Cape Peninsula University of Technology

# THE ORAL MICROBIOME AND ITS ASSOCIATION WITH CHRONIC AND SYSTEMIC DISEASE IN A SOUTH AFRICAN POPULATION.

**by**

**YVONNE PRINCE (197101968)**

**Thesis submitted in fulfilment of requirements for the award of the Degree of Doctor of Philosophy (PhD) in Biomedical Sciences**

**In the Faculty of Health and Wellness Sciences at the Cape Peninsula University of Technology**

## Supervisors:

**Professor Tandi E Matsha-Erasmus (Main Supervisor)**

**Professor Glenda M Davison (Co-Supervisor)**

**April 2021**

# DECLARATION

I, *Yvonne Prince*, hereby declare that the contents of this thesis reflect my own work and I have not, in part or its entirety, submitted it to any other university for academic examination. The opinions expressed in the thesis are my own and are not necessarily those of the Cape Peninsula University of Technology.

7th April 2021

…… ……..

**Signature** **Date**

# ABSTRACT


**Background**

The oral microbiome is a complex system that harbors a personalized microbiome. The main function is to maintain health and by doing so they interact with each other and also the human host. If any disruption occurs it will cause an ecological shift in the oral microbiota which will allow pathogens in the form of biofilms to manifest and cause oral diseases. Severe forms of oral disease may progress into systemic diseases such as Diabetes Mellites (DM), Cardiovascular disease (CVD), and Metabolic syndrome (MetS). As the relationship between the human microbiome and health becomes more apparent, more researchers are investigating these relationships as it is clear from previous research that disruptions of the oral microbiome can initiate an abnormal inflammatory response leading to periodontitis and systemic disease. However, information on the causes and changes in the oral microbiota of the oral cavity in individuals with diabetes and MetS is limited. Therefore this project aimed to investigate and characterise the oral plaque samples, using 16s rDNA, in individuals who smoked, are Diabetic, and who had MetS.

**Methods**

Dental assessment was conducted according to guidelines from the World Health Organization (WHO., 2016) and the Community Periodontal Index. Each tooth was probed for bleeding on probing (BOP) and was recorded as presence or absence of bleeding after gentle periodontal probing around each tooth circumference. For pocket depth (PD), each tooth was probed in its whole circumference, and the highest score was recorded. Plaque samples were collected using the wood toothpick method. The device was inserted in the subgingival crevice between the maxillary second premolar and the first upper molar, and 4 toothpick samples were collected from both sides of the mouth and stored separately. Plaque samples with visible presence of blood were not included in this study. The samples were immediately stored at –80 °C until DNA isolation and purification. DNA was extracted as per manufacturer instruction from each pooled toothpick using a DNA extraction kit from Zymo Quick–DNA Fungal/Bacterial Miniprep Kit (Zymo Research). Next-generation sequencing (NGS) was conducted on the 128 plaque samples using the Ion 530 Chip where massive Massively parallel sequencing was performed on the Ion S5 Gene Studio with the Ion S5 IonTorrent.

**Results**

When analysing the oral microbiota in patients across glycaemic status more than two-thirds of all subjects (63.4%, *n* = 81) had bleeding on probing. Although this was nonsignificantly distributed among the glycemic statuses, 24 (75%) subjects with known diabetes and treatment had gingival bleeding. Periodontal PD ≥4 mm was observed in 75 (58.6%) individuals, and these included 7 (5.5%) with PD ≥6 mm. The most abundant genera observed When oral microbiota analyses were performed patients across glycaemic status genera Fusobacteria and Actinobacteria were significantly enriched and Proteobacteria less enriched in subjects with DM and prediabetes. In those with gingival bleeding, Bacteroidetes were significantly more enriched. As for patients with DM and gingival bleeding reduced abundance was seen in genera Actinobacteria genera. Furthermore, in the smoking group, the oral microbiome was significantly enriched with gram-negative anaerobes. When comparing the oral microbiota within smokers and non-smokers a reduction in the abundance of the phyla *Actinobacteria* in smokers was observed. Genera *Fusobacterium* and *Campylobacter* were found in higher abundance, while genera *Leptotrichia, Actinomyces, Corynebacterium,* and *Lautropia* were found in decrease abundance were observed in smokers. As for the comparison of subjects with MetS and without MetS a significant increase in gram-positive aerobic and anaerobic microbiota was observed in those with MetS. Also in MetS subjects, the abundant genera present was Actinomyces, Corynebacterium, and Fusobacterium.

**Conclusion**

Our findings, therefore, concluded that the key to maintaining health is to maintain a well-balanced oral microbiome. Any disruption in the ecosystem due to risk factors such as smoking and other environmental factors will result in a pathogen-rich oral cavity which may provide entry of these oral bacteria into the surrounding tissue resulting in periodontal disease. This is achieved by changing the relationship between microbes and host, by increasing the relative abundance and the acquisition of virulent factors promoting oral disease. As suggested by literature the oral cavity is the primary gateway to the body and severe cases of periodontal disease may promote systemic diseases such as DM, CVD, and MetS.

# ACKNOWLEDGEMENTS

I would like to express my special thanks and gratitude to my supervisors Professor Thandi Matsha-Erasmus and Professor Glenda Davison for their continued support and encouragement. Thank you, Professor Matsha-Erasmus, for granting me the opportunity to be part of the study and your Cardiometabolic Health Research Unit. Thank you for helping me with every aspect of the study pushing and pushing me to limits I did not even think was possible.

Thank you to my Head of Department Professor Glenda Davidson for unlimited encouraging support and guidance and for assisting with improving my scientific writing skills and continuous guidance.

Dr. Dinie Hon for her wise and encouraging words and Ms. Saarah Davids for her wealth of statistical expertise and her encouraging motivational words and support with the various statistical analyses conducted in the study. Ms. Soraya Chalklen for her assistance with accessing stored samples and reagents used in the study.

My co-authors for critical critique with finalising my research papers Professor Erasmus and Professor A Kengne. Thank you to the rest of the unit colleagues Dipou, Muki, Wendy, and a special thank you to Dr. Don Matshazi, and Dr. Shanel Raugubeer for assistance with finalising the papers and thesis. A special thanks to my colleagues in the Biomedical Science Department, especially our pillar of the department Sanette Nothnagel.

Thank you to Dr. Stanton Hector for helping me optimising my methodology. Mrs. B Gaxamba for stepping in for me whenever I needed her and Mr. N Sogwagwa for his technical assistance and Ms. Fadia Alexander for encouraging words and helping with the ordering of consumables.

The Bellville South Ward 009 community for partaking in the study as well as the Bellville South Community Health Forum. The South African Medical Research Council (SAMRC) and the National Research Foundation for providing funding that enabled the study to be conducted.

The SAMRC/CPUT Cardiometabolic Health Research Unit at the Cape Peninsula University of Technology's Biomedical Sciences Department for providing the facilities for conducting the laboratory investigations.

Lastly to my family for continuous moral support. Not forgetting **GOD, THE ALMIGHTY** for carrying me true this time and always opening doors for me.

# DEDICATION

To my departed **mother, mom** thank you for raising me to be the strong, independent woman I am today. Even in your absence, your light is still shining in my life and the rest of the family's lives. Thank you, Dad, for teaching us to put God first and encouraging me to educate myself. Thank you to my husband **Duane** and daughters **Daniëlle and Tyler** for the endless love and support and for motivating me to never give up. Thank you for all your patience. I would also want to thank my sister **Henrietta Polman** for investing in my studies, your investment has brought me to where I am today. Finally thank you to the rest of my family and in-laws for encouraging me to work harder and not to give up. I love you all.

# LIST OF ABBREVIATIONS

| | |
|---|---|
| AIDS: | Acquired Immunodeficiency Syndrome |
| ATP III: | National Cholesterol Education Program Third Adult Treatment Panel. |
| BOP: | Bleeding on probing |
| BP: | Blood Pressure |
| CDC: | Centre for Disease Control and Prevention |
| CPITN: | Community periodontal index of treatment |
| CRP: | C-reactive protein |
| CS: | Cigarette smoke |
| CVD: | Cardiovascular disease |
| DBP: | Diastolic Blood Pressure |
| FBG: | Fasting Blood Glucose |
| GIT: | Gastrointestinal tract |
| GNB: | Gram-negative bacilli |
| GPB: | Gram-positive bacilli |
| HDL: | High-density lipoprotein |
| HIV: | Human Immunodeficiency Virus |
| HMP: | Human Microbiome Project |
| HNF4A: | Hepatocyte nuclear factor alpha 4 A |
| HOMIM: | Human Oral Microbe Identification Microarray |
| IDF: | International Diabetes Federation |
| IFN: | Interferon |
| IL: | Interleukins |
| JIS: | Joint-interim Statement |
| LMICs : | Low- and Middle- Income Countries |
| LPS: | Lipopolysaccharides |
| LRRs: | Leucine-Rich Repeats |
| MAP kinase: | Mitogen-activated protein kinase |
| MetS: | Metabolic syndrome |
| NCDs: | Non-communicable diseases |
| OLP: | Oral lichen planus |
| OR: | Odds Ratio |

| | |
|---|---|
| OUT: | Operational Taxonomic Unit |
| PAD: | Peripheral Arterial Disease |
| PAMP's: | Pathogen Associated Molecular Patterns |
| PD: | Pocket dept |
| PGE2: | Prostaglandin E2 |
| SA: | South Africa |
| SBP: | Systolic Blood Pressure |
| T2D: | Type two diabetes |
| TG: | Triglycerides |
| TLR: | Toll-like receptor |
| US: | United States |
| Waist-C: | Waist circumference |

# GLOSSARY

**Terms:**

| | |
|---|---|
| **Acidophilic bacteria** | Acidophilic organisms are those that thrive under highly acidic conditions (usually at pH 2.0 or below). |
| **Anaerobiosis** | Life in the absence of air or free oxygen. |
| **Archaea** | Microorganisms are similar to bacteria in size and simplicity of structure but radically different in the molecular organization. They are now believed to constitute an ancient group that is intermediate between the bacteria and eukaryotes. |
| **Atherosclerosis** | A disease of the arteries characterized by the deposition of fatty material on their inner walls. |
| **Capnophiles** | Microorganisms that thrive in the presence of high concentrations of carbon dioxide. |
| **Commensals** | A micro-organism that lives continuously on, or in certain parts of, the body, without causing disease. |
| **Dysbiosis** | Any change to the composition of resident commensal communities relative to the community found in healthy. |
| **Genome** | Genome is the genetic material of an organism. It is the complete set of DNA including all of its genes. |
| **Genus** | A principal taxonomic category that ranks above species and below family and is denoted by a capitalized Latin name. |
| **Heterogeneity** | the quality or state of being diverse in character or content. |
| **Inflammaging** | Is a chronic low-grade inflammation that develops with advanced age. |
| **Microbiome** | The microbiome refers to a community of microbes residing in a defined environment, comprising of bacteria, viruses, fungi, and protozoa, together with their genes and genomes in any given locus. |
| **Microbiota** | Refers to the microorganisms found in a specific environment by type. This includes bacteria, fungi, viruses, protozoa, and archaea |

| | |
|---|---|
| **Operational Taxonomic Unit** | The grouping of bacterial 16S rRNA gene sequences by their similarity.7 Sequences are typically grouped at a value between 97% and 99%. |
| **Periodontium** | Periodontium is the tissue including the gum, bone, cementum, and periodontal ligament -- that surrounds and supports the tooth. |
| **Species** | A group of living organisms consisting of similar individuals capable of exchanging genes or interbreeding. The species is the principal natural taxonomic unit, ranking below a genus and denoted by a Latin binomial. |

# TABLE OF CONTENT

## Contents

# LIST OF TABLES AND FIGURES

## CHAPTER 3



## CHAPTER 4

**Supplementary Appendix Table 1:** Community Periodontal Index (CPI) (WHO, 2016).

coordinate analysis (PCoA) plots for DM (red), Pre-DM (blue) and NGT (green) are shown to determine Bray–Curtis distances.

**Supplementary Appendix Figure 3:** Composition of the oral microbial community at phylum as the percentage of relative abundance in individuals with (A), normal glucose tolerance with or without gingival bleeding on probing (NGT GB+ve, NGT GB-ve); prediabetes with or without gingival bleeding on probing (Pre-DM GB+ve, Pre-DM GB-ve) versus; diabetes mellitus with or without gingival bleeding on probing (DM GB+ve, DM GB-ve). (B), normal glucose tolerance with or without pocket depth ≥ 4mm (NGT PD+ve, NGT PD-ve); Pre-DM PD+ve, Pre-DM -ve; DM PD+ve, DM PD-ve.

**Supplementary Appendix Figure 4:** Composition of the oral microbial community at genus as the percentage of relative abundance in individuals with (A), normotolerant (NGT), prediabetes (Pre-DM), and diabetes (DM) individuals; (B), gingival bleeding on probing (GB+ve) and no gingival bleeding on probing (GB-ve); (C), pocket depth ≥ 4mm (PD+ve) and pocket depth <4mm (PD-ve).

**Supplementary Appendix Figure 5:** Composition of the oral microbial community at genus as the percentage of relative abundance in individuals with (A), normal glucose tolerance with or without gingival bleeding on probing (NGT GB+ve, NGT GB-ve); prediabetes with or without gingival bleeding on probing (Pre-DM GB+ve, Pre-DM GB-ve) versus; diabetes mellitus with or without gingival bleeding on probing (DM GB+ve, DM GB-ve). (B), normal glucose tolerance with or without pocket depth ≥ 4mm (NGT PD+ve, NGT PD-ve); Pre-DM PD+ve, Pre-DM -ve; DM PD+ve, DM PD-ve.

# CHAPTER 1: INTRODUCTION

The human oral cavity is heavily colonized with over 700 microbes which consist of viruses, protozoa, fungi, archaea, and bacteria(Kilian et al., 2016). The oral cavity is divided into two types of surfaces, which include the hard surface of the teeth and the soft tissue of the oral mucosa. The entire oral anatomy includes the teeth, gingival sulcus, tongue, cheeks, hard and soft palates, and tonsils. Each of these oral niches has been colonised with the microbiota of its preference which function is to provide an ideal environment for symbiosis and to supply nutrition for the oral microbes to flourish (Xu et al., 2015; Kilian, et al., 2016). Other functions of the oral microbiota are to inhibit colonisation by oral pathogens and prevent oral diseases (Wade, 2013).

The two most well-known oral diseases are dental caries (tooth decay) and periodontal (gum) diseases (Wade, 2013). Periodontal disease is estimated to affect nearly half the global population (Petersen et al., 2005; Vos et al., 2017), and may occur due to lifestyle factors such as diet, smoking, and alcohol consumption. These factors disturb the oral equilibrium resulting in disease (Lassalle et al., 2018; Fan et al., 2018; Jiang et al., 2020). For instance, major differences in the oral microbiota have been observed in the diet intake of hunter-gatherers and traditional farmers living in close range to each other in the Philippines. In this study, it was observed that the abundance of *Neisseria* and *Haemophilus* was significantly different between the two groups. In contrast, the composition of the microbiota of people who don't eat meat was different at all taxonomic levels including oral pathogens (*Neisseria* and *Haemophilus*) and respiratory tract microbes (*Campylobacter and Porphyromonas*) (Lassalle et al., 2018). This strongly suggested that diet plays a role in initiating changes within the oral microbiome.

The effects of alcohol consumption were studied in a large cross-sectional study in the United States (US) where they found differences in the oral microbiota and the overall bacterial profile in alcohol drinkers. It was observed that the oral bacteria of order *Lactobacillales* was lower, while the genera *Actinomyces, Leptotrichia, Cardiobacterium,* and *Neisseria* were enriched. This suggested that heavy alcohol use may affect the oral microbiota (Fan et al., 2018).

Smoking has also been identified as a risk factor associated with the onset and progression of periodontal disease (Jiang et al., 2020b). In smokers, numerous mechanisms have been associated with the onset of periodontal diseases. One of these is the formation of biofilms. In those exposed to tobacco smoking, there is an increase of biofilm formation, with an increase

in specific bacteria such as *Streptococci pneumonia* (Mutepe et al., 2013) and *Staphylococci aureus* (Kulkarni et al., 2012a). Both of these bacteria contribute to the onset of disease. Other key pathogens such as *P gingivalis* together with mechanisms that allow changes to occur in the immune homeostasis may also contribute to the alteration of the subgingival microbiota in smokers. Nevertheless, not much evidence is available on populations living in Africa. Recently, a cross-sectional study examining the periodontal status and cotinine levels in an urban South African population (Chikte et al., 2019.) was performed. In this study, it was found that more than half of the study population were smokers (52.7%) with increased cotinine levels (≥15 ng/mL). This appeared to have had no significance on the periodontal variables however periodontal disease was prevalent in the studied populations and the condition was worse in male participants. It is therefore suggested that smoking may cause a disturbance in the oral microbiota and increase the level of certain periodontal pathogens through different mechanisms causing the onset of periodontal disease.

