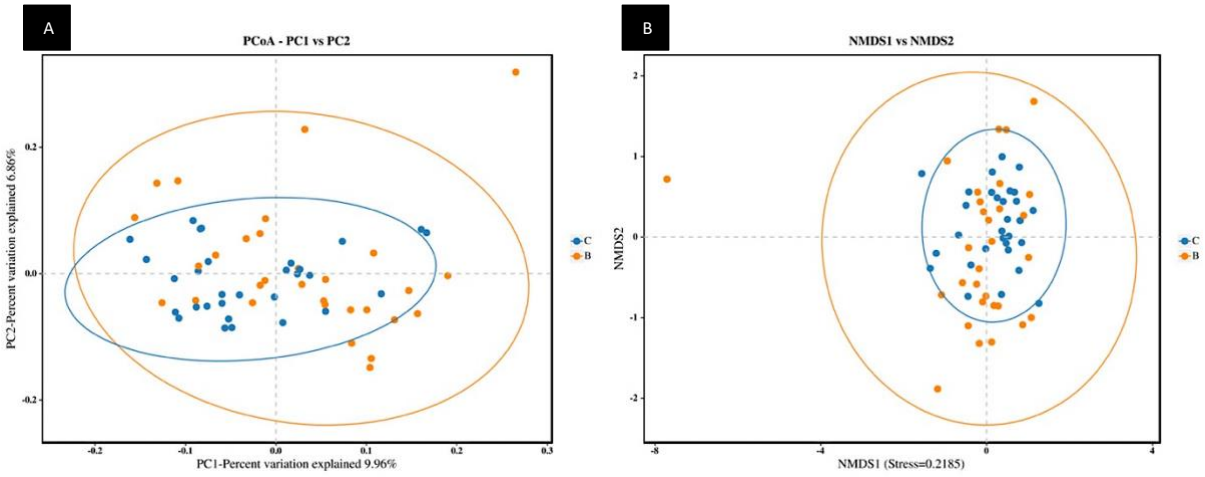


Figure 4.12 (A) Principal coordinate analysis and (B) non-metric multi-dimensional scaling plots showing the similarity relationship among the tongue microbiota between the two ethnic groups using an unweighted UniFrac distance matrix. The closer the spatial distance of the sample, the more similar the species composition of the sample. (B: Black African group; C: White European group).

4.3.6 The bacterial relative abundance and core microbiota in the low risk Black African and White European groups.

OTU statistics provided insights into bacterial relative abundance at various taxonomic levels, including phylum, class, order, family, and genus. The results revealed differences and similarities in oral bacterial composition between the two groups at different phylum levels. In subgingival plaque samples, Firmicutes dominated, constituting 43.5% and 49.1% of the microbiota in the White European and Black African groups, respectively, followed by Proteobacteria, contributing 35.3% and 39.9%, respectively (see Figs. 4.13A and 4.13B). Bacteroidota and Actinobacteriota were also significant phyla. For tongue microbiota, Firmicutes were also the primary contributors, comprising 57.5% and 58.4% of the microbiota in the White European and Black African groups, respectively, followed by Bacteroidota, contributing 22.2% and 22.1%, respectively. Proteobacteria and Actinobacteriota were notable as well. Analysis of the relative abundance of bacterial taxonomic groups revealed that both ethnic groups' oral microbiota was predominantly composed of nine phyla, as detailed in Table 4-4. Moreover, the subgingival plaque microbiota displayed substantial interindividual variability, with Firmicutes ranging from 6.2% to 84.7%, and Proteobacteria from 1.0% to 86.8% across all individuals. Similarly, the tongue microbiota exhibited high interindividual variability, with Firmicutes ranging from 38.3% to 80.8%, and Bacteroidota from 2.5% to 36.2% among all individuals (see Figs. 4.15A and 4.15B).

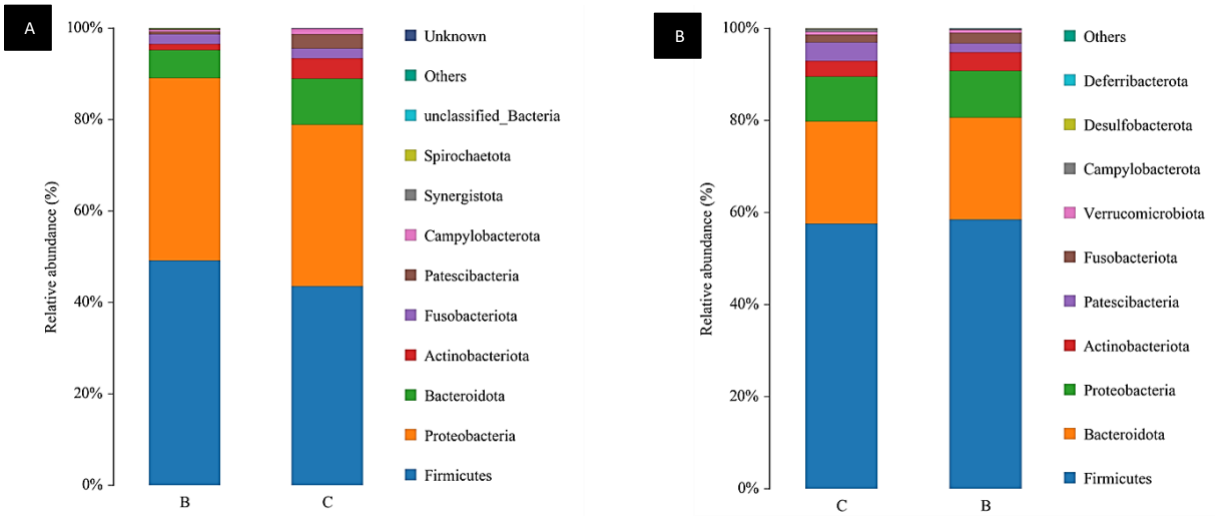


Figure 4.13 Histogram of the community composition of the oral microbiota at the phylum level in (A) subgingival plaque samples and (B) tongue samples of low risk White European and Black African groups. The abscissa represents the ethnic groups, and the ordinate represents the relative abundance. Figures show species with a relative abundance of 1% or more. All species with a relative abundance of less than 1% were classified as “others”. (B: Black African group; C: White European group).

No statistically significant differences were observed in Firmicutes (P > 0.05, Wilcoxon rank-sum test), Bacteroidetes (P > 0.05, Wilcoxon rank-sum test), and Proteobacteria (P > 0.05, Wilcoxon rank-sum test) between the two ethnic groups. In the subgingival plaque samples, Actinobacteriota and Patescibacteria were significantly more abundant in the White European group than in the Black African group (P = 0.033 and P = 0.02, respectively, Wilcoxon rank-sum test). Regarding the tongue samples, Patescibacteria exhibited lower proportions in the Black African group compared to the White European group (P = 0.001, Wilcoxon rank-sum test). For the remaining phyla, the oral microbiome sequencing data did not indicate significant differences in abundance between the White European and Black African groups. On a genus level, no significant differences were observed between the two groups across genera usually associated with periodontal health (p > 0.05 Wilcoxon rank-sum test) including *Neisseria*, *Haemophilus*, *Streptococcus*, *Rothia* and *Gemella* (data not shown). A comprehensive overview of the core microbiota between both ethnic groups is illustrated in Appendix III a & b.

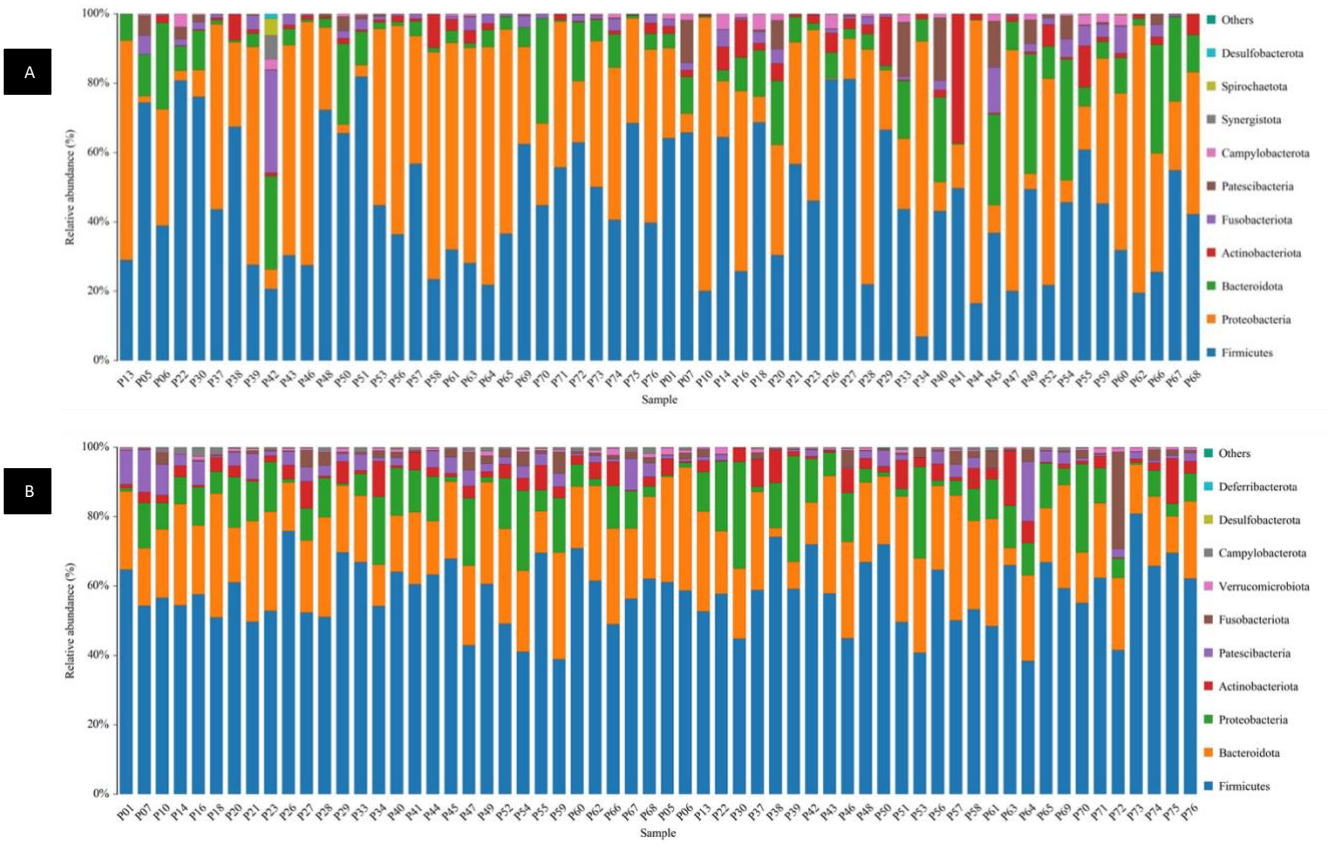


Figure 4.14 Histogram of the community composition of the oral microbiota at the phylum level in (A) subgingival plaque samples and (B) tongue samples of low-risk participants. The abscissa represents the sample, and the ordinate represents the relative abundance. Figures show species with a relative abundance of 1% or more. All species with a relative abundance of less than 1% were classified as "others". (B: Black African group; C: White European group).

Table 4-4 Relative abundance of microbial phyla in subgingival plaque and tongue samples of low-risk participants.

Phylum	Sample Type	Relative Abundance (%)
Firmicutes	Subgingival plaque	43.55 (C); 49.15 (B)
	Tongue	57.57 (C); 58.50 (B)
Proteobacteria	Subgingival plaque	35.34 (C); 39.95 (B)
	Tongue	9.74 (C); 10.13 (B)
Bacteroidota	Subgingival plaque	10.07 (C); 6.08 (B)
	Tongue	22.25 (C); 22.13 (B)
Actinobacteriota	Subgingival plaque	4.44 (C); 1.35 (B)
	Tongue	3.38 (C); 4.02 (B)
Fusobacteriota	Subgingival plaque	2.15 (C); 2.12 (B)
	Tongue	1.70 (C); 2.30 (B)
Patescibacteria	Subgingival plaque	3.10 (C); 0.59 (B)
	Tongue	4.00 (C); 1.99 (B)
Campylobacterota	Subgingival plaque	1.25 (C); 0.39 (B)
	Tongue	0.69 (C); 0.27 (B)
Synergistota	Subgingival plaque	0.00 (C); 0.17 (B)
	Tongue	0.00 (C);0.00 (B)
Spirochaetota	Subgingival plaque	0.00 (C); 0.12 (B)
	Tongue	0.00 (C); 0.00 (B)
Others	Tongue	0.00 (C); 0.04 (B)
	Subgingival plaque	0.01 (C); 0.0086 (B)

*C: White European group; B: Black African group

4.3.7 Phylogenetic profiles and functional analysis of oral microbiota in low risk White European and Black African groups.

To analyse the statistical differences in microbial communities between the Black African group and the White European group, LEfSe analysis was employed to compare OTUs between groups. The resulting histogram depicts the Linear Discriminant Analysis (LDA) scores computed for OTU-level features (see Figs. 4.15B and 4.16B). Moreover, cladograms illustrating taxa with LDA values exceeding 4.0 are presented in Figs. 4.15A and 4.16A. In the subgingival plaque microbiome, a comparison between the low-risk Black African and White European groups revealed two dominant taxa within the Black African group (**f**_Carnobacteriaceae: **g**__*Granulicatella*), while the White European group exhibited dominance from five taxa (**f**__Lactobacillaceae: **g**__*Lactobacillus*; **f**__Neisseriaceae;

p_Actinobacteriota: **c**__Actinobacteria). Within the tongue microbiome, the Black African group showcased two predominant taxa (**c**__Bacilli: **o**__Lactobacillales), whereas the White European group displayed dominance from nine taxa (**c**__Negativicutes; **o**__Veillonellales_Selenomonadales: **s**__*Veillonella_parvula*; **p**__Patescibacteria; **c**__Saccharimonadia: **o**__Saccharimonadales: **f**__Saccharimonadaceae: **g**__*TM7x*: **s**__*Candidatus_Nanosynbacter_lyticus*).

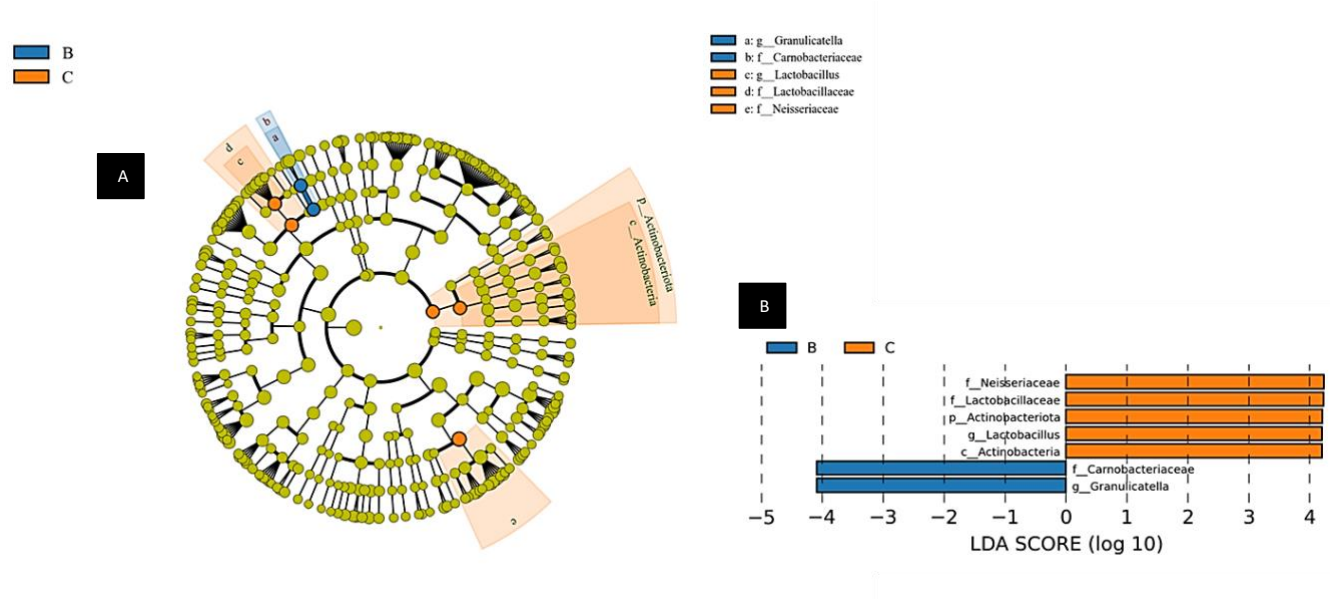


Figure 4.15 LEfSe analysis of the subgingival plaque microbiota in the two ethnic groups.

(A) The cladogram diagram shows the microbial taxa with significant differences in the two groups. Yellow nodes represent species with no significant difference and the diameter of the small circle is proportional to the relative abundance **(B)** The length of the histogram represents the LDA score; i.e., the degree of influence of taxa with significant difference between different groups. Statistical analysis was performed using the LDA effect size method. Only taxa with an alpha value of 0.05 and with absolute LDA (log10) scores >4.0 were considered significant (C: White European group; B: Black African group)

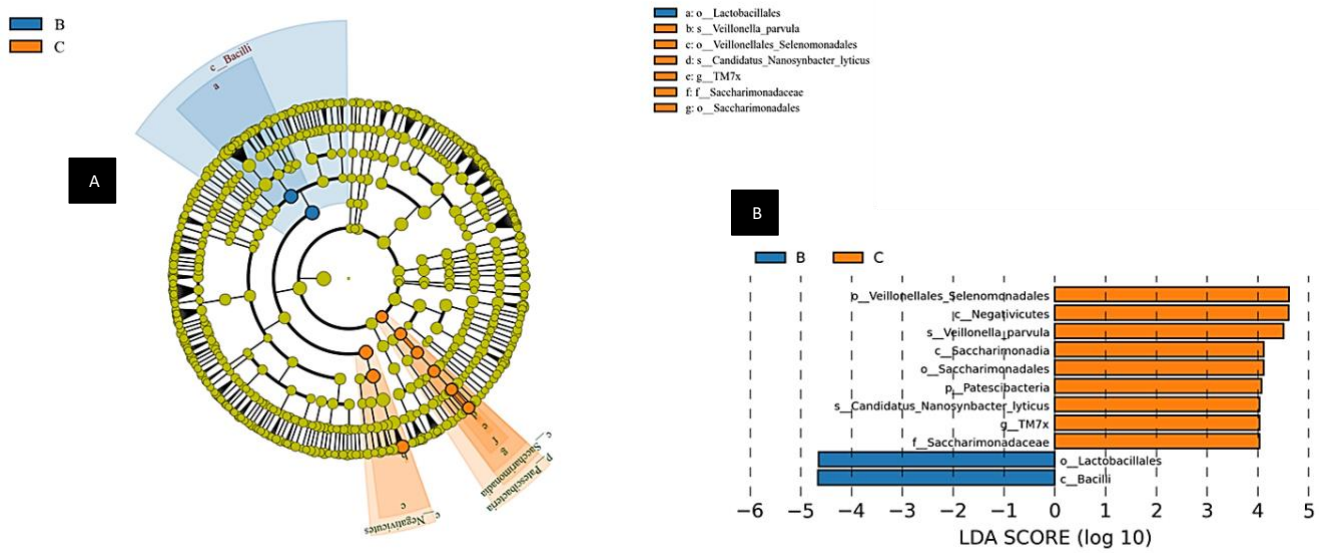


Figure 4.16 LEfSe analysis of the tongue microbiota in the two ethnic groups.

(A) The cladogram diagram shows the microbial taxa with significant differences in the two groups. Yellow nodes represent species with no significant difference and the diameter of the small circle is proportional to the relative abundance **(B)** The length of the histogram represents the LDA score; i.e., the degree of influence of taxa with significant difference between different groups. Statistical analysis was performed using the LDA effect size method. Only taxa with an alpha value of 0.05 and with absolute LDA (log10) scores >4.0 were considered significant (C: White European group; B: Black African group).