These findings have been supported by previous studies performed by (Wu et al., 2019; Hanioka et al., 2019a; Yu et al., 2017; Moon et al., 2015a), although, due to different sampling and laboratory techniques, inconsistencies have been reported with regards to the actual species involved. For example, Karabudak et al., 2019 used buccal samples and Ion Torrent IS5TM Next-Generation Sequencing system with 950 flows technique to investigate microbial differences in smokers and non-smokers. This work reported an abundance in, *Veillonella atypica, Streptococcus australis, Prevotella melaninogenica, Prevotella salivae, and Rothia mucilaginosa* in smokers whilst (Wirth et al., 2020), used saliva samples and Ion Torrent PGM TM platform, observed species predominance in *Prevotella, Veillonella,* and *Streptococcus* in both smokers and non-smokers but significant increases in the genera *Prevotella and Megasphaera*.

We can therefore conclude that the core microbiome has a similar composition with several dominant species even when different sample types are analysed. Its main function is to maintain oral health but due to factors such as diet and outside stressors such as alcohol and smoking, differences in the variable microbiome can be observed.

Periodontal disease has been reported as the 6$^{th}$ complication of diabetes mellitus (DM) and recent studies suggest that subjects with diabetes have an increased risk of developing periodontitis due to poor glycaemic control (Preshaw & Bissett, 2019). The mechanisms

underlying the relationship between these two disorders remain vague and have resulted in numerous hypotheses with the development of uncontrolled glycaemic levels being one (Winning & Linden, 2018 ). This theory has been supported by a meta-analysis study where investigators analysed the severity of periodontal disease across two statuses including diabetics and non-diabetics. They concluded that subjects with diabetes have an increasingly higher severity of periodontal disease compared to normal subjects (Santos et al., 2015) The second reason for the relationship between diabetes and periodontal disease may be due to changes in polymorphonuclear cell function and abnormalities of the inflammatory response within the tissue surrounding and supporting the tooth (periodontium). This aberrant inflammatory response may enhance the severity of periodontitis and influence the host defense (Wolff, 2014). Thirdly, Wang et al., 2019 suggested that the subgingival microflora may play a role in the association between periodontal diseases and diabetes, suggesting a direct relationship between periodontal pathogenic bacteria (such as *Porphyromonas gingivalis and Actinomyces actinomycetemcomitans*) and glycaemic control and diabetes risk (Castrillon et al., 2015). Furthermore, the microbiome of the gut, vagina, and skin are also altered in diabetes, adding evidence to the idea that altered microflora influences the development of diabetes. However, there is still limited information on the direct relationship between the oral microbiome and diabetes and therefore further clarity is required.

Oral dysbiosis is not only responsible for causing oral-related diseases but can cause systemic diseases such as cardiovascular disease (CVD), diabetes mellitus, rheumatoid arthritis, and Alzheimer's disease (Sudhakara et al., 2018). The literature describes both periodontitis and CVD as chronic diseases with slow onset starting from a younger age but the exact mechanism is still unknown. Since periodontitis has increased in prevalence, affecting 11.2% of the global population (Sanz et al., 2020) further research must be conducted. Recently oral dysbiosis was evident in a study conducted on the gut microbiome of mice, identifying the key oral pathogen *Porphyromonas gingivalis* to encourage systemic inflammation and metabolic syndrome (Arimatsu et al., 2014). This suggests that the oral microbiota may cause low-grade systemic inflammation in people, promoting the onset of Metabolic syndrome (MetS).

MetS is a worldwide concern and have been classified as a syndrome in which individuals display visceral obesity, dyslipidemia (high levels of triglycerides, and low levels of high-density lipoprotein (HDL) cholesterol), hyperglycemia, and hypertension (Alberti et al., 2005). The oral microbiome may play an important role in the onset of MetS but, as most research has

been done on the association of MetS with the gut microbiome (Vijay-Kumar et al., 2010; Zhang et al., 2010; Ussar et al., 2015), more investigation is required. Earlier studies have reported on the prospect of the clinical use of oral bacteria in various systemic diseases such as pancreatic cancer, rheumatoid arthritis, and lung cancer (Farrell et al., 2012; Zhang et al., 2015) suggesting the role of oral bacteria as potential biomarkers for metabolic syndrome.

Since MetS are still understudied and most research has been performed on gut microbiota obtained from stool samples of mice (Si et al., 2017), we aim to investigate and analyse the oral microbiome from individuals with risk factors for Mets. to characterised the microorganisms residing in the oral cavity.

It is clear from previous research that disruptions of the oral microbiome can initiate an abnormal inflammatory response leading to periodontitis and systemic disease. However, information on the causes and changes in the microorganisms of the oral cavity in individuals with diabetes and MetS are scarce. Therefore this project aimed to investigate the makeup of the oral biome, using 16s rDNA, in individuals who smoked, had diabetes, and who had MetS to characterise the microorganisms residing in the oral cavity

The objectives of the study were:

1. To determine and characterised the oral microbial diversity associated with periodontitis and diabetes.
2. To determine the effect of smoking on the oral microbiome in smokers compared to non-smokers.
3. To determine whether oral microbial diversity is associated with cardiometabolic traits such as dysglycaemia and metabolic syndrome.

# CHAPTER 2: LITERATURE REVIEW

## 2.1. THE HUMAN MICROBIOME

Humans have been described as a 'supra-organism' consisting of trillions of microorganisms. Its complex makeup consists of microbial cells made up of bacteria, viruses, fungi, and archaea which puts the total number of microorganisms count in an average human at approximately $3.0 \times 10^{13}$ outnumbering human cells in the body (Sender et al., 2016). Changes in the environment, distribution, and evolution of the essential microorganisms could be important as these factors contribute to disturbances resulting in microbial imbalance and a shift in human health to a disease state (Wang et al., 2017).

Before the start of the Human Microbiome Project (HMP) scientists projected that ~100 000 genes would be present in the human microbiome, but were astonished that only ~ 20 000 protein-coding genes were identified which were similar to the fruit fly genome (Knight et al., 2017). The Human Microbiome Project (HMP) was therefore established to better understand the physiology, genetic makeup, and factors influencing the distribution of the microbiome. The results of this project may also provide insight into how rapidly advancing technology, human lifestyles, and the environment affects the 'micro-evolution' of humans and consequently the health and the predisposition to the disease. Scientists are also studying the human microbiome to understand how these imbalances can be restored using treatment (Muszer et al., 2015). Such evidence was published in almost 350 research papers. For instance, evidence has emerged identifying novel taxa including the genera *Dorea, Oscillibacter, and Desulfovibrio*, which are associated with disease states (colorectal adenoma, dietary shifts, and opportunistic infections). Other microbes that have been discovered are from the *Barnesiella* genus and a possible novel family within *Clostridiales* (Huttenhower et al., 2012b; Morgan et al., 2008; Wylie et al, 2012). Furthermore, the HMP uncovered several novel taxa at the genus level and the abundance of these novel OTUs was <2 %, but they were present in a significant number of subjects. The Microbiome Project 2012 discovered that the human microbiome contains between 3,500 and 35,000 Operational Taxonomic Units (OTU).

The human microbiome varies between different sites of the human body with the gut and the oral cavity hosting the majority. Antonie van Leeuwenhoek, in 1680, compared his own oral and fecal microbiota and noted the remarkable differences in the microbes between these two

habitats. He also reported on the differences between samples obtained from individuals in states of health and disease and noted that a healthy gut genome consists of at least 1000 different species of which the two most common phyla were *Bacteroidetes* and *Firmicutes* (Ursell et al., 2014). These initial observations encouraged scientists to explore and characterise the distribution of the normal microbiome to identify any imbalances which could be linked to disease. This was made possible with the introduction of new technologies such as 16S rRNA analysis. These molecular studies aimed to provide a greater understanding of the host-microbiome interactions that impact disease (Amon & Sanderson, 2017; Ursell et al., 2012).

## 2.2. THE GUT MICROBIOME

The human gastrointestinal tract (GIT) microbiome develops during birth. These microbes consist of Bacteria, Eukaryotes, Viruses, and Archaea and contain approximately $10^{11}$ to $10^{12}$ cells per milliliter, making the colon the most densely populated habitat in humans. These microbiotas are encoded by over 3 million genes (Rinninella et al., 2019). The microbes live in symbiosis with the human and their primary function is to maintain health (Milani et al., 2017). During birth, infants develop their microbiome through the birth canal of the mother and thereafter while being breastfed (Dreyer & Liebl, 2018; Holgerson et al., 2013). In the case of birth through caesarean section, infants acquire their microbiome through the mother's skin (Dunn et al., 2017). As they are growing and are introduced to solid foods the composition of the microbiome may shift but thereafter remains stable (Baothman et al., 2016). Although the majority of microbiomes in humans are similar, they can vary at certain sites of colonization, and therefore each gut microbiome is unique. The main six phyla present in the gut are *Bacteroidetes, Firmicutes, Proteobacteria, Actinobacteria*, *Verrucomicrobia, and Euryarchaeota* (Bliss & Whiteside, 2018). These microbiotas reside undisturbed within the human gut until dysbiosis occurs at which time opportunistic pathogens may take over and affect the host health (Milani et al., 2017; Shreiner et al., 2015).

Thompson et al., 2017 reported that environmental factors including diet may influence the composition of the gut microbiome. This is evident in a study recently conducted by (Zou et al., 2018) where they found that a diet rich in fibres significantly improves glucose control and encourages a healthier metabolic profile in subjects with diabetes mellites type 2. They also reported that the phyla *Bacteriodetes* played a role in the digestion of animal fat, while the phyla *Prevotella* was linked to carbohydrate diets (Clemente et al., 2012). In a comparative twin study performed between a Korean cohort and a US cohort, utilising Unifrac distance

analysis, a significant difference in the gut phylogenetic microbiome composition was observed in both identical and fraternal twins. Albeit, when the alpha analysis was performed, no significant difference was noted, which suggested that the Korean cohort did not contain a greater number of OTUs than the US cohort (Moon et al., 2015b).

### 2.2.1. Risk factors influencing the Gut microbiome

The function of the gut microbiome is to maintain symbiosis, facilitate digestion, control invasion by foreign microorganisms, and regulate immunity and metabolism. Any disruption in the microbiota may result in systemic disease (Sheflin et al., 2017). Several factors can influence the composition of the gut bacteria and ultimately affect health. One of these factors includes the mode of delivery at birth (Wen & Duffy, 2017). Studies have shown that the gut bacterial community composition varies between infants born via caesarean section and infants born via vaginal delivery (Arboleya et al., 2018). Kuhle et al., 2015 suggested that infants born by caesarean have an increased risk of developing obesity and/or diabetes compared to those born vaginally because infants born vaginally are introduced to the mother's microbiota and therefore can produce white blood cells and other components of the immune system (Kulas et al., 2013). Studies supporting the suggestion that infants born via caesarean tend to be overweight have been supported by a cross-sectional study of 8900 preschool children where the odds of being overweight were 1.35 and of obesity were 1.25 in children delivered by caesarean section (Rutayisire et al., 2016). Similar findings were suggested by (Portela et al., 2015). However, inconsistencies were evident in other studies as most studies were looking at the association of mode of delivery methods were not able to adjust for vital cofounders such as maternal pre-pregnancy weight. As it has been suggested that mothers who are obese are most likely to give birth to obese children and therefore would most likely require a caesarean section (Kuhle et al., 2010; Reilly et al., 2005). This was evident when Flemming et al., 2013 did a cohort analysis looking at the association between caesarean section and childhood obesity. In this analysis, they were table to compare vaginal delivery, a caesarean section which was associated with offspring obesity, and found an odds ratio (OR) 1.49, 95% CI 1.10 to 2.00) in the univariate analysis. After adding maternal prepregnancy weight to the multiple regression model, the OR for obesity dropped from 1.48 to 1.20 (95% CI 0.87 to 1.65). When a caesarean section with and without labour was considered separately, we found no statistically significant associations relative to the vaginal delivery group (OR 1.24, 95% CI 0.84 to 1.82 and OR 1.03, 95% CI 0.58 to 1.84). Therefore, the study finding does not support a causal association between caesarean section and childhood obesity and suggested that

maternal prepregnancy weight was a vital confounder in the association between caesarean delivery and childhood obesity and needs to be considered in future studies (Flemming et al., 2013).

Toll-like Receptors (TLR) have been associated with inducing low-grade fever. Toll-like receptors are very important and form part of the innate immune response. They recognise pathogen-associated molecular patterns (PAMP's) on microbes and can influence the way the immune system responds. These pattern recognition receptors (PRRs) which initially recognise microbes initiate downstream signalling pathways that cause the innate immune response to produce inflammatory cytokines, type I interferon (IFN), and other mediators. These processes not only trigger immediate host defensive responses such as inflammation but also begin and control antigen-specific adaptive immune responses (Janeway & Medzhitov, 2002). The initiation of these responses is vital for the removal of the infected bacteria as well as crucial for the instruction of antigen-specific adaptive immune responses. A variety of Toll-like receptors have been identified, and in humans, 10 members (TLR1–TLR10) have been identified.

TLRs localises to the cell surface or intracellular compartments of macrophages and they identify distinct or overlapping PAMPs such as lipid, lipoprotein, protein, and nucleic acid. Each TLR consists of an ectodomain with leucine-rich repeats (LRRs) that mediate PAMPs recognition, a transmembrane domain, and a cytoplasmic Toll/IL-1 receptor (TIR) domain that initiates down-stream signalling. They interact with the respective PAMP or DAMPs as a homo- or heterodimer along with a co-receptor or accessory molecule (Bota et al., 2014). Upon PAMPs and DAMPs recognition, TLRs employ Toll/IL-1 receptor (TIR) domain-containing adaptor proteins such as MyD88 and TRIF, which initiate signal transduction pathways that culminate in the activation of NF-kB, IRFs, or mitogen-activated protein kinase (MAP kinases). These molecules regulate the expression of cytokines, chemokines, and type I IFNs that ultimately protect the host from microbial infection. New studies have shown that the correct cellular localization of TLRs is important in the control of the signalling, and that cell-type-specific signalling downstream of TLRs determines innate immune responses (Kawasaki & Kawai, 2014). The proper discovery and understanding of TLR genetic studies during the past decade have contributed to cell biological and biochemical approaches that have highlighted the importance of cellular localization of these receptors in the regulation of

downstream signalling. It is well known that the interaction between gut microbiota and the local TLRs helps to maintain the homeostasis of intestinal immunity (Yiu et al., 2017).

Another influential factor influencing the makeup of the gut microbiome within 24 hours is diet (Compare et al., 2016). For instance, an animal-based or high carbohydrate intake can interfere with the sleep-wake cycle (circadian rhythm) (Singh et al., 2017), while inflammation and systemic stress can further influence the characterization of the gut microbiota. The gut microbiome provides humans with the ability to digest plant polysaccharides like xylan-, pectin- and arabinose-containing carbohydrates in plant-based diets (He et al., 2015). Several human and animal studies have shown that high-calorie diets may be associated with obesity and type 2 diabetes (Field et al., 2007; Yamashita et al., 2005) and that the association between diet and obesity lies in the gut microbiota (Sonnenburg et al., 2016). Therefore, it is important to know what effect diet has on the gut microbiome. Additional factors such as genetics, physical surroundings, the use of antibiotics, and age are all mechanisms associated with and influence the gut microbiome.

### 2.3. THE ORAL MICROBIOME

The oral microbiome is known as the collective genome of microorganisms that resides in the oral cavity (Deo & Deshmukh, 2019). After the gastrointestinal tract (GIT) microbiome, the oral microbiome is the second-largest microbiome (Deo & Deshmukh, 2019) and infants develop 70% of their oral microbiome via the amniotic fluid while they are still in their mother's womb (Sampaio-Maia & Monteiro-Silva, 2014). Once established, at the age of ~3 years each distinct microbial profile has been developed (Bäckhed et al., 2015) and therefore the microorganisms maintain stable homeostasis within the host. In this symbiotic relationship, the host provides an environment to the microorganisms in which they can colonize specific surfaces according to their preference. This is dependent on the host diet and environmental factors that prevent pathogenic bacteria from flourishing (Zarco et al., 2012). The microorganisms protect themselves from the host environment by forming biofilms on the oral surfaces and converse with each other by producing and detecting tiny diffusible warning sign molecules in a process called quorum sensing. These molecules are responsible for regulating the virulence and pathogenicity of microorganisms and are important in understanding the control of bacterial infections. They further enable the biofilm microorganisms to become more accepting of the host defenses and antimicrobial agents (Kilian et al., 2016).