To investigate variations in oral microbial functional genes across ethnic groups, PICRUSt (version 2.0) was employed by comparing species composition information obtained from 16S sequencing data, then analyzing functional differences between the groups. Utilizing the KEGG database, PICRUSt unveiled six primary Level 1 biological pathways: metabolism, genetic information processing, environmental information processing, cellular processes, organismal systems, human diseases. Notably, metabolism, genetic information processing, and environmental information processing predominated, accounting for 78.9%, 9.4% and 5.6% respectively between the two groups. Furthermore, an in-depth analysis of the secondary functions associated with the predicted genes revealed a diverse array of 46 sub-functions, including membrane transport, carbohydrate metabolism, amino acid metabolism, replication and repair, energy metabolism, translation, cellular processes, and signalling (as detailed in Table 4-4). Within these 46 predicted functional categories at KEGG pathway hierarchy level 2, no significant distinctions were found between the two ethnic groups, except for carbohydrate metabolism (P = 0.04), nucleotide metabolism (P = 0.004), drug resistance: antimicrobial (P = 0.0008), infectious diseases: bacterial (P = 0.005), energy metabolism (P = 0.01), amino acid metabolism (P = 0.04), cardiovascular diseases (P = 0.006)

infectious diseases: viral (P = 0.005), and cancers: specific types (P = 0.008). The Black African ethnic group exhibited significantly higher representation of predicted functional genes in the first four mentioned categories, while the remaining six categories were overrepresented in the White European ethnic group microbiome.

Table 4-5 KEGG metabolic pathway differences between ethnic groups. (C: White European group; B: Black African group).

*P-values were adjusted for multiple comparisons by the Benjamini & Hochberg's false discovery rate.

Class 1	Class 2	C: mean rel.freq.(%)	B: mean rel.freq.(%)	p-values	*p-values (corrected)
Metabolism	Carbohydrate metabolism	8.98170989	9.21805816	0.008388	0.0428728
	Lipid metabolism	1.62779424	1.71476783	0.027924	0.0755589
	Metabolism of cofactors and vitamins	4.83942768	4.72228884	0.068782	0.1506655
	Energy metabolism	4.28974366	4.16547272	0.002605	0.0149815
	Amino acid metabolism	6.45543523	6.2437528	0.009958	0.0458081
	Nucleotide metabolism	4.33781495	4.4356274	0.000417	0.0048007
	Biosynthesis of other secondary metabolites	0.80173982	0.80845235	0.650769	0.7877729
	Metabolism of terpenoids and polyketides	0.98821959	1.0144042	0.092526	0.1702486
	Xenobiotics biodegradation and metabolism	0.72928334	0.76199131	0.025654	0.0786725
	Metabolism of other amino acids	1.40537389	1.40346893	0.762716	0.8557301
	Glycan biosynthesis and metabolism	1.81765601	1.86799329	0.178733	0.2740579
Genetic Information Processing	Translation	4.1743381	4.22021707	0.036425	0.0881857
	Folding, sorting and degradation	1.57611124	1.57908763	0.749648	0.8620951
	Transcription	0.19210357	0.19777722	0.014397	0.0551879
	Replication and repair	3.49125786	3.54350897	0.026042	0.07487
	Membrane transport	3.74328686	3.89401595	0.082102	0.1573628
	Signal transduction	1.75239316	1.82213711	0.014111	0.0590108

Environmental Information Processing	Signalling molecules and interaction	0.05040888	0.05083581	0.13242	0.2175472
Human Diseases	Drug resistance: Antimicrobial	1.06021837	1.09067456	1.77E-05	0.000816
	Drug resistance: Antineoplastic	0.00350616	0.00235623	0.081289	0.1625772
	Endocrine and metabolic diseases	0.19614419	0.1946631	0.656066	0.7738217
	Neurodegenerative diseases	0.18367714	0.17582858	0.193314	0.2868531
	Substance dependence	8.7151E-05	0.00010789	0.59356	0.737939
	Infectious diseases: Bacterial	0.45967418	0.47994398	0.000333	0.0051076
	Infectious diseases: Parasitic	0.02340215	0.0268614	0.158904	0.2520554
	Infectious diseases: Viral	0.00182956	0.00102567	0.00076	0.0058254
	Cancers: Overview	0.42994292	0.44543718	0.129144	0.2200231
	Cancers: Specific types	0.04940122	0.04480451	0.001326	0.0087146
	Immune diseases	0.04540454	0.05023943	0.20139	0.2807255
	Cardiovascular diseases	0.00061525	0.00034576	0.000739	0.0067983
Cellular Processes	Cellular community - prokaryotes	1.37237119	1.40137132	0.077576	0.1622049
	Cell motility	0.18135668	0.1555236	0.022971	0.0754777
	Cell growth and death	0.60605357	0.60751024	0.838409	0.918258
	Transport and catabolism	0.23620226	0.26942517	0.031164	0.0796419
	Cellular community - eukaryotes	0	0	1	1.0222222
Organismal Systems	Endocrine system	0.46978319	0.52566353	0.019731	0.0698157
	Aging	0.3045535	0.31304729	0.318325	0.4183706
	Circulatory system	0.00812635	0.00471908	0.056611	0.1302061
	Development	0	0	1	1.0454545
	Immune system	0.05169042	0.05443534	0.543315	0.6942354
	Environmental adaptation	0.13625802	0.136677	0.855504	0.9151899
	Nervous system	0.13226947	0.13816261	0.09653	0.1707847
	Sensory system	0	0	1	1
	Excretory system	0.02555772	0.02299098	0.196106	0.2819027

	Digestive system	0.07466312	0.0835235	0.203518	0.2753478

4.3.8 Microbial profiles related to the high risk and low risk Black African ethnic group.

OTU's with > 1% relative abundance were represented in the phyla: Actinobacteriota, Bacteroidota, Firmicutes, Fusobacteriota, Proteobacteria and Patescibacteria. The mean relative abundance of each phyla in the high risk and low risk group are Actinobacteriota (1.15% vs 1.36%), Bacteroidota (11.1% vs 6.65%), Firmicutes (60.9% vs 48.9%), Fusobacteriota (10.3% vs 2.35%), Proteobacteria (11.8% vs 39.2%) and Patescibacteria (3.67% vs 0.62%) respectively for the subgingival plaque microbiome. While for the tongue microbiome, the mean relative abundance of each phyla in the low risk and high risk group are Actinobacteriota (2.32% vs 3.9%), Bacteroidota (21.7% vs 22.2%), Firmicutes (59.2% vs 58.5%), Fusobacteriota (3.1% vs 2.3%), Proteobacteria (7.4% vs 10.1%) and Patescibacteria (5.2% vs 2.0%) respectively. The Wilcoxon rank sum statistical comparison at the phylum level showed a significantly higher proportion of Proteobacteria in the subgingival plaque microbiota of the low risk group ($p < 0.0001$). However, no significant difference in relative abundance was found in any other phyla between the two groups. Similarly, in the tongue microbiota, no significant difference was found in the relative abundance of any phyla between the two groups. Although, at species level, *Bacteroides cellulosilyticus* ($p = 0.021$), *Bacteroidia bacterium feline oral taxon 312* ($p = 0.032$), *Candidatus Nanosynbacter lyticus* ($p = 0.046$), *Dialister massiliensis* ($p = 0.008$) and *unclassified Deinococcus* ($p = 0.006$) were significantly increased in the high risk group.

4.4 Discussion

Previous studies have demonstrated the racial differences in the adult human microbiome, with most studies focusing on the microbiota of the gastrointestinal tract (McDonald *et al*., 2018 Li *et al.,* 2014; Mason *et al*., 2013), skin (Blaser *et al*., 2013), and vagina (Fettweis *et al*., 2014; Zhou *et al.,* 2007). Several studies have also implied the racial differences in the oral microbiome (Ma *et al.,* 2022; Gupta *et al.,* 2017; Nasidze *et al.,* 2009; Yang *et al.,* 2019). However, research comparing the oral microbiome of healthy Black Africans and White Europeans does not exist. The central hypothesis of this study posited that in health, the oral microbiome of the two ethnic groups, White Europeans and Black Africans, exhibits substantial similarity, with any observed differences likely attributable to other factors. In

this section, a thorough interpretation of the results is applied, emphasizing the nuanced aspects that support this hypothesis.

4.4.1 Ethnic differences in volatile organic compounds and volatile sulphur compounds.

In this study, the analysis revealed statistically significant differences in the concentrations of only one volatile organic compound group between the Black African and White European participants. Specifically, individuals from the Black African ethnic group exhibited higher levels of acids. However, there were no statistically significant differences found in the levels of other volatile organic compounds between both ethnic groups. Similarly, no statistically significant differences were found in the levels of total volatile sulphur compounds including Hydrogen sulphide, Methyl mercaptan and Dimethyl disulphide between both ethnic groups. Given that participants from both ethnic groups belonged to the healthy group with a low risk for diabetes and were periodontally healthy, this finding is consistent and expected as elevated levels of VSC's have been associated with numerous oral issues including halitosis and periodontitis (chapter 3.1). It is worth noting though, that high levels of sulphides can be produced in an otherwise orally and generally healthy individual. This is because this type halitosis, Intra-oral halitosis (IOH), originates from the tongue biofilm and improper oral hygiene practices can play a role in contributing to it (Seerangaiyan *et al.,* 2017).

The oral environment is a complex ecosystem influenced by a myriad of factors, including genetics, diet, hygiene practices, and oral microbiota (Peng *et al.,* 2022). Elevated acid levels within the oral cavity serve as potential indicators of susceptibility to periodontal issues, as acids can contribute to the acidic milieu within the oral cavity, which may favour the growth of acidophilic bacteria associated with dental caries (Campbell, 2021; He *et al.,* 2014). It is well known that a high dietary intake of carbohydrates can also lead to an increased production of acids as highlighted by Sedghi *et al.,* 2021. Moreover, African-Caribbean diets have traditionally been characterised by lower total and saturated fat contents and higher total carbohydrate content compared with that of White European adults (Apekey *et al.,* 2019; Goff *et al.,* 2015; Goff *et al.,* 2013; Vyas *et al.,* 2003) and the UK national average (Miller *et al.,* 1988). This dietary distinction underscores the likelihood that diet may play a significant role in the observed higher levels of acids within the Black African ethnic group. Additionally, the results from LEfSe analysis indicated a higher proportion of *Lactobacillus* in the Black African group. *Lactobacillus* bacteria are well-known for their ability to ferment sugars, generating lactic acid as a metabolic by product (Campbell, 2021). This lactic acid

production significantly lowers the pH level in the oral cavity, creating an acidic environment. The increased presence of *Lactobacillus* in the Black African group can be directly linked to the high carbohydrate content in the traditional Black African diet. This diet provides abundant food sources for the acid producing bacteria, supporting its proliferation and potentially contributing to the elevated acidic conditions observed in this ethnic group. However, it is essential to consider these findings in the broader context of oral health practices as dietary habits, oral hygiene routines, and access to dental care can all influence acid production and its impact on oral health.

4.4.2 Microbiological analysis and differences in low-risk ethnic groups.

The study revealed significant differences in the total tongue microbial composition, in the category of strict anaerobes, between the White European and Black African groups. However, no such distinctions were observed in subgingival plaque bacterial composition. Moreover, both ethnic groups displayed increased levels of microbes in the tongue compared to the subgingival plaque (Fig 4.7). This is in line with existing literature, indicating that distinct oral sites may harbour varying microbial communities, each with its own ecological niche and microbial composition (Giordano-Kelhoffer *et al.,* 2022; Sedghi *et al.,* 2021; Hajishengallis and Lamont, 2012). The tongue, which is constantly exposed to the oral environment, including oxygen and dietary substrates, may provide a more heterogeneous habitat compared to the relatively protected and sheltered environment of the subgingival plaque pockets (Saad *et al.,* 2013). Moreover, the differences in the prevalence of strict anaerobes in the tongue microbiome may be influenced by the local pH, oxygen tension, and substrate availability, all of which can vary between the tongue's dorsal surface and the subgingival environment (Abdulkareem *et al.,* 2023).

The increased presence of strict anaerobes in the tongue microbiome of healthy White European individuals may have significant implications for their periodontal health. This finding suggests that the oral cavity of individuals in this group might provide a favourable environment for these pathogenic bacteria, potentially heightening their susceptibility to periodontal diseases. It is also possible that differences in tongue brushing habits could have contributed to the variation in strict anaerobe prevalence between the two groups. The tongue's intricate structure, including complex papillae, pits, fissures, and crypts, provides ideal spaces for these strict anaerobes to form biofilms and thrive (Just *et al.,* 2005). These bacteria could be deep-seated within the tongue papillae, making it challenging for active

ingredients to penetrate due to the small papillae spaces, the viscosity of salivary molecules, and the low permeability of biofilms (Saad *et al.,* 2016). Depending on oral hygiene practices, tongue brushing might only remove the surface layer of the tongue coating, leaving the in-between papillae biofilm intact. The absence of significant differences in subgingival plaque bacterial composition between the two ethnic groups is noteworthy. This result suggests that, despite the differences observed in tongue microbiomes, the subgingival environment might not exhibit significant variations in bacterial composition. This finding could have important implications for periodontal disease pathogenesis. For instance, it is well-established that periodontal diseases are initiated by bacteria colonizing subgingival pockets and triggering host inflammatory responses (Hajishengallis *et al.,* 2012). If the subgingival plaque bacterial composition remains relatively similar between the ethnic groups, it might indicate that the factors contributing to periodontal disease could be more related to oral hygiene habits, host responses, genetic predispositions, or other environmental factors.

4.4.3 Ethnicity and oral microbiota composition.

The study population comprised 60 individuals at low risk for diabetes, equally distributed between the Black African and White European ethnic groups, along with an additional 10 high-risk individuals from the Black African ethnic group. While this division enabled an initial exploration of ethnic disparities in oral microbiota, it is essential to acknowledge that the low-risk group maintained a balanced ethnic composition, while the high-risk group exclusively consisted of Black African participants. Consequently, direct statistical comparisons between the risk groups was limited due to the uneven sample sizes. Nevertheless, the study conducted ethnic comparisons within the low-risk group and provided preliminary comparison data between the risk groups. This approach allowed for insights into the microbial differences and similarities within the baseline subgroups. It is important to recognize that this study serves as an important foundation for future investigations in diverse populations, paving the way for more comprehensive research in this field.

4.4.3.1 Alpha diversity between ethnic groups.

Alpha diversity metrics, including Chao1, ACE, Phylogenetic Diversity (PD) whole tree, Simpson, and Shannon indices, offer essential insights into the richness and evenness of microbial communities within samples (Li *et al.,* 2022). In the present study, analysis

revealed significant differences in only one alpha diversity metric between the two ethnic groups, both in the subgingival plaque and the tongue samples (Fig 4.10 and 4.11). The Chao1 and ACE indices, which estimate species richness, demonstrated no statistically significant differences between the low-risk White European and Black African groups across both subgingival plaque and tongue samples. In addition, no significant difference in the dominance of particular species between the ethnic groups was identified across both subgingival plaque and tongue samples as indicated by the Simpson index. These results align with the study's overarching hypothesis of substantial similarity in oral microbiota diversity between the two ethnic groups. The similarity in species richness suggests that both ethnic groups host a comparable number of bacterial taxa, which is a fundamental characteristic of alpha diversity. However, the interpretation becomes more nuanced when considering the Phylogenetic Diversity (PD) whole tree, and Shannon indices.

The PD whole tree metric assesses not only species richness but also incorporates evolutionary relationships among taxa, represented by the sum of the lengths of interconnected phylogenetic branches within a group of species on their phylogenetic tree (Subgingival plaquebs *et al.,* 2020). Within the subgingival plaque microbiome, it was observed that the White European group exhibited a significantly higher PD whole tree index than the Black African group, indicating a greater phylogenetic diversity within the former. This distinction may arise from the fact that the White European group consisted of individuals from diverse European nationalities, each contributing their unique microbiomes. Whereas, the Black African group, which primarily comprised of individuals from West African (Nigerian and Ghanaian) backgrounds, might possess a more homogenous microbial community in terms of evolutionary history. This could be attributed to shared dietary factors influencing their microbiomes in a similar manner (Li *et al.,* 2014). Furthermore, the dietary habits of the White European group, encompassing a wider variety of substances due to the diverse national backgrounds of its members, might have a greater impact on the oral ecological landscape (David *et al.,* 2014). Determining the precise impact of diet on the microbial composition of the oral cavity is undoubtedly challenging. However, it is plausible to consider that a varied diet, rich in diverse food items over an extended period, could introduce a complex mix of substrates. This variety might create an environment conducive to the proliferation of a broader spectrum of bacterial species within the oral cavity.

The Shannon index, a comprehensive measure of diversity, considers both species richness and the evenness of species abundances within a sample (Gauthier and Derome, 2021). In the tongue microbiome, the Shannon index revealed a significant difference, with the White European group demonstrating greater diversity compared to the Black African group. This finding aligns with a previous study by Li *et al.* (2014), which indicated that African populations exhibited lower alpha diversity, as measured by Shannon's index, when compared to German and Alaskan groups. One potential contributing factor to this divergence could be variations in tongue brushing practices, as different hygiene practices may influence microbial diversity and distribution in the tongue.

4.4.3.2 Beta diversity: unveiling ethnic-specific patterns.

Beta diversity analysis offers a window into the dissimilarity of microbial communities between samples. Utilizing the unweighted UniFrac analysis, which considers the presence/absence of species while considering their evolutionary relationships, this study aimed to elucidate whether distinct ethnic-specific patterns existed in oral microbial communities. The results yielded statistically significant differences between the White European and Black African groups in both subgingival plaque and tongue microbial communities, as indicated by the adonis and anosim tests. While the p-values were significant, it is vital to note that the effect sizes (R and R^2) were relatively small. To gain a more comprehensive understanding of these differences, the study explored non-metric multidimensional scaling (NMDS) and principal coordinates analysis (PCoA), relying on OTU abundance. These analyses highlighted both distinctions and commonalities in microbial composition between the two ethnic groups both in the subgingival plaque and tongue samples. The relatively small effect sizes suggest that the differences in bacterial communities between the ethnic groups are subtle, involving minor variations in the specific types of bacteria that are present. This implies that practical relevance of these distinctions may be modest in the context of overall community composition.

It is well established in the literature that there exists a core oral microbiome shared among humans, irrespective of ethnicity (Li *et al.,* 2022; Caselli *et al.,* 2020; Risely, 2020). This core microbiome consists of species that are consistently present in the oral cavity of healthy individuals (Human Microbiome Project Consortium, 2012). Since both ethnic groups in this study were classified as "healthy" and exhibited a shared core microbiota, it could explain the limited effect size in beta diversity. Meanwhile, the observed distinctions might be attributed to various factors, including dietary choices, hygiene practices, and

environmental influences, rather than ethnicity alone. Additionally, clustering analysis indicated closer spatial distances between the majority of samples in both ethnic groups. However, there were exceptions observed in a few samples from both groups. These exceptions may suggest that individual variations play a more significant role in the observed differences than a collective ethnic influence.

4.4.3.3 The relative abundance of phyla between the two ethnic groups.

The analysis of the relative abundance of bacterial phyla in subgingival plaque and tongue samples among the different ethnic groups revealed 7 common taxa between the two ethnic groups (Fig 4.19). Firmicutes, Proteobacteria, and Bacteroidota were the predominant taxa in both ethnic groups which is consistent with existing literature and the Human Oral Microbiome Database (HOMD), highlighting these phyla as core members of the oral microbiome in health (Verma *et al.,* 2018; Zaura *et al.,* 2009). Within subgingival plaque samples, Firmicutes and Proteobacteria were the predominant phyla in both ethnic groups, albeit with slight variations. Firmicutes constituted 43.5% and 49.1% of the microbiota in the White European and Black African groups, respectively, while Proteobacteria contributed 35.3% and 39.9%, respectively. Similarly, in the tongue, the Black African ethnic group showed a relatively higher proportion of the three dominant phyla mentioned above. However, no statistical differences were observed between ethnic groups in both oral sites. These minor differences may reflect individual variation (as shown in Figs. 4.20) and does not negate the hypothesis of substantial similarity in the oral microbiota between the Black African and White European ethnic groups.

Notably, Actinobacteriota and Patescibacteria displayed significant differences in relative abundance between the two ethnic groups at a phylum level. In the subgingival plaque samples, these phyla were more abundant in the White European group, however only Patescibacteria exhibited significant differences between the ethnic groups in the tongue. The variation in Actinobacteriota levels between both groups in the tongue and subgingival plaque could be attributed to differences in the microenvironments of these oral sites. Actinobacteriota includes genera such as Actinomyces, which are commonly associated with oral health. These bacteria thrive in biofilms on tooth surfaces and subgingival plaque pockets (Marsh, 2003). Therefore, the specific microenvironment of the subgingival plaque, characterized by the tooth-subgingival plaque interface, might facilitate the colonization and growth of Actinobacteriota to a greater extent than the tongue.