The oral microbiome comprises 700 bacterial species, but it has been suggested that the number of species may be about ~1200 (Jenkinson, 2011). These are composed mainly of 12 phyla and 185 genera of which about 54% have nomenclature and 14% are unknown but can be cultured. A further 32% have nomenclature but remain uncultivated (Deo & Deshmukh, 2019). The 12 main phyla known to consist of *Firmicutes, Fusobacteria, Proteobacteria, Actinobacteria, Bacteroidetes, Chlamydiae, Chloroflexi, Spirochaetes,* SR1*, Synergistetes, Saccharibacteria* (TM7), and *Gracilibacteria* (GN02) (Perera et al., 2016).

Other non-bacterial pathogens such as protozoa also reside in the oral cavity. For example, *Entamoeba gingivalis* is an opportunistic amoebic parasite that resides inside the mouth and the gingival pocket biofilm near the base of the teeth and in periodontal pockets. Other protozoa include *Trichomonas tenax* which also lives as an oral saprophyte within the oral cavity. Fungi have also been identified in the oral microbiome and are composed mainly of Candida species. Other species include *Cladosporium, Aureobasidium, Saccharomycetales, Aspergillus, Fusarium, and Cryptococcus species* (Deo & Deshmukh, 2019).

Therefore, even though there are differences in the composition of the variable microbiomes between persons, it is important to note that overall (core) microbiota is relatively stable especially at the genus level (Deo & Deshmukh, 2019; Kilian et al., 2016). Furthermore, although similarities in individuals do occur, the diversity of the microbiome is site-specific. (Wu et al., 2018).

### *2.3.1. The Oral Microbiome in Health*

Once the oral microbiome has been established its main function is maintaining physiology, metabolism, and immunological responses by protecting the oral cavity and inhibiting disease. It does this by living in a symbiotic relationship with one another and the host immune system. Other functions are to digest foods and carbohydrates, maintain energy, preserve skin and mucosa barrier function, as well as to promote progression and regulation of the immune system. The balance between pro-inflammatory, anti-inflammatory processes and inhibition of invasion and growth of disease-promoting microorganisms is considered an important function of the oral biome (Deo & Deshmukh, 2019; Kilian et al., 2016). Microbes have been performing these metabolic activities in animals for almost 500 million years (Cho & Blaser, 2012). Resident oral microbiota exists in the form of biofilms which can perform functions

such as maintaining health or promoting disease. They protect the oral cavity and prevent disease (Kilian et al., 2016) by inhibiting the colonisation of pathogenic bacteria and the prevention of dysbiosis. The importance of dysbiosis is seen when commensals are disturbed by factors such as antibiotics (Sullivan, 2001), and as a result, microbial opportunistic bacteria such as Candida species and *Staphylococcus aureus* begin to colonise. Other oral bacteria such as *Streptococcus salivarius* strain K12 produce a bacteriocin that inhibits Gram-negative species which are associated with periodontitis and halitosis in vitro (Masdea et al., 2012; Wescombe et al., 2009). Also, the oral microbiome reduces nitrate to nitrite which is taken up into the bloodstream via gastric absorption and converted into nitric oxide. Nitric oxide is important for vascular health and helps to maintain healthy blood vessels by playing a role in vasodilation. It, therefore, has an anti-hypertensive effect that helps maintain cardiovascular health (Gee & Ahluwalia, 2016).

The oral microbiota is nevertheless controlled by the host regardless of the production of toxic products such as proteases, overgrowth, and pathogenicity. Importantly, the microbiota of these communities differs over time, from person to person, and from site to site. These dynamics may influence the onset of inflammatory disease which is stimulated by the virulence of key pathogens such as *P. gingivalis.* These pathogens have interactive communications with accessory pathogens such as the mitis group of streptococci, thereby initiating an inflammatory cascade. Therefore, the chronic inflammatory disease is initiated by a remodeling of normally benign microbiota into a dysbiotic one (Hajishengallis & Lamont, 2012; Hajishengallis et al., 2012).

### *2.3.2. Factors Influencing the oral microbiome*

Aging is one of the risk factors influencing the oral microbiome, as people age, they suffer severe memory loss which results in poor oral hygiene and poor diets and therefore, the oral microbiome also changes (Thomas et al., 2017). Therefore, aging is accompanied by the onset of multiple clinical changes, predisposing older people to diseases such as periodontitis and Alzheimer's (Viganò et al., 2018; López-otín et al., 2013). This condition occurs because of chronic inflammation which increases morbidity and mortality in older people due to the interference of the natural microbiota in the oral and gut.

Aging is also associated with a deteriorating immune system resulting in more infections (Viganò et al., 2018). Various studies have reported that aging changes the composition and

number of microbial communities, leading to a decrease in the variety of microbiota, as well as an upsurge of pathogens that could result in prolonged and chronic inflammation (An et al., 2018; López-otín et al., 2013). For example, Ogawa et al., 2018 did a comparison of the oral microbiome in older people living in a nursing home with elderlies living in their own homes and found that the older people living in a nursing home had a less diverse oral microbiome at phyla level but not at the genus level. They also had an increased abundance of *Actinomyces*, *Streptococcus*, *Bacilli*, *Selenomonas*, *Veillonella*, *Haemophilus*, with a lower relative abundance of *Prevotella*, *Leptotrichia*, *Campylobacter*, and *Fusobacterium*. In another study done by Singh et al., 2019 which compared healthy aging individuals to a similar aged unhealthy group, it was found that the healthy group had an increased alpha diversity compared to those who were unhealthy. Enrichment in the genera *Neisseria. Haemophilus, Fusobacterium*, and *Capnocytophaga* were observed in healthy aging but were found to be at a lower abundance (Singh et al., 2019). These findings were however contradicted by(García-Peña et al., 2017) who reported that the main species which constitute the mature microbiota are not altered in elders. Despite this, bacterial changes have been demonstrated in many geriatric diseases, like Alzheimer's and Parkinson's (Viganò et al., 2018). Inflammation increases morbidity and mortality in older people mostly because it is chronic and systemic without the presence of infection (Franceschi et al., 2000). For example, epidemiology studies have suggested that noticeable changes can be seen in the body shape of the elderly which is accompanied by low energy levels and impaired immune responses. These physical changes are all associated with increases in inflammatory markers such as C-reactive proteins and interleukin-6 (Franceschi & Campisi, 2014).

Other factors such as diet have also been associated with alteration in the oral microbiome, although the bacterial taxonomy remains stable. Studies by Lassalle et al, 2018 have suggested that the changes observed between a hunter-gatherer diet and a western diet may be the result of vitamin B5 autotrophy and urease-mediated pH regulation (Lassalle et al., 2018; Hernandez et al., 2017). Other elements identified as influencing the oral microbiome include the external environment mode of delivery and feeding methods of infants (Dreyer & Liebl, 2018).

Alcohol has also been associated with changes in oral microbiota diversity. This was evident in both animal (Kantorski et al., 2007) and human studies and it has been hypothesised that consumption thereof may impact the oral microbiota either through the direct cytotoxic effect on bacteria (Jansson, 2008) or by disrupting the saliva-bacterium interactions (Enberg et al.,

2001) by providing ethanol as a substrate for bacterial metabolism (Homann et al., 2000). For instance, the amount of alcohol consumed per day may influence the diversity of oral microbiota present as reported by researchers when they performed both small and large scale studies on alcohol consumption. Investigators reported that the consumption of at least one glass of red wine per day exposes individuals to reduced species richness and reduction of certain anaerobic bacteria in sub- and supra-gingival plaque (Signoretto et al., 2010). Besides the direct effect alcohol has on the oral biome it may also affect the host defense therefore encouraging periodontitis (Shepherd, 2011).

Smoking another risk factor that promotes changes in the oral microbiota. People that smoke cigarettes are exposed to numerous toxins and therefore their oral cavity, are exposed to toxins such as carbon monoxide, oxidizing radicals, carcinogens (such as nitrosamine), and nicotine (which is addictive). These chemicals cause adverse conditions in the periodontium resulting in dysbiosis (Enersen, 2011; Feres et al, 2017). The harmful effects of smoking can be divided into different categories according to the effect cigarettes have on the microbiology (microbiota/periodontal pathogens), gingival blood flow, polymorphonuclear neutrophil phagocytosis, cytokine production (e.g., interleukin-1), CD3, CD4, and CD8+ T-cell subsets and periodontal healing (Enersen, 2011). Sapkota et al., 2019 found that smoking causes an increase in the proliferation of anaerobic microorganisms due to a lack of oxygen caused by a potential oxidation-reduction which results in the formation of periodontal pockets. Recent studies have also suggested that smoking gives rise to an increase in acidophilic bacteria and archaea due to an increase in the acidity of the saliva (Karabudak et al., 2019). Furthermore, It was also found that specific pathogens such as *P. gingivalis, Treponema denticola,* and *T. forsythia* were associated with smoking and the development and progression of periodontal disease (Jiang et al., 2020; Shchipkova et al., 2010a; Hanioka et al., 2019). This was also evident in previous studies performed by (Bodet et al., 2007).

However conflicting results have also been found in earlier studies on the effect of smoking on oral microbiota. For instance, differences in the oral microbiome composition were found when different sample types were used when comparing the results of experiments utilising different samples from different sites within the oral cavity. For example, Genera such as *Prevotella and Veilonella* are predominant in the saliva in periodontal conditions with an abundance of *Neiserria* genera in healthy conditions (Yamashita & Takeshita, 2017). On the other hand, genera that dominate the buccal are *Streptococci, Haemophilus parainfluenzae,* S*imonsiella*

*spp., Granulicatella spp., Gemella spp., Veillonella spp. and Prevotella spp* (Karabudak et al., 2019). Differences were reported in the study of 62 swabs from the tonsillar pillars (Charlson et al., 2010), 200 subgingival samples (Mason et al., 2015a), 30 marginal and subgingival plaque and gingival crevicular fluid samples (Kumar et al., 2011) oral wash samples (Wu et al., 2016) 64 saliva samples (Morris et al., 2013) and a study of 292 stimulated oral samples performed by the Human Oral Microbe Identification Microarray (HOMIM).

Although many of these studies noticed that taxa were altered due to smoking status, others showed no correlation between the oral microbiome and smoking (Boström et al., 2001; Gomes et al., 2008). Thus, the conflicting results reported may be due to variation in sampling and methodology, samples collected from different oral sites, ethnicity, and all these factors may account for the inconsistency in reporting on the effects of smoking on the oral microbiome. Therefore, larger-scale studies should be conducted to compare the normal oral microbiome to a disease state, and to observe how these microbial changes in the oral microbiome could be linked to disease.

### *2.3.3. Diseases associated with the oral microbiome*

Gingivitis and periodontitis are the two most well-known and prevalent periodontal diseases. Gingivitis a milder form of inflammatory disease is caused by bacterial plaque that forms in the gingival margin. The most predominant genera associated with gingivitis are TM7, *Leptotrichia, Selenomonas, Streptococcus, Veillonella, Prevotella, Lautropia,* and *Haemophilus,* and may serve as biomarkers of the disease (Huang et al., 2011; Igic et al., 2012). Gingivitis progresses into a more severe form known as periodontitis when left untreated.

Periodontitis is a biofilm -induce chronic inflammation that causes loss and destruction of tissue and bone. During the progresion of periodontal disease, the composition and abundance of individual species change from a healthy to a disease state. The species known as red complex are associated with the progression of periodontitis and include *Porphyromonas gingivalis (P. gingivalis), Treponema denticola (T. denticola) and Tannerella forsythia (T. forsythia)* (Boutin et al., 2017). Even though these species may be present in a lower abundance in healthy individuals they are associated with the progression of periodontitis (Costalonga & Herzberg, 2014). This was evident in a study where a shift in the oral community was noticed and an enrichment of Veillonella species was seen in the subgingival crevice in oral health while enrichment of *Filifactor alocis* was observed in subjects with periodontal diseases (Kumar et al., 2006)

During the progression of periodontal disease, the species composition and abundance of dominant species allow the virulent plaque bacteria to flourish (Lamont et al., 2019; Abusleme et al., 2013). It has therefore been hypothesised that by controlling the environment which predisposes the enrichment of these pathogens, a disease state can be prevented (Rengo et al., 2015). For example, biofilm formation is stimulated when there is an increase in sugar intake or a decrease in saliva flow. This results in a lower pH within the oral cavity and creates an environment favourable for pathogenic bacteria to grow. Therefore, periodontitis is promoted by the amount or degree of biofilm aggregation which can differ between individuals according to their risk profile (Meyle & Chapple, 2015).

In subjects with gingivitis, the condition is self-resolving, but if the patient is susceptible to periodontitis, the inflammation may be initiated by various risks or external factors such as tobacco, diet, alcohol, uncontrolled diabetes, and a genetic predisposition. All these factors can result in more aggressive or chronic periodontitis which may lead to inflammation of the connective tissue supporting the teeth (Meyle & Chapple, 2015). In diabetes mellitus, tissue destruction becomes worse due to the metabolic dysregulation which controls the inflammatory response (Meyle & Chapple, 2015). Although to date, current literature has shown that the microbial communities shift in response to disease, it remains unclear if there is a 'healthy amount' of these bacteria that are consistent across individuals (Schwarzberg et al., 2014), or whether this varies between individuals due to diverse thresholds for activation and or deregulation of the host response. Therefore, longitudinal studies need to be conducted the notate these shifts in bacteria communities in our study population.

## 2.4. SMOKING AND THE ORAL MICROBIOME

Diseases related to smoking have become a major concern globally and despite numerous efforts to educate people on smoking habits, it has been suggested that an estimated by 2020 that 10% of all deaths will be directly related to smoking. This, therefore, carries a huge burden on the health care system globally (Marrero & Adashi, 2015; Mattiuzzi & Lippi, 2020) Also, growing evidence shows the exposure of the oral microbiome to cigarette smoke may promote a risk of diseases such as cancer, cardiovascular disease, chronic obstructive pulmonary disease, and periodontitis (Yu et al., 2017; Leite et al., 2018a; Huang & Shi, 2019; Wu et al., 2016), yet factors that affect the oral microbiome are poorly understood.

Cigarette smoking contains several toxins that have direct access to the oral bacteria and therefore, cause oral dysbiosis via various mechanisms which include antibiotic effects and oxygen deprivation (Sapkota et al., 2010)Lee et al., 2012)Thus depletion of vital oral pathogens is lost due to cigarette smoking resulting in colonisation of oral pathogens which initiates the onset of periodontal disease (Nociti et al., 2015). Earlier studies have demonstrated the changes in the richness of preferred oral microbiota in smokers compared to non-smokers (Morris et al., 2013; Mason et al., 2015a). For instance, a large meta-analysis study done by (Wu et al., 2016) on the oral microbiome of smokers demonstrated significant differences from non-smokers, especially at the phylum level. They observed a significant reduction of the phylum *Proteobacteria* and an abundance of *Firmicutes* and *Actinobacteria* in smokers compared to non-smokers. However, contradictions across studies were observed due to different sample sizes, methodology, and sampling sites. These variations may have imposed limitations on oral bacterial profiling and therefore warrant more investigation characterising the smoking profile of the South African population.

*2.4.1. Possible Mechanisms contributing to oral microbial changes*

Smoking contributes to the modification of the oral microbial ecology through various mechanisms. Fresh tobacco leaves contain microorganisms such as *Pantoe, Acinetobacter, Pseudomonas fluorescence, and Stenotrophomonas maltophilia* (Larsson et al., 2008). Before 16S rRNA analysis, microarray, and cloning, these microorganisms were identified through culture techniques. With these high throughputs, technologies' the identification of these unculturable species mentioned ranging from *Acinetobacter, Bacillus, Burkholderia, Clostridium, Klebsiella, and Pseudomonas aeruginosa* were made possible. These microorganisms can be associated with diseases such as pneumonia, bacteraemia, foodborne diseases, and urinary tract infections (Sapkota et al., 2010). Therefore, smoking can be linked to a different microbial profile when compared to non-smokers due to tobacco leaves containing microorganisms that are introduced into the oral microbiome of smokers.