In the tongue, the LEfSe analysis (Fig. 4.21) highlighted TM7x as the major genus contributing to the significant differences between the two groups within the Patescibacteria phylum level, accounting for 1% and 3% in the Black African and White European oral microbiomes, respectively (see Appendix III a & b). TM7x, often characterized by its low abundance (typically representing around 1% of the entire oral microbiome), has earned the moniker "microbial dark matter" due to the challenges associated with its laboratory cultivation (Bor *et al.,* 2019). It has been linked to potential pathogenic associations, particularly with inflammatory mucosal diseases, including periodontitis (Nie *et al.,* 2022). However, no definitive causative relationship between TM7x and periodontitis has been established. Despite its low prevalence, TM7x has garnered interest because of its potential functional significance in relation to biofilm formation and periodontal health (Bor *et al.,* 2022). Nevertheless, further research is imperative to elucidate its metabolic capabilities, interactions with other bacteria, and its implications for oral health and disease, particularly within distinct racial groups.

4.4.3.4 Functional capabilities of the microbial communities within both ethnic groups.

The PICRUSt analysis was used to infer the functional capacity of the microbiota by prediction of functional genes that typically are associated with different taxa. Across majority of the 46 predicted functional categories, no significant differences were observed, reinforcing the study hypothesis of substantial similarity of the oral microbial structure between both ethnic groups. Moreover, the minor variations in the specific KEGG pathways highlighted may be due to individual variations in genetics, dietary habits, oral hygiene and lifestyle. For instance, carbohydrate metabolism involves the breakdown and utilization of carbohydrates, and differences in carbohydrate sources and consumption can impact the oral microbiome (Sedghi *et al.,* 2021). For example, individuals with diets rich in sugary carbohydrates may harbour bacteria specialized in utilizing these substrates potentially leading to differences in metabolic pathways. As mentioned earlier, African-Caribbean diets have traditionally been characterised by higher total carbohydrate content compared with that of White European adults (Apekey *et al.,* 2019; Goff *et al.,* 2015) and as such might explain the enrichment of carbohydrate metabolism functional genes in the Black African group. However, PICRUSt is only a means of predicting functional genes; thus, further research is required to confirm the accuracy of gene function information by metagenomic analysis and gene expression studies.

4.4.4 Differences in the oral microbial profiles of the high risk and low risk Black Africans

The comparative analysis of oral microbial profiles between individuals at low and high risk for type II diabetes yielded intriguing findings. Notably, the phylum Proteobacteria exhibited a significant reduction in the subgingival plaque microbiota of the high-risk group, prompting questions about its potential impact on periodontal health. Interestingly, no significant differences were found in other phyla between the two groups, both in the tongue and sub gingival samples. This suggests a nuanced interplay of microbial factors contributing to the oral health dynamics in individuals at different diabetes risk levels. Although, on a species level, specific species such as *Bacteroides cellulosilyticus*, *Bacteroidia bacterium feline oral taxon 312*, *Candidatus Nanosynbacter lyticus*, *Dialister massiliensis*, and *unclassified Deinococcus* displayed higher prevalence in the high-risk group. These findings hint at potential biomarkers associated with diabetes-related oral health issues. For instance, *Dialister* species are often found in periodontal pockets and have been associated with chronic periodontitis (Oswal *et al*., 2020; Hiranmayi *et al.,* 2017; Colombo *et al.,* 2016; Ferraro *et al.,* 2007). They can produce harmful enzymes and toxins, contributing to tissue damage and inflammation (Kemp *et al.,* 2021). Its higher prevalence in the high-risk group may suggest a potential link between this species and the increased risk of periodontal disease associated with type II diabetes. Additionally, *Candidatus Nanosynbacter lyticus*, though poorly studied, demonstrated significant presence in the high-risk group. While its specific role in periodontal health remains unclear, its prevalence could also be a potential indicator of its role in the transition between oral health to disease. However, it is hard to draw any definitive conclusions due to the small and unequal sample sizes between the groups. These results, serving as preliminary data, necessitate further in-depth investigations with larger sample sizes to establish robust correlations and fully comprehend the intricate relationship between oral microbial composition, diabetes risk, and periodontal health.

4.5 Conclusion

In summary, this study offers valuable insights into the oral microbiome of healthy Black Africans and White Europeans. By addressing a significant gap in current oral microbiology research, it contributes to our understanding of ethnic-specific oral microbiota profiles. The central hypothesis, which posits a substantial similarity in oral microbiota between these

ethnic groups, finds support in a comprehensive analysis covering volatile organic compounds, microbial composition, alpha and beta diversity, phyla relative abundance, and functional capabilities. These findings emphasize the need for careful approaches to oral health, considering individual microbiome variations and the interplay of numerous factors, such as dietary choices, oral hygiene practices, and individual uniqueness. This study serves as a vital step towards unravelling the intricate dynamics of oral health within ethnically diverse populations. However, further research is essential to unveil the functional significance of the observed microbial differences and their potential implications for both oral and systemic health in distinct racial groups. By refining our understanding of the multifaceted factors influencing oral microbiota and their health-related outcomes, we can pave the way for more targeted and effective interventions.

Chapter 5 General discussion, limitations and future work

5.1 General discussion

This study has explored the understanding of the human oral microbiome, examining its significance and potential implications for oral and systemic health. Each chapter has offered insights, improving the understanding of this intricate microbial ecosystem.

Chapter 2 undertook a systematic review, focusing on the utility of the Human Oral Microbe Identification Microarray (HOMIM), a 16S rRNA gene sequencing method in quantifying the oral microbiome within the context of periodontal disease. This critical review showed HOMIM's moderate effectiveness in identifying and quantifying oral bacterial species, marking a notable advancement beyond earlier methodologies such as DGGE. HOMIM, with its expanded scope of species identification and comprehensive species probes, has raised the bar for characterizing the oral bacterial community. However, it is imperative to acknowledge the inherent limitations of this molecular profiling technology. To further advance our understanding, future investigations should explore the effectiveness of Human Oral Microbe Identification using Next-Generation Sequencing (HOMINGS), which offers promising advancements beyond HOMIM. Additionally, considering the ever-evolving landscape of microbial profiling techniques, the exploration of MTG and MTT methods for quantifying the human oral microbiome remains an important avenue for future research.

Chapter 3 focused on the testing of oral care products, with a focus on a toothpaste containing 5% potassium nitrate and 0.24% sodium fluoride as a prospective alternative to Chlorhexidine for managing halitosis and reducing the risk of periodontal disease. The findings of this study provided evidence regarding the efficacy of the test toothpaste. In an *in-vitro* flat-bed biofilm model, it demonstrated the capacity to significantly reduce volatile sulphur compounds (VSC) and volatile organic compounds (VOC). The mechanism underlying this reduction appeared to involve the control of biofilm cells responsible for odorous gas production and disruption of the biotransformation process. Furthermore, the test toothpaste displayed promise in reducing biofilm density, including both facultative and strict anaerobes, through the disruption of bacterial cell membranes. The *in-vitro* model's potential to simulate real tongue biofilm microbial activities suggests that these findings may have clinical relevance. However, the transition from *in-vitro* to *in-vivo* settings is

complex and multifaceted, warranting further research to validate the test toothpaste's effectiveness in real-world clinical scenarios and its long-term effects. It is paramount to consider the myriad factors at play in oral health, including individual variations in oral microbiota, dietary habits, and oral hygiene practices. Nevertheless, the study's results are promising, hinting at the test toothpaste's potential as a viable alternative to Chlorhexidine for managing oral malodour and periodontal disease, which merits further exploration and validation in clinical contexts.

Chapter 4 extends the scope of this study by comparing the oral microbiota profiles of Black Africans and White Europeans. This research addresses a critical gap in the existing literature, shedding light on ethnic-specific oral microbiota profiles. The central hypothesis of substantial similarity between these ethnic groups in oral microbiota composition receives support across various analytical dimensions, including volatile organic compounds, microbial composition, diversity metrics, phyla relative abundance, and functional capabilities. These findings emphasize the importance of thoughtful approaches to oral health, recognizing the unique microbial variations among individuals and the impact of factors such as dietary choices, oral hygiene practices, and individual genetic diversity. This study represents a further step towards exploring the intricate dynamics of oral health within ethnically diverse populations. However, this research represents a foundational exploration, and further investigation is warranted to understand the functional implications of the observed microbial disparities and their potential consequences for both oral and systemic health in distinct racial groups. By advancing our understanding of the multifaceted factors shaping oral microbiota and their health-related outcomes, we can facilitate more tailored and effective interventions.

In summary, this study's collective findings underscore the significance of sustained research efforts in the field of oral microbiology. Each chapter has contributed distinct insights, raising pertinent questions and indicating promising avenues for future research. The quest for advanced microbial identification technologies, the development of innovative oral care products, and the exploration of ethnic-specific microbial profiles all represent compelling areas of inquiry. These endeavours have the potential to improve our comprehension of oral health, enabling more targeted interventions and ultimately enhancing the well-being of individuals across diverse populations. Exploring the complexities of the human oral microbiome reveals significant implications for oral and

systemic health, emphasizing the importance of continued research and innovation in this field.

5.2 Study limitations

This study was conducted in two phases: an *in-vitro* phase and a subsequent clinical trial, aimed at gaining insights into the oral microbial community in healthy African and White European groups. While the research provided valuable and novel findings, certain limitations were encountered during its execution. This section discusses the main limitations that may have influenced the research outcomes.

5.2.1 Impact of the COVID-19 pandemic

One of the primary limitations of this study was the unprecedented impact of the COVID-19 pandemic. During the pandemic, there was sudden closure of research laboratories, and the biological safety committee did not approve any protocols involving collection of samples from the mouth. As a result, significant delays were experienced in the recruitment process and data collection, which ultimately affected the study's timeline and objectives. Furthermore, in response to the COVID-19 restrictions and safety concerns, certain aspects of the study had to be adapted. For the safety of investigators and participants, the use of organoleptic assessment by a trained organoleptic judge was removed from the study. This method plays a critical for assessing halitosis, an indicator of periodontal disease; thus, this adaptation may have limited the comprehensive understanding of the oral health parameters under investigation.