Smoking may also affect the peripheral immune system. For instance, smoking decreases or lowers the active function of natural killer cells, increases the white blood cells and therefore smokers have increased exposure to infections (Jaspers, 2014). Although there are increases in macrophages and neutrophils, they have reduced phagocytic functions, resulting in pathogens or bacteria not being efficiently cleared. This is evident in the reduced bacterial stimulated production of neutrophil superoxide and surface expression of TLR2 which is crucial for the

recognition and response to gram-positive bacteria (Matthews et al., 2012; Droemann et al., 2005). Therefore, the protection against bacteria in individuals who smoke may be different due to impaired antimicrobial defences caused by the immunosuppressive nature of tobacco.

Biofilm formation another mechanism associated with smoking. Cigarette smoke (CS) also exposes the oral microbiome of smokers to colonisation by specific microbiota due to increased biofilm formation. Biofilms are communities of dense surface multi-specie bacteria or fungal species enclosed within a microbially self-derived matrix that facilitates colonization and survival of specific bacteria due to smoking exposure (Mutepe et al., 2013; Kulkarni et al., 2012a). Studies have found an enrichment of *Streptococcus pneumonia* in the presence of biofilm formation and the toxin, pneumolysin, to be deactivated in smokers (Mutepe et al., 2013). Similarly, enrichment of *Staphylococcus aureus* and human cell adherence in the presence of a cigarette (CS) suggests that bioactive substances present in cigarettes affect the inhabitant microbiota and play an important role in the pathogenesis of respiratory infection. (Kulkarni et al., 2012b).

Furthermore, several other mechanisms have been described. These include factors such as oxygen, pH, and acid production. For instance, oxygen tension promotes microaerophilic and anaerobic bacteria over aerobes due to lower oxygenation (Mason et al., 2015b; Ganesan et al., 2017) and by increasing the amount of free iron (Ghio et al., 2008). This results in the inhibition of oral peroxidase which is an important enzyme in the salivary antioxidant system and is vital in the oral defence against the free radicals which are present in cigarette smoke (CS) (Reznick et al., 2003).

Cigarette exposure increases anaerobic glycolysis in salivary cells and, studies on humans, have indicated a reduction in periodontal pocket oxygen tension after cigarette smoking (Eichel & Shahrik, 1969; Hanioka et al., 2000; Eichel B & Shahrik HA., 1969)Other research on the oral microbiome has shown an increased abundance of *Firmicutes (Streptococcus spp), Veilonella spp, and Rothia (Actinobacteria)* in the upper gastrointestinal tract of smokers when compared with non-smokers (Shanahan et al., 2018). These differences in *Neisseria*, *Streptococcus*, and *Rothia* spp. in smokers suggest that they are the result of changes in oxygen tension. Alterations in duodenal bicarbonate secretion and lower pH in smokers may also induce selective pressure on the growth of *Neisseria*, which is one of the capnophiles and is

sensitive to acid conditions. *Streptococcus and Rothia spp.* On the other hand, are acidogenic and acid tolerant. (Murthy et al., 1978; Ainsworth et al., 1993).

*2.4.2. Smoking Associated with Periodontitis*

After Dental carriers, Periodontitis is the second most common chronic oral disease in adults. Environmental risk factors such as tobacco smoking in the form of cigarettes have been identified as an important risk factor for causing periodontitis and is responsible for 80% of myocardial infarction and 70% of chronic respiratory lung infections (Gautam et al., 2011). It has been understood that when tobacco smoke is introduced to the oral cavity the immune response is weakened causing inflammation in the periodontal tissues and the development of periodontal disease (Williams & Genco, 2010). However, it has been suggested that due to nicotine and vasoconstriction, smokers appear to have less gingival bleeding while pocket depth measurements are higher due to an increase of alveolar bone loss (Gautam et al., 2011).

Supporting that smoking has a worsened effect on the progression of periodontitis (Leite et al., 2018b) and co-workers performed a meta-analysis study where twenty-eight studies were included in the review of those only 14studies presented with data that fit the criteria for meta-analysis. They concluded that risk ratios estimate that smoking increases the risk of periodontitis by 85% (risk ratio¼1.85, 95% CI¼1.5, 2.2). Meta-regression demonstrated that age explained 54.2% of the variability between studies, time of follow-up explained 13.5%, loss to follow-up 10.7%, criteria used to assess the periodontal status explained 2.1%, and severity of periodontitis explained 16.9%, they, therefore, concluded that smoking has a detrimental effect on the progression of periodontitis. These findings were supported by a study were 66 subjects from a University clinic. The subjects were divided according to the self-report on smoking statutes and divided into smokers, non-smokers, and passive-smokers. Cotinine levels were determined from saliva samples and dental examination compromised of plaque-index, probing depth (PD), clinical attachment level (CAL), and bleeding of probing (BP) and this was performed on six sites per tooth. As expected, the smokers had a significant increase in PD and CAL value ($p<0.05$), whereas passive smokers, showed lesser values, but the value was more than non-smokers with no significance (Kanmaz et al., 2019).

## 2.5. DIABETES IN SOUTH AFRICA

Diabetes mellitus, a chronic inflammatory condition is characterised by increased blood glucose levels due to the body's inability to produce enough insulin or insulin resistance. There are various categories of diabetes and include type 1 diabetes, mainly present in children of any age and normally inherited, Type 2 diabetes, which accounts for 90% of diabetes globally, and thirdly Gestational diabetes, which normally occurs during pregnancy (Chatterjee et al., 2017; Dias et al., 2019). People with prediabetes are often unaware of their condition and they do not present with signs and symptoms. Prediabetes is usually characterised by impaired glucose tolerance or impaired fasting glucose and without any intervention will evolve into diabetes within 10 years. According to the International Diabetes Federation (IDF's) 2019 Diabetes Atlas statistics, an estimated 463 million adults between the ages of 20-79 years are presently living with diabetes (Atlas, 2019). This accounts for 9.3% of the global population in this age group. It has been projected that by 2045 the total number of diabetic cases would have increased to 700 million (10.9%) globally (Atlas, 2019). In South Africa, reports from the IDF's Diabetes Atlas indicate that 12.7% of adults in SA have diabetes, which is 137% more than 2017 statistics of 5.4% (Atlas, 2019).

### *2.5.1. Changes in the Oral Microbiota Caused by Diabetes*

Diabetes has an important influence on gut microbiota however, how it influences the oral microbiome remains unclear. Research utilising 16s rRNA platforms, after transferring the oral microbiota from diabetic mice to healthy germ-free mice, has shown that diabetes causes an alteration in the oral microorganism composition (Xiao et al., 2017). This study has shed some light on the relationship between diabetes and the oral microbiome.

The primary function of the oral microbiome is to maintain health and to regulate blood sugars however, the link between diabetes and the oral microbiome still requires investigation (Long et al., 2017). Individual reports have demonstrated that periodontal disease can be linked to an increased risk of developing diabetes and that when treated, blood glucose levels and glycated haemoglobin levels improved (Morita et al., 2012; Bharti et al., 2013). Therefore, it was suggested that diabetic patients have a greater chance of having periodontitis especially when the diabetes is not well managed. This may be explained by the fact that both conditions are associated with inflammation leading to a bidirectional link between the two (Long et al., 2017).

When Inflammation and infection develop, oral microorganisms move from one oral site to another (Han &Wang 2013). It has further been reported by Long et al., 2017 that virulent periodontal microorganisms such as *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* are linked to poor blood glucose control and diabetes. Nevertheless, the direct association or relationship between the oral microbiome and diabetes still requires further investigation. In a study, of 29 obese individuals and 13 individuals with diabetes, the genus Bifidobacteria in the phylum Actinobacteria had a lower abundance. This finding was considered significant and in a further study, Bifidobacteria was significantly associated with decreased obesity prevalence (Yang et al., 2019). Others comparing 20 participants with diabetes and 11 non-diabetic controls, the phylum *Firmicutes and* the two genera, *Streptococci* and *Lactobacilli* were found to be in abundance. Nevertheless, these patients were on diabetic treatment which could have influenced the results. Therefore, more studies are needed with a bigger cohort to support these findings.

*2.5.2. Periodontal Disease and Diabetes Mellitus*

Both Periodontitis and Diabetes mellitus are chronic inflammatory diseases. Periodontitis is induced by biofilm formation and affects 740 million people globally in its chronic form (Kassebaum et al., 2014) whereas diabetes is defined as a group of chronic metabolic disorders characterise by irregular or poorly controlled glucose levels (Rengo et al., 2015) The most common microorganism associated with periodontal infections is gram-negative anaerobes which contain various virulent factors assisting them to escape the host immune system (Minty et al., 2019). These include virulent factors such as lipopolysaccharides (LPS, endotoxins), lipoteichoic acids, toxins, proteinases, short-chain fatty acids, capsules, and cell membrane products (Dennison et al, 1997; Baehni et al., 1981). Once this occurs these microorganisms occupy the oral cavity and become subgingival inhabitants.

Subjects with diabetes are three to four times more susceptible to developing periodontitis due to an impaired immune response and vice-versa (Casanova et al., 2014; Pérez-Losada et al., 2016; Preshaw et al., 2012; Grossi, 2001) These findings have been supported by (Blasco-Baque et al., 2016) who demonstrated the association between DM and periodontal disease in both humans and animals. For instance, these investigators provided evidence of the role of oral dysbiosis in the progression of insulin resistance. In addition, Liu and co-workers demonstrated in diabetic mice how periodontal disease worsens pancreatic β-cell failure in these mice (Liu & Zhang, 2016). Even more recently in a study of subjects 40 years of age, it

was demonstrated that periodontitis is more prevalent in subjects with diabetes (de Miguel-Infante et al., 2019)

The role of the inflammatory response is important in the relationship between periodontal disease and diabetes. Periodontal tissue destruction occurs due to the interaction of bacterial virulent products with mononuclear phagocytic cells. Macrophages secrete cytokines including fibroblast activating factor and inflammatory mediators such as primarily Interleukins (IL-lß), Prostaglandin E2 (PGE2), Tumor necrosis factor (TNF-a), and Interleukins (IL-6) (Carolina et al., 1996; Kornman et al., 2000). Further, it is believed that diabetic periodontal disease develops through glucose-mediated AGE accumulation.

The periodontium is highly vascularized, and the increased accumulation of the advanced glycation end products (AGE) causes increased oxidant stress on the surrounding tissue and alters the endothelial cell function which increases the activity of the metalloproteinases (references). Similar modifications have been seen in the tissues affected by the periodontal tissue of diabetic individuals. Therefore, the level of AGE products found or present in blood serum in humans has been associated with the degree of periodontitis in diabetes mellitus type 2 (Takeda et al., 2006). Some studies have suggested that the presence of AGE products could be utilised as a biomarker for periodontal status in diabetic subjects (Xu et al., 2015; Lalla & Papapanou, 2011). The oral pathogen *Porphyromonas gingivalis* and its lipopolysaccharide are also responsible for the development of periodontitis in diabetics by activating the innate immune response via activation of Toll-like receptors (TLRs) (Xu et al., 2020). This correlates well with a recent study performed by (Mei et al., 2020) who is an experiment on mice that showed that *Porphyromonas gingivalis* can induce insulin resistance in diabetic patients.

## 2.6. THE ORAL MICROBIOME AND METABOLIC SYNDROME

Metabolic syndrome (MetS) is described as a condition that consists of clusters or group of risk factors for CVD and diabetes that often occurs together rather than alone. These risk factors include dyslipidaemia (raised levels of triglycerides [TGs] and low levels of high-density lipoprotein [HDL] cholesterol), raised fasting glucose, raised high blood pressure, and central obesity (Alberti et al., 2005). Besides, dyslipidaemia, high blood pressure, and hyperglycaemia, the disorder includes a prothrombotic state and a proinflammatory state and also puts subjects with DM type 2 at risk of developing CVD. Therefore, many efforts have been made in investigating all these risk factors (lifestyle, obesity). Furthermore, an association between the

oral microbiome and an increased prevalence in CVD and DM type 2 has been observed (Liccardo et al., 2019)

Studies have associated the oral pathogen *Porphyromonas gingivalis* with systemic inflammation and MetS (Arimatsu et al., 2014). These findings suggest that the oral bacteria can cause low-grade inflammation in subjects, that may progress to the development of MetS. This was evident in a previous Korean study where researchers identified the oral pathogens associated with the interplay of the gut, the oral microbiome, and MetS. They identified that genera *Granulicatella* and *Neisseria* were enriched in subjects with MetS and that the genus *Peptococcus* as were enriched in healthy controls (Si et al., 2017). Although much research has been done on the gut microbiome, the oral microbiome and its role in Mets is not well established (Vijay-kumar et al., 2013; Wang et al., 2019b; Zhang et al., 2010; Mason et al., 2013). Therefore, future studies are needed to identify these oral biomarkers in the South African population which can be associated with metabolic syndrome.

### *2.6.1. Periodontal disease and Cardiovascular Disease (CVD)*

Cardiovascular disease (CVD) in general is termed a disease affecting the heart or blood vessels, and often develops due to the build-up of fatty deposits inside the arteries known as atherosclerosis which is associated with a high risk of blood clots. CVD may be responsible for several heart diseases such as coronary artery disease, heart attacks, arrhythmia, heart failure, etc, all of which are responsible for 17.8 million mortalities per year globally (Virani et al., 2020). The pathogenesis of CVD has been linked to various risk factors such as smoking, hypertension, increased sodium intake, hyperlipidaemia, and obesity (Francula-Zaninovic & Nola, 2018; Carbone et al., 2019; Psaltopoulou et al., 2017). The WHO in 2007, reported that the prevalence of CVD is increasing annually even though modern society has been educated on risk factors and therapies. Therefore, researchers have put much focus on the association of periodontists with CVD.

The association of Periodontitis with CVD has been linked in two ways. Firstly, it is believed that when moderate or severe periodontitis is present the levels of systemic inflammation increase, and therefore the periodontal bacteria can enter the bloodstream. This can occur due to normal day-to-day routine brushing of teeth or eating an apple (Tomás et al., 2012; Tonetti et al., 2008). Secondly, bacteria such as *P. gingivalis*, *B. forsythus*, *P. intermedia,* and *A. actinomycetemcomitans* gain entry by invading and damaging the periodontal tissue allowing

them access into the bloodstream causing bacteraemia and possibly CVD (Haraszthy et al., 2000; Padilla et al., 2006). Eke in 2012 reported a 64% increase in moderate to severe periodontitis in individuals over the age of 65. This was supported by a United States (US) study where the incidence of periodontitis has increased by 6 to 20% in the last era. (Eke et al., 2012).

Furthermore, the link between periodontitis and CVD was evident in a meta-analysis performed by (Janket et al., 2003; Scannapieco et al., 2003; and Khader et al., 2004). Researchers used the Medline search engine to analyse all published literature including abstracts since 1980 to provide a measurable summary of periodontal disease as a risk factor for CVD and to discover the possible causes for contradictory results in the literature. The results indicated that periodontal disease seemed to be associated with a 19% increase in the risk of future cardiovascular disease. This increase in relative risk is more prominent (44%) in subjects aged >65 years. Therefore, based on these analyses, investigators have concluded that there could be a link between periodontitis and CVD (Genco et al., 2002; Hujoel, 2002; Hansen et al., 2016). Thus, our investigation will characterise these oral microbiotas and associate them with the different cardiometabolic traits.

## 2.7. Reference


Abusleme, L., Dupuy, A.K., Dutzan, N*., et al*. 2013. The subgingival microbiome in health and periodontitis and its relationship with community biomass and inflammation. *ISME J*, 7(5): 1016–1025.

Ainsworth, M.A., Hogan, D.L., Koss, M.A. & Isenberg, J.I. 1993. Cigarette smoking inhibits acid-stimulated duodenal mucosal bicarbonate secretion. *Ann Intern Med*, 119(9): 882–886.

Alberti, K.G.M.M., Zimmet, P. & Shaw, J. 2005. The metabolic syndrome - A new worldwide definition. *Lancet*, 366(9491): 1059–1062.

Amon, P. & Sanderson, I. 2017. What is the microbiome? *Arch Dis Child: Educ Pract Ed*, 102(5): 258–261.

An, J.Y., Darveau, R. & Kaeberlein, M. 2018. Oral health in geroscience: animal models and the aging oral cavity. *GeroScience*, 40(1): 1–10.

Arboleya, S., Suárez, M., Fernández, N., *et al*. 2018. C-section and the Neonatal Gut Microbiome Acquisition: Consequences for Future Health. *Ann Nutr Metab*, 73(suppl 3): 17–23.

Arimatsu, K., Yamada, H., Miyazawa, H.,*et al.* 2014. Oral pathobiont induces systemic inflammation and metabolic changes associated with alteration of gut microbiota. *Sci Rep*, 4: 1–9.

Atlas, 2019. Global and regional diabetes prevalence estimates for 2019 and projections for 2030 and 2045: Results from the International Diabetes Federation Diabetes Atlas, Diabetes Res Clin Pract. 9$^{th}$edition

Bäckhed, F., Roswall, J., Peng, Y., *et al.* . 2015. Dynamics and stabilization of the human gut microbiome during the first year of life. *Cell Host and Microbe*, 17(5): 690–703.

Baehni, P.C., Tsai, C.C., McArthur, W.P., Hammond, B.F., Shenker, B.J. & Taichman, N.S. 1981. Leukotoxic activity in different strains of the bacterium Actinobacillus actinomycetemcomitans isolated from juvenile periodontitis in man. *Arch Oral Biol*, 26(8): 671–676.

Baothman, O.A., Zamzami, M.A., Taher, I., Abubaker, J. & Abu-Farha, M. 2016. The role of Gut Microbiota in the development of obesity and Diabetes. *Lipids Health Dis*, 15(1): 1–8.

Bharti, P., Katagiri, S., Nitta, H., *et al* . 2013. Periodontal treatment with topical antibiotics improves glycemic control in association with elevated serum adiponectin in patients with type 2 diabetes mellitus. *Obes Res Clin Pract*, 7(2): e129–e138.

Blasco-Baque, V., Garidou, L., Pomié, C., Escoula, Q., Loubieres, P., Le Gall-David, S., et al. 2016. Periodontitis induced by Porphyromonas gingivalis drives periodontal microbiota dysbiosis and insulin resistance via an impaired adaptive immune response. *Gut*, 66(5): 872–885.

Bliss, E.S. & Whiteside, E. 2018. The gut-brain axis, the human gut microbiota and their integration in the development of obesity. *Front Physiol*, 9(JUL).

Bodet, C., Chandad, F. & Grenier, D. 2007. Pathogenic potential of Porphyromonas gingivalis, Treponema denticola and Tannerella forsythia, the red bacterial complex associated with periodontitis. *Pathol Biol*, 55(3–4): 154–162.

Bota, I., Segal, D. & Davies, D. 2014. The Structural Biology of Toll-Like Receptors. *Structure* 23(1):

Boutin, S., Hagenfeld, D., Zimmermann, H., *et al*. 2017. Clustering of subgingival microbiota reveals microbial disease ecotypes associated with clinical stages of periodontitis in a cross-sectional study. *Front Microbiol*, 8(MAR): 1–13.

Boström,L., Bergström,J., Dahle,G., LE.L. 2001. Smoking and subgingival microflora in periodontal diease. *J Clin Periodontol* : 212–219.

Carbone, S., Canada, J.M., Billingsley, H.E., Siddiqui, M.S., Elagizi, A. & Lavie, C.J. 2019. Obesity paradox in cardiovascular disease: Where do we stand? *Vasc Health Risk Manag*, 15: 89–100.

Carolina, N., Offenbacher, S., Carolina, N. & Hill, C. 1996. Periodontal Diseases : Pathogenesis. *Ann Periodontol*, 1(1): 821–878.

Casanova, L., Hughes, F.J. & Preshaw, P.M. 2014. Diabetes and periodontal disease: A two-way relationship. *Br Dent J*, 217(8): 433–437.

Castrillon, C.A., Hincapie, J.P., Yepes, F.L., Roldan, N., Moreno, S.M., Contreras, A. & Botero, J.E. 2015. Occurrence of red complex microorganisms and Aggregatibacter actinomycetemcomitans in patients with diabetes. *J Investig Clin Dent*, 6(1): 25–31.

Charlson, E.S., Chen, J., Custers-Allen, R., *et al*. 2010. Disordered microbial communities in the upper respiratory tract of cigarette smokers. *PLoS ONE*, 5(12): 1–10.

Chatterjee, S., Khunti, K. & Davies, M.J. 2017. Type 2 diabetes. *Lancet*, 389(10085): 2239–2251. http://dx.doi.org/10.1016/S0140-6736(17)30058-2.

Chikte, U., Pontes, C.C., Karangwa, I. & Kimmie-dhansay, F. 2019. Periodontal Disease Status among Adults from South Africa. *Int J Environ Res Public Health*.16(19):3662.

doi: 10.3390/ijerph16193662.

Cho, I. & Blaser, M.J. 2012. The human microbiome: at the interface of health and disease. *Nat Rev Genet*, 13(4): 260–270.

Clemente, J.C., Ursell, L.K., Parfrey, L.W. & Knight, R. 2012. The impact of the gut microbiota on human health: An integrative view. *Cell*, 148(6): 1258–1270.

Compare, D., Rocco, A., Sanduzzi Zamparelli, M. & Nardone, G. 2016. The Gut Bacteria-Driven Obesity Development. *Dig Dis*, 34(3): 221–229.

Costalonga, M. & Herzberg, M.C. 2014. The oral microbiome and the immunobiology of periodontal disease and caries. *Immunol Lett*, 162(2): 22–38. http://dx.doi.org/10.1016/j.imlet.2014.08.017

Dennison, D.K. & Van Dyke, T.E. 1997. The acute inflammatory response and the role of phagocytic cells in periodontal health and disease. *Periodontol 2000*, 14(1): 54–78.

Deo, P.N. & Deshmukh, R. 2019. Oral microbiome: Unveiling the fundamentals. *J Oral Maxillofac Pathol*, 23(1): 122–128.

Dias, S., Adam, S., Rheeder, P. & Pheiffer, C. 2019. Prevalence of and risk factors for gestational diabetes mellitus in South Africa. *SAML*, 109(7): 463–467.

Dreyer, J.L. & Liebl, A.L. 2018. Early colonization of the gut microbiome and its relationship with obesity. *Human Microbiome Journal*, 10(August): 1–5. https://doi.org/10.1016/j.humic.2018.08.002.

Droemann, D., Goldmann, T., Tiedje, T., Zabel, P., Dalhoff, K. & Schaaf, B. 2005. Toll-like receptor 2 expression is decreased on alveolar macrophages in cigarette smokers and COPD patients. *Respir Res*, 6: 2–9.

Dunn, A.B., Jordan, S., Baker, B.J. & Carlson, N.S. 2017. The Maternal Infant Microbiome: Considerations for Labor and Birth. *MCN Am J Matern Child Nurs*, 42(6): 318–32

Eichel, B. & Shahrik, H.A. 1969. Tobacco smoke toxicity: Loss of human oral leukocyte function and fluid-cell metabolism. *Science*, 166(3911): 1424–1428.

Eke, P.I., Dye, B.A., Wei, L., Thornton-Evans, G.O. & Genco, R.J. 2012. Prevalence of periodontitis in adults in the united states: 2009 and 2010. *J Dent Res*, 91(10): 914–920.

Embery, G., Waddington, R.J., Hall, R.C. & Last, K.S. 2000. Connective tissue elements as diagnostic aids in periodontology. *Periodontol 2000*, 24(1): 193–214.

Enberg, N., Alho, H., Loimaranta, V. & Lenander-Lumikari, M. 2001. Saliva flow rate, amylase activity, and protein and electrolyte concentrations in saliva after acute alcohol consumption. *Oral Surg, Oral Med, Oral Pathol, Oral Radiol, and Endod*, 92(3): 292–298.

Enersen, M. 2011. Risk factors for periodontal disease. *Oral Infections and Cardiovascular Disease*, 62: 17–25.

Fan, X., Peters, B.A., Jacobs, E.J.,*et al*. 2018. Drinking alcohol is associated with variation in the human oral microbiome in a large study of American adults. *Microbiome*, 6(1): 59.

Farrell, J.J., Zhang, L., Zhou, H., *et al.* 2012. Variations of oral microbiota are associated with pancreatic diseases including pancreatic cancer. *Gut*, 61(4): 582–588.

Feres, F., Teles, R., Teles, L. C ., Figueiredo, M.F. 2017. The subgingival periodontal microbiota in the aging mouth Magda. *Physiology & behavior*, 176(1): 139–148.

Field, A.E., Willett, W.C., Lissner, L. & Colditz, G.A. 2007. Dietary fat and weight gain among women in the nurses' health study. *Obesity*, 15(4): 967–976.

Flemming, K., Woolcott, C.G., Allen, A.C., Veugelers, P.J. & Kuhle, S. 2013. The association between caesarean section and childhood obesity revisited: A cohort study. *Arch Dis Child*, 98(7): 526–532.

Franceschi, C., Bonafè, M., Valensin, S., Olivieri, F., de Luca, M., Ottaviani, E. & de Benedictis, G. 2000. Inflamm-aging. An evolutionary perspective on immunosenescence. *Ann New Y Acad of Sci*, 908: 244–254.

Franceschi, C. & Campisi, J. 2014. Chronic inflammation (Inflammaging) and its potential contribution to age-associated diseases. *J Gerontol A Biol Sci and Med Sci*, 69: S4–S9.

Francula-Zaninovic, S. & Nola, I.A. 2018. Management of Measurable Variable Cardiovascular Disease' Risk Factors. *Curr Cardiol Rev*, 14(3): 153–163.

Ganesan, S.M., Joshi, V., Fellows, M., *et al*. 2017. A tale of two risks: Smoking, diabetes and the subgingival microbiome. *ISME J*, 11(9): 2075–2089.

García-Peña, C., Álvarez-Cisneros, T., Quiroz-Baez, R. & Friedland, R.P. 2017. Microbiota and Aging. A Review and Commentary. *Arch Med Res*, 48(8): 681–689.

Gautam, D.K., Jindal, V., Gupta, S.C., Tuli, A. & Kotwal, B. 2011. Effect of cigarette smoking on the periodontal health status : A comparative, cross-sectional study. *J Indian Soc Periodontol*, 15(4): 4–8.

Gee, L.C. & Ahluwalia, A. 2016. Dietary Nitrate Lowers Blood Pressure: Epidemiological, Pre-clinical Experimental and Clinical Trial Evidence. *Curr Hypertens Rep*, 18(2): 1–14.

Genco, R., Offenbacher, S. & Beck, J. 2002. Periodontal disease and cardiovascular disease: epidemiology and possible mechanisms. *J Am Dent Assoc (1939)*, 133 Suppl(June): 14S-22S. http://dx.doi.org/10.14219/jada.archive.2002.0375.

Gomes, S.C., Nonnenmacher, C., Susin, C., Oppermann, R. v., Mutters, R. & Marcantonio, R.A.C. 2008. The Effect of a Supragingival Plaque-Control Regimen on the Subgingival Microbiota in Smokers and Never-Smokers: Evaluation by Real-Time Polymerase Chain Reaction. *J Periodontol*, 79(12): 2297–2304.

Grossi, S.G. 2001. Treatment of periodontal disease and control of diabetes: an assessment of the evidence and need for future research. *Ann Periodontol*, 6(1): 138–145.

Hajishengallis, G., Darveau, R.P. & Curtis, M.A. 2012. The keystone-pathogen hypothesis. *Nat Rev Microbiol*, 10(10): 717–725. http://dx.doi.org/10.1038/nrmicro2873.

Hajishengallis, G. & Lamont, R.J. 2012. Beyond the red complex and into more complexity: The polymicrobial synergy and dysbiosis (PSD) model of periodontal disease etiology. *Mol Oral Microbiol* 27(6): 409–419.

Han, Y, Wang, X. 2013. Mobile Microbiome : Oral Bacteria in Extra-oral Infections and Inflammation. *J Dent Res*: 485–491.

Hanioka, T., Morita, M., Yamamoto, T., Inagaki, K., Wang, P.L., Ito, H., et al. 2019a. Smoking and periodontal microorganisms. *Jpn Dent Sci Rev*, 55(1): 88–94. https://doi.org/10.1016/j.jdsr.2019.03.002.

Hanioka, T., Tanaka, M., Takaya, K., Matsumori, Y. & Shizukuishi, S. 2000. Pocket Oxygen Tension in Smokers and Non-Smokers With Periodontal Disease. *J Periodontol*, 71(4): 550–554.

Hansen, G.M., Egeberg, A., Holmstrup, P. & Hansen, P.R. 2016. Relation of Periodontitis to Risk of Cardiovascular and All-Cause Mortality (from a Danish Nationwide Cohort Study). *Am J Cardiol*, 118(4): 489–493. http://dx.doi.org/10.1016/j.amjcard.2016.05.036.

Haraszthy, V.I., Zambon, J.J., Trevisan, M., Zeid, M. & Genco, R.J. 2000. Identification of Periodontal Pathogens in Atheromatous Plaques. *Journal of Periodontology*, 71(10): 1554–1560.

He, J., Li, Y., Cao, Y., Xue, J. & Zhou, X. 2015. The oral microbiome diversity and its relation to human diseases. *Folia Microbiol,* 60(1): 69–80.

Hernandez, B.Y., Zhu, X., Goodman, M.T., Gatewood, R., Mendiola, P., Quinata, K. & Paulino, Y.C. 2017. Betel nut chewing, oral premalignant lesions, and the oral microbiome. *PLoS ONE*, 12(2): 1–19.

Holgerson, P.L., Vestman, N.R., Claesson, R., *et al*. 2013. Oral microbial profile discriminates breast-fed from formula-fed infants. *J Pediatr Gastroenterol Nutr* 56(2): 127–136.

Huang, C. & Shi, G. 2019. Smoking and microbiome in oral, airway, gut and some systemic diseases. *J Transl Med,* 17(1): 1–15.

Huang, S., Yang, F., Zeng, X., *et al*. 2011. Preliminary characterization of the oral microbiota of Chinese adults with and without gingivitis. *BMC Oral Health*, 11(1): 33. http://www.biomedcentral.com/1472-6831/11/33.

Hujoel, P.P. 2002. Does chronic periodontitis cause coronary heart disease? A review of the literature. *J Am Dent Assoc (1939)*, 133 Suppl(June): 31S-36S. http://dx.doi.org/10.14219/jada.archive.2002.0377.

Huttenhower, C., Gevers, D., Knight, R*., et al*. 2012a. Structure, function and diversity of the healthy human microbiome. *Nature*, 486(7402): 207–214.

Homann, N., Tillonen, J., Meurman, J.H., *et al*. 2000. Increased salivary acetaldehyde levels in heavy drinkers and smokers: A microbiological approach to oral cavity cancer. *Carcinogenesis*, 21(4): 663–668.

Igic, M., Kesic, L., Lekovic, V., Apostolovic, M., Mihailovic, D., Kostadinovic, L. & Milasin, J. 2012. Chronic gingivitis: The prevalence of periodontopathogens and therapy efficiency. *Eur J Clin Microbiol Infect Dis*, 31(8): 1911–1915.

Janeway, C.A. & Medzhitov, R. 2002. Innate immune recognition. *Annu Rev Immunol*, 20(2): 197–216.

Janket, S.J., Baird, A.E., Chuang, S.K. & Jones, J.A. 2003. Meta-analysis of periodontal disease and risk of coronary heart disease and stroke. *Oral Surg Oral Med Oral Pathol, Oral Radiol Endod*, 95(5): 559–569.

Jansson, L. 2008. Association between alcohol consumption and dental health. *J Clin Periodontol,* 35(5): 379–384.

Jaspers, I. 2014. Cigarette smoke effects on innate immune mechanisms in the nasal mucosa: Potential effects on the microbiome. *Ann Am Thorac Soc*;11 Suppl 1:S38-42.

doi: 10.1513/AnnalsATS.201306-154MG

Jenkinson, H.F. 2011. Beyond the oral microbiome. *Environ Microbiol*, 13(12): 3077–3087.

Jiang, Y., Zhou, X., Cheng, L. & Li, M. 2020. The Impact of Smoking on Subgingival Microflora: From Periodontal Health to Disease. *Front Microbiol*, 11(January): 1–13.

Kanmaz, B., Lamont, G., Danaci, G., Gogeneni, H., Buduneli, N. & Scott, D.A. 2019. Microbiological and biochemical findings in relation to clinical periodontal status in active smokers, non-smokers and passive smokers. *Tob Induc Dis*, 17(March).