At the study's inception, one of the primary objectives of the study was to compare the effects of a novel oral formulation developed by Colgate Palmolive between healthy and diabetic groups from a Black African population in Nigeria and a White European population in the UK. This was to be assessed by a blinded randomised 6-week clinical trial involving the two clinical and ethnic groups. However, due to the limited timeframe left on the study after the prolonged pandemic-related delays, recruitment of the type II diabetic participants proved to be challenging. Consequently, the diabetic cohort could not be adequately represented in the study, and only baseline measurements with the healthy group were conducted during the trial, which hindered the fulfilment of one of the original objectives.

5.2.2 Potential bias in the diabetes risk assessment tool

Another limitation arose from the use of the current NHS screening tool to assess diabetes risk within the healthy group. The tool's original research (Gray *et al.,* 2010), which informed the scoring system, predominantly involved White European (76%) and South Asian participants (22%), with limited representation from other ethnic groups (3%). This lack of diversity and inadequate representation may have introduced bias in the diabetes risk assessment scores, especially for participants from Black African origins. The application of the diabetes risk assessment tool revealed disparities in the risk scores between different ethnic groups. For instance, the cut off points for the low-risk group varied between the Black African and White European participants. Black African females required a maximum of 6 points to be classified as low risk, while Black African males required 7 points. In contrast, White European females and males required 0 and 1 point, respectively, to qualify as low risk. Moreover, the cut off for the high-risk group was set at above 16 points, presenting challenges in recruiting White European participants, as it would require morbid obesity for eligibility. Consequently, the recruitment process favoured participants from the Black African group, which may have introduced unintended biases in the final cohort. The study faced constraints in recruiting an adequate number of participants within the high-risk cohort. The limited sample size, particularly in the Black African group, might have impacted the study's statistical power and limited the depth of information available to understand the oral microbial community within different ethnic populations fully.

5.2.3 Generalizability of Findings

The study's findings may have limited generalizability to broader populations due to the narrow focus on specific ethnic groups and the challenges faced in recruiting a diverse sample. The underrepresentation of other ethnicities and the lack of a diabetic cohort further limit the extrapolation of the results to other communities or clinical conditions.

Furthermore, as with any clinical trial, several variables may have remained uncontrolled, leading to potential confounding effects on the study outcomes. Factors such as participants' dietary habits, socio economic status and individual variations in microbiota could have influenced the results, but they were not fully accounted for in the study design. The study's reliance on self-reported data and the use of a single diabetes risk assessment tool may have introduced measurement biases and limitations in capturing participants' accurate health information. Additionally, the lack of specific details on participants' lifestyle factors may have also impacted the interpretation of the study's findings.

5.2.4 Conclusion

In conclusion, while this study offers valuable insights into the oral microbial community among healthy individuals, it is essential to acknowledge and consider the limitations mentioned above when interpreting the results. The impact of the COVID-19 pandemic, limited representation of diabetic participants, potential bias in the diabetes risk assessment tool, and the narrow focus on specific ethnic groups all contribute to the study's scope and generalizability. By addressing these limitations, future research can not only enhance the applicability and robustness of the findings in informing oral health interventions across diverse populations but also foster a more comprehensive understanding of oral health among different ethnic groups.

5.3 Further work and recommendations

While this study has contributed to the understanding of the oral microbiome and its implications for oral health, there are several areas for future research that can build upon the limitations and expand the knowledge base in this field.

The current investigation has laid the groundwork by revealing the promising antimicrobial effects of the tested oral formulation *in-vitro*. To provide a comprehensive understanding, future research should embark on extensive long-term double blinded clinical trials with the test toothpaste. These trials, conducted over an extended period, can gauge the sustained impact of the oral formulation on oral health. They should encompass diverse aspects, such as the persistence of its antimicrobial effects, its influence on the development of oral malodor, and its efficacy in preventing biofilm formation. A meticulous analysis of these aspects in a clinical context would yield invaluable insights into the formulation's real-world utility and potential advantages over existing oral care products. Moreover, in the pursuit of advancing the field of oral care, it is imperative to undertake comparative studies involving various oral formulations with distinct active ingredients. Such studies would be instrumental in discerning the most efficacious agents for combatting oral malodor and suppressing biofilm formation. By systematically comparing multiple formulations under standardized conditions, researchers can elucidate nuanced differences in their performance, potentially identifying synergistic combinations and optimal concentrations. These findings could substantially inform the development of advanced and superior oral care products, ultimately benefitting public health. While this study has offered preliminary

evidence of the efficacy of the test toothpaste, a comprehensive understanding of its mechanism of action remains elusive. To bridge this knowledge gap, future research should undertake a meticulous exploration of the formulation's mechanisms of action. Particular attention should be devoted to unravelling the intricate interactions between potassium nitrate and other compounds within the formulation. Through rigorous experimentation and in-depth analysis, researchers can delineate the precise molecular pathways through which the formulation exerts its antimicrobial effects. This mechanistic insight holds the potential to enhance the formulation's targeted action and refine its composition for maximal efficacy.

Another study that showed promise in this work was the comparison of oral microbiota profiles between White European and Black African ethnic groups. It is important to note that this current study employs a cross-sectional design, which captures a singular, static snapshot of the oral microbiota at a specific point in time. While this design is valuable for establishing initial associations, it necessitates further exploration through longitudinal research. To advance understanding in this field, future research should consider the adoption of longitudinal studies, which offer a dynamic perspective on the oral microbiome's evolution over time. Such investigations could play a pivotal role in uncovering how alterations in the oral microbiota may contribute to the development of type 2 diabetes. A promising research avenue involves the continuous monitoring of individuals from both ethnic groups at high risk for diabetes, allowing researchers to trace shifts in their oral microbiota and discern potential correlations with the onset of the disease. While this current study focuses on healthy cohorts characterized by low risk for type II diabetes, broadening the research horizon to encompass individuals diagnosed with type 2 diabetes holds substantial clinical relevance. Therefore, it will prove useful for future investigations to introduce direct comparisons between ethnic groups within the healthy control cohort, individuals diagnosed with type 2 diabetes without periodontal disease and individuals diagnosed with type 2 diabetes with periodontal disease. This comparative framework facilitates the identification of specific oral microbiome alterations closely linked to the onset of diabetes. Such an approach would enable the identification of distinct oral microbiome signatures intricately associated with the disease. The subsequent analysis would explore the nuanced variations of these signatures across different ethnic groups, highlighting potential implications for the management and treatment of diabetes.

Furthermore, the field of oral microbiome research should expand to encompass interventional studies targeting clinical and ethnic groups. These interventions may encompass a wide spectrum of innovative approaches, including novel oral formulations, probiotics, prebiotics, or other pioneering strategies aimed at modulating the oral microbiome. The efficacy of these interventions can be rigorously evaluated by assessing alterations in the oral microbiota composition before, during, and after the intervention periods. Such investigations are poised to yield critical insights into the viability and impact of these novel approaches on oral and systemic health. Finally, a deeper comprehension of the functional role played by the oral microbiome in health and disease is essential. To address this, future research should incorporate functional metagenomics—a robust analytical technique. This methodology provides the potential to elucidate the intricate metabolic capabilities of specific microbial taxa residing within the oral ecosystem. Through functional metagenomics, researchers can delve into the intricate interactions between these microbial communities, revealing their contributions to both oral and systemic health.

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Publications

- Effectiveness of the Human Oral Microbe Identification Microarray in Identifying Periodontal Pathogens: A Systematic Review (2022) DOI: 10.3390/applmicrobiol2030047
- Recent Advances in the Application of Essential Oils as Potential Therapeutic Candidates for Candida-Related Infections (2022) DOI: 10.3390/applmicrobiol2020030

Appendix

I. Clinical trial documents relating to ethics and permissions

a. Consent form

Study number:
Patient Identification Number for this trial:

CONSENT FORM

Title of Project: "Oral microflora in health and in Type 2 diabetes, a comparison across the African and White European population."
IRAS reference:

Project Manager: Stephanie Udoh
Email: stephanie.udoh@uwe.ac.uk
Tel: 01173281085

Please tick the checkboxes after reading and agreeing with the following statements:

1. I confirm that I have read and understand the participant information sheet version …… for the above study; dated …… and I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily; ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason; ☐

3. I understand that data collected during the study, may be looked at by individuals from Colgate-Palmolive and the University of the West of England, Bristol from regulatory authorities where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. I agree that anonymized quotes may be used in the final Report of this study; ☐

4. I understand that the information collected about me will be used to support other research in the future, and may be shared anonymously with other researchers; ☐

5. I agree to take part in the research. ☐

_____	_____	_____
Name of Participant	Date	Signature
_____	_____	_____
Name of Person taking consent (if different from researcher)	Date	Signature

b. Health questionnaire

BREATH AND ORAL RESEARCH STUDY

MAKE A DIFFERENCE IN ORAL HEALTH.

We are the Centre of Oral Malodour Research at UWE. Join our 30-min clinical trial on oral health and be a part of shaping the future of oral health care.

Contact us at stephanie.udoh@uwe.ac.uk if you have any questions.

* Indicates required question

1. Email *

2. Date of birth? *

Example: January 7, 2019

3. What is your gender? *

Mark only one oval.

- Female
- Male
- Other: _____

4. What is your ethnic group? *

Mark only one oval.

- Black African / African / Caribbean / Black African British
- White
- Asian / Asian British Mixed /
- Multiple ethnic groups Other:
- _____

5. Height (cm) *

_____

6. Weight (kg) *

_____

7. Waist size (cm or inches, please specify) *

_____

8. Does your mom, dad or sibling have a Diabetes Diagnosis? *

Check all that apply.

- [ ] No
- [ ] Yes (Please state below which relative, type of diabetes and age of diagnosis) Other:
- [ ] _____

9. Do you have high blood pressure or take any blood pressure medication? *

Mark only one oval.

- Yes
- No

10. Do you agree to be contacted by the researcher with more information about this study ? *

Mark only one oval.

- Yes
- No

c. Participant information sheet

UREC reference: HAS.20.03.148

Project Manager:
Stephanie Udoh
University of the West of England
Email: stephanie.udoh@uwe.ac.uk
Tel: 01173281085

Project Title: Oral microflora in health and in Type 2 diabetes, a comparison across the African and White European population.

This is an invitation to take part in the study "Oral microflora in health and in Type 2 diabetes, a comparison across the African and White European population."

Before you decide, it is important for you to understand, why we are conducting this study and what it will involve. Please take the time to read the following information carefully and discuss it with others if you wish. Please ask us if there is anything that is unclear, or if you would like more information. Please also take the time to decide whether you wish to take part.