Kantorski, K.Z., de Souza, D.M., Yujra, V.Q., *et al*. 2007. Effect of an alcoholic diet on dental caries and on Streptococcus of the mutans group: Study in rats. *Braz Oral Res*, 21(2): 101–105.

Karabudak, S., Ari, O., Durmaz, B., Dal, T., Basyigit, T., Kalcioglu, M.T. & Durmaz, R. 2019. Analysis of the effect of smoking on the buccal microbiome using next-generation sequencing technology. *J Med Microbiol*, 68(8): 1148–1158.

Kassebaum, N.J., Bernabé, E., Dahiya, M., Bhandari, B., Murray, C.J.L. & Marcenes, W. 2014. Global burden of severe periodontitis in 1990-2010: A systematic review and meta-regression. *J Dent Res*, 93(11): 1045–1053.

Kawasaki, T. & Kawai, T. 2014. Toll-like receptor signalling pathways. *Front Immunol*, 5(SEP): 1–8.

Khader, Y.S., Albashaireh, Z.S.M. & Alomari, M.A. 2004. Periodontal Diseases and the Risk of Coronary Heart and Cerebrovascular Diseases: A Meta-Analysis. *J Periodontol*, 75(8): 1046–1053.

Kilian, M.;, Chapple, I.L.C.; Hannig, M., *et al.* 2016. The oral microbiome – an update for oral healthcare professionals. *Br Dent j*, 221(10): 657–666.

Knight, R., Callewaert, C., Marotz, C., Hyde, E.R., Debelius, J.W., McDonald, D. & Sogin, M.L. 2017. The Microbiome and Human Biology. *Annu Rev Genomics Hum Genet*, 18(March): 65–86.

Kuhle, S., Allen, A.C. & Veugelers, P.J. 2010. Perinatal and childhood risk factors for overweight in a provincial sample of Canadian Grade 5 students. *Int J Pediatr Obes*, 5(1): 88–96.

Kuhle, S., Tong, O.S. & Woolcott, C.G. 2015. Association between caesarean section and childhood obesity: A systematic review and meta-analysis. *Obes Rev*, 16(4): 295–303.

Kulas, T., Bursac, D., Zegarac, Z., Planinic-Rados, G. & Hrgovic, Z. 2013. New Views on Caesarean Section, its Possible Complications and Long-Term Consequences for Children's Health. *Med Arch (Sarajevo, Bosnia and Herzegovina)*, 67(6): 460–463.

Kulkarni, R., Antala, S., Wang, A., *et al*. 2012a. Cigarette smoke increases Staphylococcus aureus biofilm formation via oxidative stress. *Infect Immun*, 80(11): 3804–3811.

Kumar, P.S., Leys, E.J., Bryk, J.M., Martinez, F.J., Moeschberger, M.L. & Griffen, A.L. 2006. Changes in periodontal health status are associated with bacterial community shifts as assessed by quantitative 16S cloning and sequencing. *J Clin Microbiol*, 44(10): 3665–3673.

Kumar, P.S., Matthews, C.R., Joshi, V., de Jager, M. & Aspiras, M. 2011. Tobacco smoking affects bacterial acquisition and colonization in oral biofilms. *Infect Immun* 79(11): 4730–4738.

Lalla, E. & Papapanou, P.N. 2011. Diabetes mellitus and periodontitis: A tale of two common interrelated diseases. *Nat Rev Endocrinol*, 7(12): 738–748. http://dx.doi.org/10.1038/nrendo.2011.106.

Lamont, R.J., Koo, H., Hajishengallis, G., Diseases, I., Health, C.O., Medicine, P.D. & Medicine, P.D. 2019. The oral microbiota : dynamic communities and host interactions. *Nat Rev Microbiol,* 16 (12):745-759. doi: 10.1038/s41579-018-0089-x.

Larsson, L., Szponar, B., Ridha, B.,*et al*. 2008. Identification of bacterial and fungal components in tobacco and tobacco smoke. *Tob Induc Dis*, 4(1): 4.

Lassalle, F., Spagnoletti, M., Fumagalli, M., *et al*. 2018. Oral microbiomes from hunter-gatherers and traditional farmers reveal shifts in commensal balance and pathogen load linked to diet. *Mol Ecol* 27(1): 182–195.

Lee, J., Taneja, V. & Vassallo, R. 2012. Cigarette smoking and inflammation: Cellular and molecular mechanisms. *J Dent Res*, 91(2): 142–149.

Leite, F.R.M., Nascimento, G.G., Scheutz, F. & López, R. 2018a. Effect of Smoking on Periodontitis: A Systematic Review and Meta-regression. *Am J Prev Med*, 54(6): 831–841. http://dx.doi.org/10.1016/j.amepre.2018.02.014.

Liccardo, D., Cannavo, A., Spagnuolo, G., Ferrara, N., Cittadini, A., Rengo, C. & Rengo, G. 2019. Periodontal disease: A risk factor for diabetes and cardiovascular disease. *Int J Mol Sci*, 20(6).

Liu, Y. & Zhang, Q. 2016. Periodontitis aggravated pancreatic β-cell dysfunction in diabetic mice through interleukin-12 regulation on Klotho. *J Diabetes Investig* 7(3): 303–311.

Long, J., Cai, Q., Steinwandel, M., *et al.* 2017. Association of oral microbiome with type 2 diabetes risk. *J Periodontal Res*, 52(3): 636–643.

López-otín, C., Blasco, M.A., Partridge, L., Serrano, M. & Kroemer, G. 2013. The Hallmarks of Aging Longevity. *Cell*, 153(6): 1194.

Marrero, S. & Adashi, E.Y. 2015. Noncommunicable diseases. *Semin Reprod Med*, 33(1): 35–40.

Masdea, L., Kulik, E.M., Hauser-Gerspach, I., Ramseier, A.M., Filippi, A. & Waltimo, T. 2012. Antimicrobial activity of Streptococcus salivarius K12 on bacteria involved in oral malodour. *Arch Oral Biol*, 57(8): 1041–1047. http://dx.doi.org/10.1016/j.archoralbio.2012.02.011.

Mason, M.R., Nagaraja, H.N., Camerlengo, T., Joshi, V. & Kumar, P.S. 2013. Deep Sequencing Identifies Ethnicity-Specific Bacterial Signatures in the Oral Microbiome. *PLoS ONE*, 8(10): 1–7.

Mason, M.R., Preshaw, P.M., Nagaraja, H.N., Dabdoub, S.M., Rahman, A. & Kumar, P.S. 2015a. The subgingival microbiome of clinically healthy current and never smokers. *ISME J*, 9(1): 268–272.

Matthews, J.B., Chen, F.M., Milward, M.R., Ling, M.R. & Chapple, I.L.C. 2012. Neutrophil superoxide production in the presence of cigarette smoke extract, nicotine and cotinine. *J Clin Periodontol*, 39(7): 626–634.

Mattiuzzi, C. & Lippi, G. 2020. Estimation of Worldwide Burden of Smoking-Related Asthma. *Int Arch Allergy Immunol*, 181(4): 292–295.

Mei, F., Xie, M., Huang, X., Long, Y., Lu, X., Wang, X. & Chen, L. 2020. Porphyromonas gingivalis and its systemic impact: Current status. *Pathogens*, 9(11): 1–23.

Meyle, J. & Chapple, I. 2015. Molecular aspects of the pathogenesis of periodontitis. *Periodontol 2000*, 69(1): 7–17.

de Miguel-Infante, A., Martinez-Huedo, M.A., Mora-Zamorano, E., *et al*. 2019. Periodontal disease in adults with diabetes, prevalence and risk factors. Results of an observational study. *Int J Clin Pract*, 73(3): 1–9.

Milani, C., Duranti, S., Bottacini, F., et al., 2017. The First Microbial Colonizers of the Human Gut: Composition, Activities, and Health Implications of the Infant Gut Microbiota. *Microbiol Mol Biol Rev*, 81(4): 1–67.

Minty, M., Canceil, T., Serino, M., Burcelin, R., Tercé, F. & Blasco-Baque, V. 2019. Oral microbiota-induced periodontitis: a new risk factor of metabolic diseases. *Rev Endocr Metab Disord*, 20(4): 449–459.

Moon, J.H., Lee, J.H. & Lee, J.Y. 2015a. Subgingival microbiome in smokers and non-smokers in Korean chronic periodontitis patients. *Mol Oral Microbiol*, 30(3): 227–241.

Morgan, X, Segata, N. 2008. Biodiversity and Functional Genomics in the Human Microbiome. *Bone*, 23(1): 1–7. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3624763/pdf/nihms412728.pdf.

Morita, I., Inagaki, K., Nakamura, F., *et al.* 2012. Relationship between periodontal status and levels of glycated hemoglobin. *J Dent Res* 91(2): 161–166.

Morris, A., Beck, J.M., Schloss, P.D., *et al*. 2013. Comparison of the respiratory microbiome in healthy nonsmokers and smokers. *Am J Respir Crit Care Med*, 187(10): 1067–1075.

Murthy, S.N.S., Dinoso, V.P., Clearfield, H.R. & Chey, W.Y. 1978. Serial pH Changes in the Duodenal Bulb During Smoking. *Gastroenterology*, 75(1): 1–4. http://dx.doi.org/10.1016/0016-5085(78)93754-X.

Muszer, M., Noszczyńska, M., Kasperkiewicz, K. & Skurnik, M. 2015. Human Microbiome: When a Friend Becomes an Enemy. *Arch Immunol Ther Exp*, 63(4): 287–98.

Mutepe, N.D., Cockeran, R., Steel, H.C., Theron, A.J., Mitchell, T.J., Feldman, C. & Anderson, R. 2013. Effects of cigarette smoke condensate on pneumococcal biofilm formation and pneumolysin. *Eur Respir J*, 41(2): 392–395.

Nociti, F.H., Casati, M.Z. & Duarte, P.M. 2015. Current perspective of the impact of smoking on the progression and treatment of periodontitis. *Periodontol 2000*, 67(1): 187–210.

Ogawa, T., Hirose, Y., Honda-Ogawa, M., et al. 2018. Composition of salivary microbiota in elderly subjects. *Sci Rep*, 8(1): 1–8. http://dx.doi.org/10.1038/s41598-017-18677-0.

Padilla, C., Lobos, O., Hubert, E., *et al*. 2006. Periodontal pathogens in atheromatous plaques isolated from patients with chronic periodontitis. *J Periodontal Res*, 41(4): 350–353.

Perera, M., Al-Hebshi, N.N., Speicher, D.J., Perera, I. & Johnson, N.W. 2016. Emerging role of bacteria in oral carcinogenesis: A review with special reference to perio-pathogenic bacteria. *J Oral Microbiol*, 8(1): 1–10.

Pérez-Losada, F. L., Jané-Salas, E., Sabater-Recolons, M., *et al.* 2016. Correlation between periodontal disease management and metabolic control of type 2 diabetes mellitus. A systematic literature review. *Med Oral Patol Oral Cir Bucal*, 21(4): e440–e446.

Petersen, P.E., Bourgeois, D., Ogawa, H., Estupinan-Day, S. & Ndiaye, C. 2005. The global burden of oral diseases and risks to oral health. *Bull World Health Organ*, 83(9): 661–669.

Portela, D.S., Vieira, T.O., Matos, S.M.A., de Oliveira, N.F. & Vieira, G.O. 2015. Maternal obesity, environmental factors, caesarean delivery and breastfeeding as determinants of overweight and obesity in children: Results from a cohort. *BMC Pregnancy Childbirth*, 15(1): 1–10. doi: 10.1186/s12884-015-0518-z.

Preshaw, P.M., Alba, A.L., Herrera, D., Jepsen, S., Konstantinidis, A., Makrilakis, K. & Taylor, R. 2012. Periodontitis and diabetes: a two-way relationship Matrix metalloproteinase NHANES National Health and Nutrition Examination Survey. *Diabetologia*, 55: 21–31.

Preshaw, P.M. & Bissett, S.M. 2019. Periodontitis and diabetes. *Br Dent J*, 227(7): 577–584.

Psaltopoulou, T., Hatzis, G., Papageorgiou, N., Androulakis, E., Briasoulis, A. & Tousoulis, D. 2017. Socioeconomic status and risk factors for cardiovascular disease: Impact of dietary mediators. *Hellenic J Cardiol*, 58(1): 32–42. http://dx.doi.org/10.1016/j.hjc.2017.01.022.

Reilly, J.J., Armstrong, J., Dorosty, A.R., *et al*. 2005. Early life risk factors for obesity in childhood: Cohort study. *BMJ*, 330(7504): 1357–1359.

Rengo, G., Pagano, G., Paolillo, S., *et al*. 2015. Impact of diabetes mellitus on lymphocyte GRK2 protein levels in patients with heart failure. *Eur J Clin Invest*, 45(2): 187–195.

Reznick, A.Z., Klein, I., Eiserich, J.P., Cross, C.E. & Nagler, R.M. 2003. Inhibition of oral peroxidase activity by cigarette smoke: In vivo and in vitro studies. *Free Radic Biol Med*, 34(3): 377–384.

Rinninella, E., Raoul, P., Cintoni, M., *et al*. 2019. What is the healthy gut microbiota composition? A changing ecosystem across age, environment, diet, and diseases. *Microorganisms*, 7(1).

Rutayisire, E., Wu, X., Huang, K., Tao, S., Chen, Y. & Tao, F. 2016. Caesarean section may increase the risk of both overweight and obesity in preschool children. *BMC Pregnancy Childbirth*, 16(1): 1–8. http://dx.doi.org/10.1186/s12884-016-1131-5.

Sampaio-Maia, B. & Monteiro-Silva, F. 2014. Acquisition and maturation of oral microbiome throughout childhood: An update. *Dent Res J*, 11(3): 291–301.

Santos, C.M.M.L., Lira, R., Fischer, R.G., Santos, A.P.P. & Oliveira, B.H. 2015. Systemic antibiotics in periodontal treatment of diabetic patients: A systematic review. *PLoS ONE*, 10(12): 1–11.

Sanz, M., Marco del Castillo, A., Jepsen, S.,*et al*. 2020. Periodontitis and cardiovascular diseases: Consensus report. *J Clin Periodontol*, 47(3): 268–288.

Sapkota, A.R., Berger, S., Vogel, T.M., Reynolds, C.R.J. & Co, T. 2010. Human Pathogens Abundant in the Bacterial Metagenome of Cigarettes. *, Environ Health* Perspect,118(3): 351–356.

Scannapieco, F.A., Bush, R.B. & Paju, S. 2003. Associations between periodontal disease and risk for nosocomial bacterial pneumonia and chronic obstructive pulmonary disease. A systematic review. *Ann Periodontol*, 8(1): 54–69.

Schwarzberg, K., Le, R., Bharti, B., *et al*. 2014. The personal human oral microbiome obscures the effects of treatment on periodontal disease. *PLoS ONE*, 9(1): 1–8.

Sender, R., Fuchs, S. & Milo, R. 2016. Revised Estimates for the Number of Human and Bacteria Cells in the Body. *PLoS Biol*, 14(8): 1–14.

Shanahan, E.R., Shah, A., Koloski, N., Walker, M.M., Talley, N.J., Morrison, M. & Holtmann, G.J. 2018. Influence of cigarette smoking on the human duodenal mucosa-associated microbiota. *Microbiome*, 6(1): 1–12.

Shchipkova, A.Y., Nagaraja, H.N. & Kumar, P.S. 2010. Subgingival microbial profiles of smokers with periodontitis. *J Dent Res*, 89(11): 1247–1253.

Sheflin, A.M., Melby, C.L., Carbonero, F. & Weir, T.L. 2017. Linking dietary patterns with gut microbial composition and function. *Gut Microbes*, 8(2): 113–129.

Shepherd, S. 2011. Alcohol consumption a risk factor for periodontal disease. *Evid*

*Based Dent,* 12(3): 76.

Shreiner, A.B., Kao, J.Y. & Young, V.B. 2015. The gut microbiome in health and in disease. *Curr Opin Gastroenterol*, 31(1): 69–75.

Si, J., Lee, C. & Ko, G.P. 2017. Oral microbiota: Microbial biomarkers of metabolic syndrome independent of host genetic factors. *Front Cell Infect Microbiol*, 7(DEC): 1–11.

Signoretto, C., Bianchi, F., Burlacchini, G., *et al.* 2010. Drinking habits are associated with changes in the dental plaque microbial community. *J Clin Microbiol*, 48(2): 347–356

Singh, H., Torralba, M.G., Moncera, K.J., DiLello, L., Petrini, J., Nelson, K.E. & Pieper, R. 2019. Gastro-intestinal and oral microbiome signatures associated with healthy aging. *GeroScience*, 41(6): 907–921.

Singh, R.K., Chang, H.W., Yan, D., *et al*. 2017. Influence of diet on the gut microbiome and implications for human health. *J Transl Med*, 15(1).

Sonnenburg, E.D., Smits, S.A., Tikhonov, M., Higginbottom, S.K., Wingreen, N.S. & Sonnenburg, J.L. 2016. generations. *Nature*, 529(7585): 212–215.

Sudhakara, P., Gupta, A., Bhardwaj, A. & Wilson, A. 2018. Oral dysbiotic communities and their implications in systemic diseases. *Dent Jl*, 6(2): 1–14.

Sullivan, Å. 2001. Effect of antimicrobial agents on the ecological balance of human microflora. *Lancet Infect Dis*, 1(2): 101–114.

Takeda, M., Ojima, M., Yoshioka, H., *et al*. 2006. Relationship of Serum Advanced Glycation End Products With Deterioration of Periodontitis in Type 2 Diabetes Patients. *J Periodontol*, 77(1): 15–20.

Thomas, S., Izard, J., Walsh, E., et al. 2017. The Host Microbiome Regulates and Maintains Human Health: A Primer and Perspective for Non-Microbiologists. *Cancer Res.*, 77(8): 1783–1812.

Thompson, S. V., Hannon, B.A., An, R. & Holscher, H.D. 2017. Effects of isolated soluble fiber supplementation on body weight, glycemia, and insulinemia in adults with overweight and obesity: A systematic review and meta-analysis of randomized controlled trials. *Am J Clin Nutr*, 106(6): 1514–1528.

Thorstensson, H., Dahlén, G. & Hugoson, A. 1995. Some suspected periodontopathogens and serum antibody response in adult long-duration insulin-dependent diabetics. *J Clin Periodontol*, 22(6): 449–458.

Tomás, I., Diz, P., Tobías, A., Scully, C. & Donos, N. 2012. Periodontal health status and bacteraemia from daily oral activities: Systematic review/meta-analysis. *J Clin Periodontol*, 39(3): 213–228.

Tonetti, M.S., D'Aiuto, F. & Nibali, L. 2008. Treatment of periodontitis and endothelial function. *N Engl J Med*, 67(4): 353.

Ursell, L.K., Metcalf, J.L., Parfrey, L.W. & Knight, R. 2012. Defining the human microbiome. *Nut Rev*, 70Suppl 1(Suppl 1):S38-44 doi: 10.1111/j.1753-4887.2012.00493.x.

Ussar, S., Griffin, N.W., Bezy, O., *et al.* 2015. Interactions between gut microbiota, host genetics and diet modulate the predisposition to obesity and metabolic syndrome. *Cell Metab*, 22(3): 516–530.

Viganò, L., Ferraro, S., Casu, C. & Fanuli, M. 2018. The Oral Microbiome in the Context of Aging and Cognitive Impairment. *Acta Scientific Microbiology*, 1(8): 2581–3226.

Vijay-Kumar, M., Aitken, J.D., Carvalho, F.A.,*et al*. 2010. Metabolic syndrome and altered gut microbiota in mice lacking toll-like receptor 5. *Science*, 328(5975): 228–231.

Vijay-kumar, M., Aitken, J. D., Frederic, A., Cullender, Tyler, C. & Mwangi, S. 2013. Metabolic Syndrome and Altered Gut Microbiota in Mice Lacking Toll-Like receptor 5. *Science*, 328(August): 228–231.

Virani, S.S, Alonso A, Benjamin, E.J., *et al.* 2020 Heart Disease & Stroke Statistical Update Fact Sheet Global Burden of Disease High Blood Cholesterol and Other Lipids. *AHA J*, (Cvd): 9–11.

Vos, T., Abajobir, A.A., Abbafati, C., *et al.* 2017. Global, regional, and national incidence, prevalence, and years lived with disability for 328 diseases and injuries for 195 countries, 1990-2016: A systematic analysis for the Global Burden of Disease Study 2016. *Lancet*, 390(10100): 1211–1259.

Wade, W.G. 2013. The oral microbiome in health and disease. *Pharmacol Res*, 69(1): 137–143.

Wang, B., Yao, M., Lv, L., Ling, Z. & Li, L. 2017. The Human Microbiota in Health and Disease. *Engineering*, 3(1): 71–82.

Wang, R.R., Xu, Y.S., Ji, M.M., *et al.* 2019. Association of the oral microbiome with the progression of impaired fasting glucose in a Chinese elderly population. *J Oral Microbiol*, 11(1).

Wen, L. & Duffy, A. 2017. Factors influencing the gut microbiota, inflammation, and type 2 diabetes. *J Nutri*, 147(7): 1468S-1475S.

Wescombe, P.A., Heng, N.C.K., Burton, J.P., Chilcott, C.N. & Tagg, J.R. 2009. Streptococcal bacteriocins and the case for. *Future Microbiol*: 819–835.

Williams, R.C. & Genco, R.J. 2010. *Periodontal Disease and Overall Health : A Clinician's Guide*.

Winning, L. & Linden, G.J. 2018. A review of the relationship between chronic periodontitis and diabetes. *US Endocrinology*, 14(2): 80–85.

Wirth, R., Maróti, G., Mihók, R., Simon-Fiala, D., Antal, M., Pap, B., Demcsák, A., Minarovits, J. & Kovács, K.L. 2020. A case study of salivary microbiome in smokers and non-smokers in Hungary: analysis by shotgun metagenome sequencing. *J Oral Microbiol*, 12(1). https://doi.org/10.1080/20002297.2020.1773067.

Wolff, L.F. 2014. Diabetes and periodontal disease. *Am J Dent*, 27(3): 127–128.

Wu, H., Zeng, B., Li, B., *et al.* 2018. Research on oral microbiota of monozygotic twins with discordant caries experience - In vitro and in vivo study. *Sci Rep*, 8(1): 1–8.

Wu, J., Li, M. & Huang, R. 2019. The effect of smoking on caries-related microorganisms. *Tob Induc Dis*, 17(April).

Wu, J., Peters, B.A., Dominianni, C.,*et al.* 2016. Cigarette smoking and the oral microbiome in a large study of American adults. *The ISME J*, 10: 2435–2446.

Xiao, E., Mattos, M., Vieira, G.H.A., et al. 2017. Diabetes Enhances IL-17 Expression and Alters the Oral Microbiome to Increase Its Pathogenicity. *Cell Host and Microbe*, 22(1): 120-128.e4.

Xu, J., Xiong, M., Huang, B. & Chen, H. 2015. Advanced Glycation End Products Upregulate the Endoplasmic Reticulum Stress in Human Periodontal Ligament Cells. *J Periodontol*, 86(3): 440–447.

Xu, W., Zhou, W., Wang, H. & Liang, S. 2020. Roles of Porphyromonas gingivalis and its virulence factors in periodontitis. Elsevier Ltd. https://doi.org/10.1016/bs.apcsb.2019.12.001.

Xu, X., He, J., Xue, J., *et al.* 2015. Oral cavity contains distinct niches with dynamic microbial communities. *Environ Microbiol*, 17(3): 699–710.

Yamashita, K., Nishiyama, T., Yokoyama, T., Abe, H. & Manabe, M. 2005. A comparison of the rate of bacterial contamination for prefilled disposable and reusable oxygen humidifiers. *J Crit Care.* 20: 172–175. http://10.0.3.248/j.jcrc.2005.01.002.

Yamashita, Y. & Takeshita, T. 2017. The oral microbiome and human health. *J Oral Sci*, 59(2): 201–206.

Yang, Y., Cai, Q., Zheng, W., Steinwandel, M. & Blot, W.J. 2019. Oral microbiome and obesity in a large study of low-income and African-American populations. *J Oral Microbiol*, 11(00).

Yiu, J.H.C., Dorweiler, B. & Woo, C.W. 2017. Interaction between gut microbiota and toll-like receptor: from immunity to metabolism. *J Mol Med*, 95(1): 13–20. http://dx.doi.org/10.1007/s00109-016-1474-4.

Yu, G., Phillips, S., Gail, M.H., et al. 2017. The effect of cigarette smoking on the oral and nasal microbiota. *Microbiome*, 5(1): 3.

Zarco, M.F., Vess, T.J. & Ginsburg, G.S. 2012. The oral microbiome in health and disease and the potential impact on personalized dental medicine. *Oral Dis*, 18(2): 109–120.

Zhang, C., Zhang, M., Wang, S., *et al*. 2010. Interactions between gut microbiota, host genetics and diet relevant to development of metabolic syndromes in mice. *ISME Journal*, 4(2): 232–241.

Zhang, X., Zhang, D., Jia, H., Feng, Q., Wang, D., Liang, D et al., 2015. The oral and gut microbiomes are perturbed in rheumatoid arthritis and partly normalized after treatment. *Nat Med* 21(8): 895–905. http://dx.doi.org/10.1038/nm.3914.

Zou, J., Chassaing, B., Singh, V., et al. 2018. Fiber-Mediated Nourishment of Gut Microbiota Protects against Diet-Induced Obesity by Restoring IL-22-Mediated Colonic Health. *Cell Host Microbe*, 23(1): 41-53.e4.

# CHAPTER 3: MANUSCRIPT 1

Published in Journal of Dental Research

**Oral Microbiome Signatures in Diabetes Mellitus and Periodontal Disease**

Yvonne Prince[1], Saarah Davids[1], Rajiv T Erasmus[3], Andre P Kengne [4,5], Glenda M Davison[1], Tandi E Matsha [1*]

[1]SAMRC/CPUT Cardiometabolic Health Research Unit, Department of Biomedical Sciences, Faculty of Health and Wellness Science, Cape Peninsula University of Technology, Cape Town, South Africa

[2]Division of Chemical Pathology, Faculty of Health Sciences, National Health Laboratory Service (NHLS) and Stellenbosch University, Cape Town, South Africa

[3]Non-Communicable Diseases Research Unit, South African Medical Research Council, Cape Town, South Africa

[4]Department of Medicine, University of Cape Town, Cape Town, South Africa

**Corresponding Author:**

T.E. Matsha, SAMRC/CPUT/Cardiometabolic Health Research Unit, Department of Biomedical Sciences, Faculty of Health and Wellness Sciences, Cape Peninsula University of Technology, PO Box 1906, Bellville, Cape Town 7530, South Africa.

Email: matshat@cput.ac.za

Word count, 3616

Number of figures and Tables: 5

Number of supplementary files: 7

**Abstract**

Disturbances in the oral microbiome are associated with periodontal disease initiation and progression and diabetes mellitus (DM), but how this contributes to the cause-and-effect relationship between periodontal disease and DM is poorly understood. We examined the bacterial composition in plaque samples from 128 South Africans with periodontal disease across glycemic statuses using 16S rDNA sequencing of regions 2, 3, 4, 6-7, 8, and 9. Of the 9 phyla identified, Firmicutes, Proteobacteria, Bacteroidetes, Fusobacteria, and Actinobacteria made up >98%. Fusobacteria and Actinobacteria were significantly more abundant in subjects with diabetes, while Proteobacteria were less abundant. However, in the presence of gingival bleeding and DM, as compared with DM without gingival bleeding, Actinobacteria were markedly reduced while Bacteroidetes were more abundant. In contrast, no differences in Actinobacteria or Bacteroidetes abundance were observed between DM with and without pocket depth (PD) ≥4 mm. At the genus level, similar changes in relative abundance were observed in the presence of DM and periodontal disease. Our findings remained in conditional logistic regression models adjusted for age, sex, waist circumference, and the 5 most dominant phyla. For example, Actinobacteria significantly increased the odds of diabetes by 10% in subjects with gingival bleeding, while Fusobacteria increased this odd by 14%; yet, among subjects with PD ≥4 mm, Fusobacteria decreased the odds of DM by 47%. Our findings have confirmed the alterations in the composition of the oral microbiota across glycemic statuses as well as different stages of periodontal disease. However, it is not clear whether these differences were the consequence of hyperglycemia or the presence of periodontal diseases. Therefore, we recommend further investigations in a longitudinal study design.

**Keywords: Africa, Actinobacteria, diabetes, 16S rDNA, microbiome, periodontitis**

## 1. Introduction

The Human Microbiome Project was initiated as an attempt to investigate and characterize the genetic diversity, distribution, and biogeography of the microorganisms inhabiting the human microbiome. The information generated from this has led to a more comprehensive understanding of the predisposition to certain diseases and to the possibility of developing therapeutic strategies that target the human microbiome (Turnbaugh et al.2007). Trillions of microorganisms inhabit the human microbiome and can be found on the internal and external surfaces of humans (Grice and Segre 2012). Each microorganism plays a vital role in the physiology of the human body, including the development of the immune system and the control of digestion and absorption (D'Argenio and Salvatore 2015). However, if the balance, interaction, and diversity of these microorganisms are disrupted, it can lead to an unhealthy state and disease (Young 2017). The oral microbiome is populated by a diverse population of oral microorganisms, which comprise >700 bacterial species. Studies have demonstrated that the oral microbiota in healthy individuals remains stable and is usually nonpathogenic. Some may, however, become destructive and lead to the development of oral diseases such as periodontitis (Griffen et al. 2012; Liu et al. 2012; Abusleme et al. 2013; Li et al.2015; Park et al. 2015). In 2017, the International Diabetic Federation listed periodontitis as a risk factor in individuals with diabetes. The association between the conditions, though, remains unclear and is believed to be bidirectional (Taylor 2001; Negrato et al. 2013). These studies have been supported by evidence that has shown that the risk of periodontitis is increased threefold in diabetic individuals as compared with nondiabetic individuals (Mealey and Oates 2006) and that adults with a glycated hemoglobin (HbA1c) level >9% have a significantly higher prevalence of severe periodontitis than those without diabetes (Preshaw et al. 2012). Our previous findings showed a high prevalence of diabetes and periodontitis in a South African population from the Western Cape, Cape Town (Erasmus et al. 2012; Chikte et al. 2019). Therefore, in this study, we aimed to investigate the composition of the oral microbiome across all glucose tolerance statuses and periodontal diseases in South African adults from Cape Town.

## 2. Materials and Methods

### *2.1. Ethical Considerations*

The current study forms part of the Vascular and Metabolic Health (VMH) Study registered at the Cape Peninsula University of Technology. Ethical approval for the study was obtained from the research ethics committees of the Cape Peninsula University of Technology and Stellenbosch University (NHREC: REC-230 408-014, CPUT/HW-REC 2015/H01, CPUT/HW-REC 2017/H31, and N14/01/003). The study was conducted according to the code of ethics of the World Medical Association (Declaration of Helsinki).

### *2.2. Study Design and Procedures*

This was a case-control study involving participants from the ongoing VMH Study, which is an extension of the Cape Town Bellville South study and has been described in detail previously (Kengne et al. 2017). The sample consisted of 128 randomly selected individuals—32 normotolerant, 32 prediabetes, 32 screen-detected type 2 diabetes mellitus (T2DM), 32 known T2DM receiving treatment—from the VMH cohort of 2,000 who participated in a diabetes mellitus screening program with an oral glucose tolerance test between 2014 and 2016. All participants except those with known diabetes and treatment underwent an oral glucose tolerance test. Plasma glucose, HbA1c, lipids, insulin, serum cotinine, gamma-glutamyl transferase, and C-reactive protein were measured in an ISO 15189–accredited pathology practice (PathCare **Reference** Laboratory).

### *2.3. Periodontal Assessment and Plaque*

#### *Sample Collection*

Bleeding on probing was recorded for each tooth as presence or absence of bleeding after gentle periodontal probing around each tooth circumference. For pocket depth (PD), each tooth was probed in its whole circumference, and the highest score was recorded. The examination and classification were conducted according to guidelines from the World Health Organization (WHO; 2016) and the Community Periodontal Index, as shown in Appendix Table 1. Plaque samples were collected with a wood toothpick because this method has been shown to yield a higher or similar proportion of bacteria when compared with saliva or supragingival plaque sampling (Hsuet al. 2010; Okada et al. 2017). Briefly, plaque sampling was achieved by inserting the device in the subgingival crevice between the maxillary second premolar and the first upper molar, and 4 toothpick samples were collected from both sides of the mouth and

stored separately. Plaque samples with visible presence of blood were not included in this study. The samples were immediately stored at –80 °C until DNA isolation and purification.