1. Aim of the study

This study aims at looking at the baseline microbiota profile of healthy individuals as well as the potential changes in the type and number of germs on the subgingival plaque, tongue and in bad breath (oral malodour) levels following the use of two selected toothpaste provided by Colgate-Palmolive.

2. Who are we, who is funding this study and why we are asking for your help?

We are the Centre of Oral Malodour Research at UWE and have been working in this field for the last 23 years. This study is funded by Colgate-Palmolive, USA and the university of the West of England, Bristol.

Periodontitis (subgingival plaque disease) can affect any individual during their life, causing a lot of distress and in some cases, could lead to tooth loss. Subgingival plaque disease may arise from germs on the surface of the subgingival plaque. It is believed that certain types of germs have the capacity to transform certain sorts of food into smelly gases. By measuring breath levels and sampling the subgingival plaque and tongue-scrape for the numbers of germs, an association may be seen between breath odour and quantity or types of germs present on the surface of the tongue. The results obtained from this study could substantially influence the development of oral hygiene formulations (e.g. mouthwashes, lozenges or toothpastes) that could be potentially used to reduce subgingival plaque disease and bad breath, and subsequently relieve the stress and discomfort that this condition may cause in many people.

3. Who can participate?

We welcome participation from anybody who fits the following criteria:

- Aged between 18 and 65
- White European and African origin
- Able to give informed consent
- Has the ability to attend trial dates
- Has twenty or more permanent teeth
- Has no active dental caries

Participants who fall under the following criteria will not be eligible to participate:

- Is experiencing advanced subgingival plaque disease or has active and severe caries
- Has used antibiotics within one month prior to the commencement of the study

- Has recently (within 48 hours prior to the trial) consumed or used any of the following:
  antimicrobial agents such as medicated sweets/lozenges or topical creams; food with a very strong odour such as garlic; alcoholic beverages or food; cigarettes or vaping devices
- Has a history of severe infectious diseases, or an active respiratory infection during the trial period.

4. Do I have to take part?

Your participation in this study is voluntary. If you decide to take part, you will be given this information sheet and will be asked to sign a consent form. If you decide to participate, you are still free to withdraw at any time without giving a reason.

5. What are we testing?

We are testing two different toothpastes. One toothpaste will contain the active ingredient and the other one will not have it. Both toothpastes contain Fluoride at the same concentration as most of the shop-bought brands, which will protect you against tooth decay. One toothpaste may or may not have any effect on subgingival plaque disease.

6. What are the possible benefits of taking part?

Participation in this study **will not** benefit you personally. However, longer-term the results of this study may help develop new oral formulations and help other individuals in the future.

7. What will happen to me if I take part?

This cross-over study will take place at the University of the West of England, Bristol in our laboratory (2K7) on the Frenchay Campus. Before the start of the trial, participants will be examined by a qualified professional who will assess their oral and dental health in a similar manner to a routine check- up at the dental practice. The Professional will advise each participant on the best way to brush their teeth in preparation for the trial. Participants will be asked to abstain from consuming any food or beverages on the morning of the trial, as well as being asked not to brush their teeth. The participant will be brought into the laboratory, where subgingival plaque and tongue scrape samples will be collected, after which they will be asked to keep their mouth closed and breath normally through their nose for two minutes. After this, the participants will be requested to continue breathing nasally with their mouth closed for the duration of the experiment, after which they will have their breath measured by the SIFT-MS machine. Participants will be asked to visit the laboratory on one occasions. On the day of sampling, parking space will be reserved for participants travelling by car. Each participant will receive vouchers to cover for their travel and food expenses.

The study will be conducted as follows:

Prior to the study, you should continue with your normal oral hygiene routine (brushing your teeth and flossing using dental floss). The night before your visit to

the laboratory, you must not consume any strong food (e.g. garlic, spices) and you must not drink alcohol. You can brush your teeth at night before going to bed. On the day of the study, you must not brush your teeth, drink or eat anything as this will invalidate the sample we collect from you.

Visit to the laboratory: Baseline sampling

12 hours after last brushing:

- You must not consume any food or drink or brush your teeth
- You will have your breath measured by a breath machine
- A professional will collect your subgingival plaque-scrape sample using a sterile cocktail stick
- You will give a tongue-scrape sample using a sterile toothbrush under supervision of the PI

You will have finished the study

8. How will anonymized samples be collected and mouth-air analyzed?

Each participant will be contacted by email about the day and time of the visit at their convenience. On the day of the study, you will be asked to fill in a health questionnaire prior to your samplings and assessments and sign an informed consent form which will be kept confidential and secure. This will be administered by the principal investigator.

Once you have consented to participate in the study, you will be asked to give a sample of your subgingival plaque and tongue scrape for microbiological analysis and have your breath assessed by an instrument. Each sample taken will be labelled with a code allocated to you for samples to remain anonymized and confidential from here on in through the study.

All these procedures will be performed under instruction and supervision of the investigator. The subgingival plaque scrape sample will be collected by a dental professional. All the samples and tests together should not take more than 30 minutes in total, so we hope that this should not interfere too much with your normal working day.

On the sampling day you will be asked NOT to wear strongly perfumed cosmetics, nor to consume food associated with bad breath (for example garlic, onions, curry) on the day prior to and on the day of sampling. Your normal oral hygiene practice should continue the day before the study, but on the morning of testing you should NOT perform any oral hygiene (e.g., brush your teeth or use mouthwash) or ingest food prior to the tests.

9. What will happen next to my sample?

Coded microbial samples will be analysed using conventional microbiology methods and results (data) will be stored. The microbial sample from the surface of the tongue will be stored in a -80°C freezer. After completion of the samples by all participants, all frozen samples will be shipped to Colgate-Palmolive in the USA for potential further analysis. Each sample will be coded. No personal information in relation to the participants will be given to the research team in the USA. At

completion of the study all stored samples are sterilised and disposed of, in accordance with the Human Tissue Act. Any samples which are not correctly labelled with the code will be destroyed in accordance with the Human Tissue Act.

10. What happens next if I wish to participate in the trial?

If having read the information (and any outstanding questions answered fully from our discussions) and you are happy to participate in the study, you may sign the consent form. This form states that you have read and understood this information sheet, that the participation is entirely voluntary, and that the samples are completely anonymised. You may also, if you prefer, take this information sheet away and think about whether you wish to take part**.** Please do not hesitate to contact me via the details below with any further questions and/ or when you are happy to consent.
Once consented you will be provided with; your protocol, your diary and appointment date & time for attending the laboratory, your informed consent code which needs to be written on any donated sample, and my contact details.
Consent forms will be kept for the duration of the study in a locked cabinet in a secure office. If at any point you wish to formally withdraw consent, please contact me (details below) and I will ensure your consent form is immediately destroyed and samples withdrawn and destroyed in accordance with the Human Tissue Act.

In the very unlikely event that an adverse reaction occurs in response to the procedure the University of the West of England, Bristol will consider the possibility of no-fault compensation without admitting liability. UWE confirms that it has in place all appropriate Professional Indemnity Insurance and Public Liability Insurance to cover any claims for negligence on the part of UWE staff and students in performing UWE's role in the study.

11. What if something goes wrong?

In case you are not happy about the way the team dealt with you on the day of your assessment, or about the procedure used in this study, please do not hesitate to contact an independent person, the Research and Innovation Associate Dean: Prof. Olena Doran, University of the West of England, Frenchay Campus, Coldharbour Lane, Bristol e-mail: olena.doran@uwe.ac.uk. Tel: 0117 3281670.

12. Who has ethically approved this research?

The project has been reviewed and approved by [the Faculty/University of the West of England University Research Ethics Committee. Any comments, questions or complaints about the ethical conduct of this study can be addressed to the Research Ethics Committee at the University of the West of England at: Researchethics@uwe.ac.uk

Where will the results of the research study be published?

The results will be published in internal reports, reports submitted to the funder, in conference presentations and in peer-reviewed journals. No participant will be identified from the results presented.

What if I have more questions or do not understand something?

If you would like any further information about the research please contact in the first instance:

Stephanie Udoh: stephanie.udoh@uwe.ac.uk; Telephone (0117) 3281085
Thank you for agreeing to take part in this study.

You will be given a copy of this Participant Information Sheet and your signed Consent Form to keep.

II. Diabetes risk score for participants

LOW RISK GROUP (0-7)

	Age	Ethnicity	Height (cm)	Height (m)	Weight (kg)	BMI	Blood Pressure	Risk Score	Waist Circ (cm)	Family History of Diabetes
001	25-34	White	180.0	1.8	80.0	24.69136	No	1	86	No
005	25-34	Black African	178.0	1.8	79.0	24.93372	No	7	82	No
006	18-24	Black African	161.0	1.6	53.7	20.71679	No	6	70	No
007	25-34	White	182.0	1.8	77.0	23.24598	No	0	76	No
010	25-34	White	185.0	1.9	75.0	21.91381	No	1	78	No
013	25-34	Black African	173.0	1.7	66.2	22.11902	No	7	86	No
014	45-54	White	162.0	1.6	51.0	19.43301	No	0	73	No
016	25-34	White	173.5	1.7	56.0	18.60326	No	0	74	No
018	35-44	White	158.0	1.6	82.5	33.04759	No	5	86	No
020	25-44	White	152.0	1.5	52.0	22.50693	No	5	64	Yes: Mom, gestational diabetes at 35
021	18-24	White	172.0	1.7	63.0	21.29529	No	0	79	No
022	25-34	Black African	162.0	1.6	54.0	20.57613	No	6	71.12	No

023	35-44	White	177.8	1.8	66.7	21.0927	No	1	81.28	No
026	55-64	White	170.0	1.7	72.0	24.91349	No	5	82	No
027	35-44	White	155.0	1.6	55.0	22.89282	No	0	77	No
028	25-34	White	175.0	1.8	70.0	22.85714	No	0	74	No
029	25-34	White	152.0	1.5	52.0	22.50693	No	5	69	Yes: Dad, Type 2 diabetes at 45
030	25-34	Black African	177.0	1.8	75.7	24.16292	No	7	86	No
033	18-24	White	192.0	1.9	107.0	29.02561	No	4	83	No
034	25-34	White	171.0	1.7	69.0	23.597	No	0	73.66	No
037	25-34	Black African	170.0	1.7	70.0	24.22145	No	6	86	No
038	25-34	Black African	178.9	1.8	79.0	24.68348	No	7	86.36	No
039	25-34	Black African	181.0	1.8	80.0	24.41928	No	7	86.36	No
040	18-24	White	168.0	1.7	60.0	21.2585	No	0	68	No
041	35-44	White	168.0	1.7	53.0	18.77834	No	0	73	No
042	18-24	Black African	176.5	1.8	77.0	24.71732	No	7	86	No
043	18-24	Black African	166.0	1.7	61.0	22.13674	No	6	71.12	No
044	18-24	White	168.0	1.7	70.0	24.80159	No	0	78	No
045	18-24	White	169.0	1.7	60.0	21.00767	No	0	72	No
046	25-34	Black African	154.0	1.5	59.0	24.87772	No	6	79	No
047	18-24	White	174.0	1.7	75.0	24.7721	No	0	78	No
048	25-34	Black African	180.0	1.8	80.0	24.69136	No	7	86.36	No
049	18-24	White	174.0	1.7	58.0	19.15709	No	1	73.66	No
050	18-24	Black African	180.0	1.8	73.3	22.62346	No	7	85	No
051	18-24	Black African	162.0	1.6	60.0	22.86237	No	6	73	No
052	18-24	White	172.0	1.7	65.0	21.97134	No	5	69	Yes, dad with type 2 at 24.
053	25-34	Black African	168.0	1.7	70.0	24.80159	No	6	77	No
054	25-34	White	155.0	1.6	75.0	31.21748	No	5	81	No
055	25-34	White	160.0	1.6	63.0	24.60938	No	0	76	No

056	25-34	Black African	160.0	1.6	62.0	24.21875	No	6	82.5	No
057	25-34	Black African	185.0	1.9	75.0	21.91381	No	7	81.28	No
058	18-24	Black African	170.0	1.7	67.0	23.18339	No	6	68.58	No
059	35-44	White	162.0	1.6	56.0	21.33821	No	4	92	No
060	35-44	White	167.6	1.7	73.0	25.97572	No	3	84	No
061	25-34	Black African	184.0	1.8	65.0	19.19896	No	7	82	No
062	18-24	White	160.0	1.6	60.0	23.4375	No	0	71	No
063	18-24	Black African	178.0	1.8	65.0	20.51509	No	6	73	No
064	25 - 34	Black African	171.0	1.7	72.0	24.62296	No	7	78	No
065	25 - 34	Black African	160.0	1.6	62.0	24.21875	No	7	81.28	No
066	25 - 34	White	172.0	1.7	61.6	20.82207	No	0	72	No
067	25 - 34	White	172.0	1.7	58.6	19.808	No	0	78.74	No
068	25 - 34	White	159	1.6	60	23.73324	No	0	83.82	No
069	18-24	Black African	160.02	1.6	60	23.43164	No	6	72	No
070	18-24	Black African	168.0	1.7	54.2	19.20351	No	6	73	No
071	35-44	Black African	182.0	1.8	82.0	24.75546	No	7	88	No
072	25-34	Black African	174.0	1.7	72.0	23.78121	No	7	82	No
073	25-34	Black African	172.0	1.7	65.2	22.03894	No	7	78.0	No
074	25-34	Black African	165	1.7	60	22.03857	No	6	76.2	No
075	18-24	Black African	170	1.7	70	24.22145	No	6	81	No
076	18-24	Black African	167	1.7	53.6	19.21905	No	6	67	No

HIGH RISK GROUP (16 -47)

	Age	**Ethnicity**	**Height (cm)**	**Height (m)**	**Weight (kg)**	**BMI**	**Blood Pressure**	**Risk Score**	**Waist Circ (cm)**	**Family History of Diabetes**
003	25-34	Black African	185.0	1.9	97.0	28.34186	No	19	110	No
004	25-34	Black African	176.0	1.8	89.0	28.73192	Yes	19	90	No
008	25-34	Black African	185.0	1.9	140.0	40.90577	No	24	133	No

009	18-24	Black African	177.8	1.8	96.0	30.36741	No	16	91.44	No
011	25-34	Black African	171.0	1.7	91.7	31.36008	No	16	97	No
017	25-34	Black African	162.6	1.6	98.0	37.08504	No	25	109.22	Yes, mom at 43 (gestational diabetes)
032	35-44	Black African	177.0	1.8	94.0	30.00415	No	16	98	No
077	24-35	Black African	157.0	1.6	78.9	32.00941	No	20	96	Yes
078	24-35	Black African	183	1.8	100	29.86055	Yes	19	91.44	No

III. Comprehensive detail of overall microbiota in White European and Black African ethnic group

a. Low risk subgingival plaque samples
KRONA INTERACTIVE

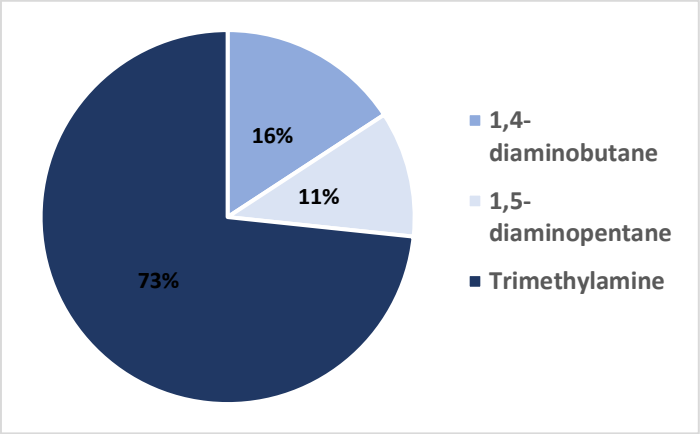
b. Low risk tongue samples
KRONA INTERACTIVE

c. High risk subgingival plaque samples
KRONA INTERACTIVE

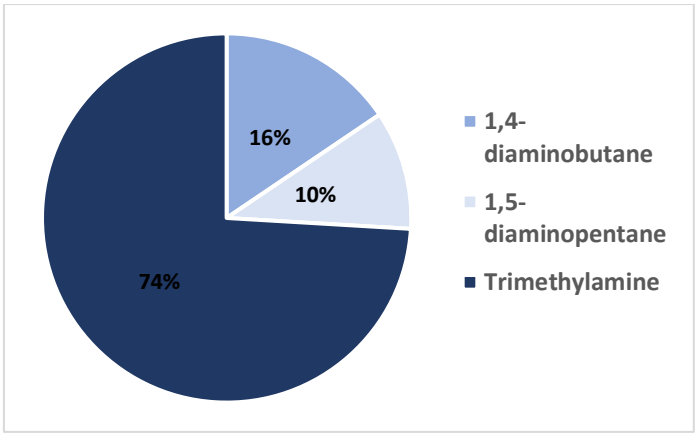
d. High risk tongue samples
KRONA INTERACTIVE

IV. Representation of VOC classes measured in participants

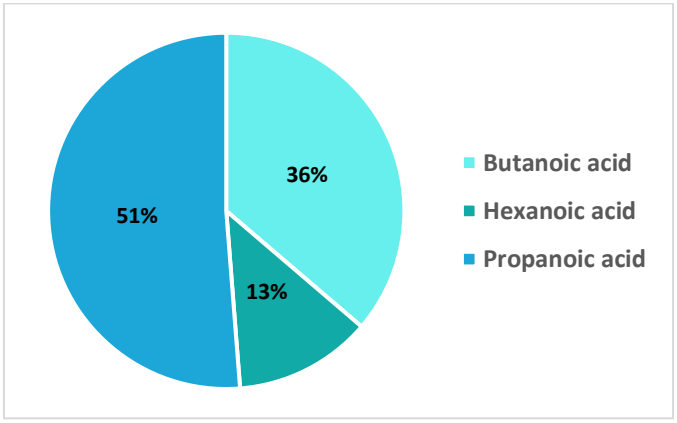
a. Representation of Amines in healthy participants from White European group (n=30)



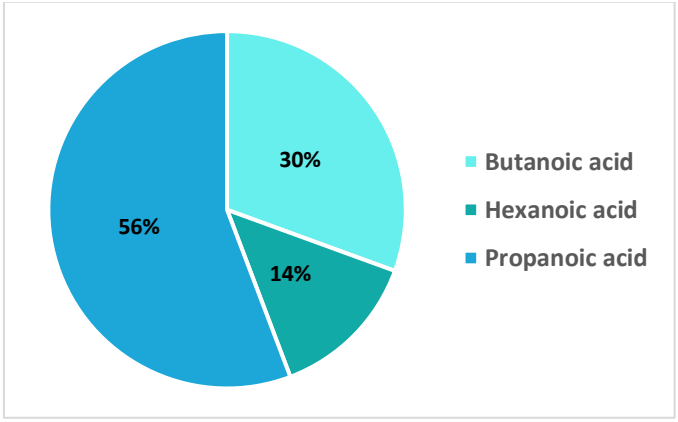
b. Representation of Amines in healthy participants from Black African group (n=30)



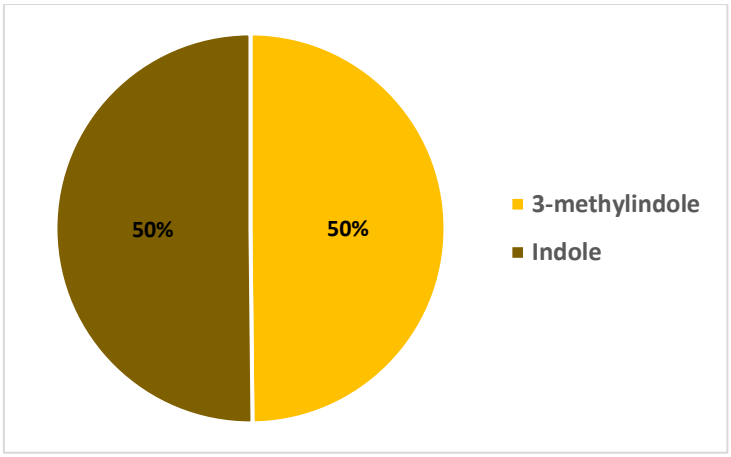
c. Representation of Acids in healthy participants from White European group (n=30)



d. Representation of Acids in healthy participants from Black African group (n=30)



e. Representation of Indoles in healthy participants from White European group (n=30)



f. Representation of Indoles in healthy participants from Black African group (n=30)