*2.4. 16S Metagenomic Sequencing*

*DNA Isolation and Quality Control.* A minimum of 2 picks were selected for each participant, and the DNA extracted from each toothpick was pooled and sequenced. DNA was extracted from plaque samples with the Zymo Quick–DNA Fungal/Bacterial Miniprep Kit (Zymo Research) per the manufacturer's instructions, and all samples met a 260/280 value >1.8. A concentration ≥20 μg/μL was used for 16S rDNA sequencing. Metagenomic DNA (mgDNA) from these samples were quantified on the Qubit 4.0 Fluorometer with the Qubit 1x dsDNA HS assay kit according to the protocol (MAN0017455 Rev. A.0). Spectrophotometry was performed on the NanoDrop ND-1000 to determine the purity of the mgDNA samples. Genomic quality scores were determined on the LabChip GXII Touch with the DNA Extended Range LabChip and Genomic DNA Reagent Kit (PerkinElmer) according to the protocol (CLS140166 Rev. C; Supplementary Report: Genomic DNA [gDNA] Quality Control). The genomic quality score is a value between 0 and 5, with 5 indicating high-quality gDNA. *Metagenomics.* The Ion 16S Metagenomics Kit was used to amplify hypervariable regions from polybacterial samples according to the protocol (MAN0010799 REV C.0). Target regions were amplified from 2 μL of mgDNA across 25 cycles with 2 primers pools on the SimpliAmp Thermal Cycler (ThermoFisher Scientific). Following verification, polymerase chain reaction products from the 2 primer pools were combined for each sample, purified with Agencourt AMPure XP reagent, and eluted in 15 μL of nuclease-free water. Purified amplicons were quantified on the Qubit 4.0 Fluorometer with the Qubit 1x dsDNA HS assay kit according to the protocol (MAN001 7455 Rev. A.0).

*2.5. Library Preparation.*

Library preparation was performed from 100 ng of pooled amplicons for each sample with the NEXTflex DNA Sequencing Kit according to the protocol (v 15.12; Bio Scientific Corporation). Library fragment size distributions were assessed on the LabChip GXII Touch (PerkinElmer), with the X-mark chip and HT DNA NGS 3K reagent kit according to the manufacturer's protocol (CLS145098 Rev. E).

*2.6. Template Preparation, Enrichment, Sequencing, and Analysis.*

Libraries were diluted to a target concentration of 10 pM. The diluted and barcoded 16S libraries were combined in equimolar amounts for template preparation with the Ion 510, Ion 520, and Ion 530 Chef Kit. In brief, 25 μL of the pooled library was loaded on the Ion Chef liquid handler with reagents, solutions, and supplies according to the protocol (MAN0016854, REV.C.0). Enriched template-positive ion sphere particles were loaded onto an Ion 530 Chip. Massively parallel sequencing was performed on the Ion S5 Gene Studio with the Ion S5 Sequencing Solutions and Sequencing Reagents Kits according to the protocol (MAN0016854, REVC.0). Flow space calibration and BaseCaller analyses were performed with default analysis parameters in the Torrent Suite software (v 5.12.0). Raw sequence data and taxonomy assignment were as previously described (Saeb et al. 2019). Alpha diversity was computed by Chao1, observed species, Shannon, and Simpson indices. EMPeror (v 0.9.60) was used to visualize beta diversity and transformed operational taxonomic unit counts were used for principal coordinate analysis.

*2.7. Calculations and Definitions*

Body mass index was calculated as weight per square meter (kg/m2). Categories of glucose tolerance were defined by applying the 1998 WHO criteria (Alberti and Zimmet 1998). PD ≥4 mm was indicative of periodontal disease. Gingival bleeding was defined as the presence of ≥30% of bleeding on probing sites.

*2.8. Statistical Analysis*

The software program SPSS (v 25; IBM Corp) and R statistical software (v 3.6.2; R Foundation for Statistical Computing Platform) were used for data analyses. The results were reported as number (prevalence) for periodontal variables (gingival bleeding and PD ≥4 mm), and the chi-square test was used to compare results. Independent *t* test was used to determine statistically significant differences in the relative abundances between cases and controls for the phylum and genus. We used a conditional logistic regression to estimate odds ratio (OR) and 95% CIs for the association between glycemic status and periodontal diseases. The analysis was performed for each glycemic status and adjusted for age, sex, and waist circumference.

## 3. Results

As expected, the glycemic indices (plasma glucose and HbA1c) were significantly increased in subjects with hyperglycemia (prediabetes or diabetes; all $P \leq 0.0001$). Similarly, anthropometric measurements were significantly increased in females and in subjects with hyperglycemia ($P \leq 0.0069$); however, there was no significant interaction between sex and glycemic status. More than two-thirds of all subjects (63.4%, $n = 81$) had bleeding on probing. Although this was nonsignificantly distributed among the glycemic statuses, 24 (75%) subjects with known diabetes and treatment had gingival bleeding. Periodontal PD ≥4 mm was observed in 75 (58.6%) individuals, and these included 7 (5.5%) with PD ≥6 mm (Table 1). Based on Chao1 index, the alpha diversity was decreased in individuals with diabetes as compared with those with prediabetes or normoglycemia (Appendix Table 2). Beta diversity indicated a dissimilarity between diabetes and normoglycemia (Appendix Fig. 1). From a total of 9 phyla (Firmicutes, Proteobacteria, Bacteroidetes, Fusobacteria, Actinobacteria, Spirochaetes, Synergistetes, Chloroflexi, and Tenericutes), 5 (Firmicutes, Proteobacteria, Bacteroidetes, Fusobacteria, Actinobacteria) made up >98% across all categories of glycemic status and periodontal disease, as shown in Figure 1 and Appendix Figure 2. Fusobacteria were significantly less in prediabetes ($P = 0.018$), while Proteobacteria were reduced in those with diabetes ($P = 0.002$). Firmicutes were significantly more abundant in prediabetes and diabetes ($P = 0.001$). In individuals with gingival bleeding, the Bacteroidetes were significantly more abundant than in those without bleeding ($P = 0.022$), while Actinobacteria were slightly less abundant ($P = 0.081$; Fig.1B). No significant differences were observed between phyla and the presence of PD ≥4 mm (Fig. 1C). Actinobacteria were more abundant in individuals with diabetes and without gingival bleeding, while Bacteroidetes were more abundant in subjects with diabetes or prediabetes and gingival bleeding ($P \leq 0.03$). Proteobacteria were relatively less abundant in subjects with diabetes with or without gingival bleeding as compared with those with prediabetes and no gingival bleeding or normoglycemia with or without gingival bleeding ($P \leq 0.04$; Fig. 2A). A similar trend was observed in subjects with prediabetes and PD ≥4 mm, except Actinobacteria were markedly increased in diabetes irrespective of periodontal PD ($P \leq 0.01$; Fig. 2B, Appendix Fig. 3). At the genus level, *Actinomyces, Corynebacterium, Leptotrichia, Olsenella, Selenomonas*, and *Tannerella* were more abundant in subjects with diabetes or prediabetes, while *Aggregatibacter, Campylobacter, Fusobacterium, Haemophilus, Mannheimia*, and *Neisseria* were relatively less abundant (Fig. 3A). *Bergeyella* was <5% in subjects with normoglycemia and prediabetes but absent in those with diabetes. *Streptococcus,*

*Actinomyces, Haemophilus*, and *Granulicatella* were less abundant in subjects with gingival bleeding than in those without, while *Prevotella, Fusobacterium, Neisseria, Porphyromonas, Olsenella*, and *Gemella* were more abundant (Fig. 3B). In subjects with PD ≥4 mm, *Leptotrichia* and *Selenomonas* were more abundant than in those with PD <4 mm. *Haemophilus, Corynebacterium, Porphyromonas, Gemella*, and *Rothia* were relatively less abundant in those with PD ≥4 mm than in those with PD <4 mm (Fig. 3C, Appendix Fig. 4). *Neisseria* was increased in those who were normotolerant or had prediabetes with gingival bleeding, while *Haemophilus* was decreased in these groups. However, *Actinomyces* and *Prevotella* were relatively more abundant in subjects with prediabetes or diabetes, but a marked increase was observed in *Actinomyces* in diabetes without gingival bleeding while *Prevotella* was markedly increased in those with gingival bleeding (Fig. 4A). When we compared genera in subjects with gingival bleeding or PD ≥4 mm and prediabetes or diabetes, reduced *Porphyromonas* were observed in the presence of PD ≥4 mm and prediabetes versus those without PD ≥4 mm or diabetes with or without PD ≥4 mm. *Selenomonas* and *Leptotrichia* (*P* = 0.027) were relatively more abundant in the presence of pockets ≥4 mm and diabetes (Fig. 4B, Appendix Fig. 5). In conditional logistic regression models adjusted for age, sex, waist circumference, and the 5 most dominant phyla (Firmicutes, Proteobacteria, Bacteroidetes, Fusobacteria, Actinobacteria), waist circumference (OR, 1.02 [95% CI, 1.01 to 1.04], *P* = 0.024), Actinobacteria (1.10 [1.03-1.16], *P* =0.003), and Fusobacteria (1.14 [1.02 to 1.27], *P* = 0.02) were associated with prevalent diabetes in subjects with gingival bleeding as compared with those without gingival bleeding. However, in subjects with diabetes and PD ≥4 mm, only waist circumference (1.04 [1.01 to 1.02], *P* = 0.023) was significantly associated with diabetes (i.e., none of the phyla). In similar conditional logistic regression models adjusted for age, sex, and waist circumference and replacing the phyla with genera, the following were associated with diabetes among individuals with gingival bleeding: age (OR, 1.05 [95% CI, 1.01 to 1.08], *P* = 0.012), *Veillonella* (0.73 [0.54 to 0.99], *P* = 0.041), *Neisseria* (0.47 [0.22 to 1.01], *P* = 0.054), and *Porphyromonas* (1.55 [1.00 to 2.51], *P* = 0.076). In subjects with diabetes and PD ≥4 mm, the OR for age was 1.05 (95% CI: 1.01 to 1.09, *P* = 0.012); *Capnocytophaga*, 1.82 (1.03 to 3.331, *P* = 0.038); *Neisseria*, 0.64 (0.46 to 0.88, *P* = 0.007); *Porphyromonas*, 1.84 (1.08 to 3.14, *P* = 0.025); and *Streptococcus*, 0.81 (0.68 to 0.97, *P* = 0.023).

## 4. Discussion

This study of the oral microbiome in 128 participants across all glycemic groups has demonstrated that the distribution of the microbiome differs according to glycemic status and/or the presence of periodontal disease. Across all glucose tolerance statuses or the presence of periodontal disease, the 5 dominant phyla that constituted >98% of the 9 phyla were Firmicutes, Proteobacteria, Bacteroidetes, Fusobacteria, and Actinobacteria. In individuals with prediabetes or DM, Fusobacteria and Actinobacteria were significantly more abundant and Proteobacteria less abundant, while in subjects with gingival bleeding, Bacteroidetes were significantly more abundant. Generally, Actinobacteria were more abundant in those with prediabetes or DM but markedly reduced in those with gingival bleeding and DM as compared with those with DM without gingival bleeding. Although this pattern was not observed in the presence of PD ≥4 mm and DM, it was evident in those with prediabetes and PD ≥4 mm. These findings remained in conditional logistic regression models adjusted for age, sex, and waist circumference, whereby Actinobacteria significantly increased the odds of diabetes by 10% in subjects with gingival bleeding while Fusobacteria increased this by 14%. Emerging data derived from high throughput metagenomic sequencing of the oral microbiome with 16S rDNA or rRNA support the role of these microorganisms in the development of DM, but the findings remain controversial (Long et al. 2017; Ogawa et al. 2017; Tam et al. 2018; Saeb et al. 2019). Similar to our findings, these studies showed differences between glycemic statuses and the proportions of several phyla. A study comprising 3 elderly subjects with DM, 12 without DM, and healthy young cohort of 9 found similar phyla to our study, but hierarchal distributions of taxa were different in that the reported descending order of abundance was Firmicutes, Actinobacteria, Proteobacteria, Bacteroidetes, and Fusobacteria in subjects with diabetes (Ogawa et al. 2017). Contrary to our findings, a study involving 98 participants with incident diabetes, 99 who were obese without diabetes, and 97 with normal weight and without diabetes reported a decreased abundance of Actinobacteria among patients with DM versus controls, and this was significantly associated with a decreased risk of DM while Firmicutes were associated with an increased risk of DM (Long et al. 2017). A recent case-control study involving 15 patients with DM, 10 with impaired glucose tolerance, and 19 controls found 11 classes in saliva samples but differences in the order of abundance in subjects with impaired glucose tolerance or DM as compared with controls (Saeb et al. 2019). In all subjects, class Bacteroidia was the most abundant; the second-most common was Bacilli in controls and DM and Gammaproteobacteria in impaired glucose tolerance, and the abundance of Actinobacteria

was similar among the groups (Saeb et al. 2019). The differences in these studies possibly lie in the failure to account for the duration and control of DM as well as the degree of inflammation and the PD at which the plaque sample is collected. Recently, plaque samples taken at periodontal pockets of 4 mm, 5 to 6 mm, and ≥7 mm were analyzed separately, and significant distribution in the microbiome was observed among the groups (Shi et al. 2018). In our study, we included subjects with periodontal diseases (gingival bleeding and/or PD >4 mm) across all glycemic statuses, which allowed us to investigate changes in the oral microbiome within each glycemic group and the presence of periodontal diseases. We observed a markedly increased abundance of Bacteroidetes in subjects with diabetes or prediabetes and gingival bleeding or prediabetes with PD ≥4 mm, as opposed to their counterparts with PD ≥4 mm, suggesting a role of this phyla in the development of DM and the early stages of periodontal disease. Subgingival microorganisms play an important role in the initiation and progression of periodontitis, and differences in the microbiome have been observed between chronic periodontitis, a slowly progressive disease, and aggressive periodontitis (Shi et al. 2018). This has been linked to pH, temperature, and the oxygen tension changes in the progression of PD (Forscher et al. 1954; Loesche et al. 1983; Mettraux et al. 1984; Haffajee et al. 1992). It has also been reported that DM with periodontitis had an increase in the bacteria *Porphyromonas gingivalis*, which belongs to the phylum Bacteroides (Wang et al. 2013; Padmalatha et al. 2016). Similarly, we observed a relatively higher abundance of *Porphyromonas* in subjects with diabetes and gingival bleeding. Hyperglycemia has been shown to increase the expression of innate immunity receptors and to potentially affect the immune reactions in periodontal tissues (Sonnenschein and Meyle 2015; Zekeridou et al. 2017). For example, in individuals with T2DM and periodontal diseases, increased levels of interleukin 1B, interleukin 6, and tumor necrosis factor alpha have been reported as compared with those with only periodontal diseases (Andriankaja et al. 2009; Duarte et al. 2014; Yang et al. 2014). Although the mechanisms underlying the bidirectional relationship between periodontal diseases and DM remain unknown, these reports lend credible evidence that the presence of hyperglycemia promotes an inflammatory state in the infected periodontal tissue, which thereby worsens the destruction of the tissue. Limitations of this study include the cross-sectional study design, as it did not allow the investigation of the long-term effects of periodontitis and the predominant phyla on the progression of the disease. Further longitudinal studies are recommended to address these issues and to possibly analyze the effects of glycemic control on the oral microbiota. A further limitation of this study was the low number of participants in each glycemic group; however, our study numbers are comparable to published reports. In our study,

we did not include the PD of each plaque sample, although, based on the low number of participants with PD ≥6 mm ($n$ = 7), participants with periodontitis would have had a PD of 4 to 5 mm. We also did not use a current periodontal disease classification system (Caton et al. 2018) due to missing information on samples collected between 2014 and 2016. Although the Community Periodontal Index has been recommended for screening of periodontal disease and treatment needs by the WHO, it has also been shown to misclassify gingivitis (Cutress et al. 1986). It is well known that poor glycemic control increases risk for periodontal diseases; however, due to the small number (25%) of participants with HbA1c <7% in the DM group with treatment, meaningful statistical comparisons could not be performed. In summary, our findings have demonstrated that the composition of the oral microbiota differs across glycemic statuses as well as in the presence of periodontal diseases, with the phyla Actinobacteria and Fusobacteria increasing the risk of DM in subjects with gingival bleeding by ≤14%.

We recommend further investigations in a longitudinal study design to elucidate the causal relationship between the microbiota and hyperglycemia.

## 5. Conflict of interest

*The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest*.

## 6. Author contributions

Conceptualization, T.E.M., contributed to conception and data acquisition and interpretation, methodology. U. Chikte., contributed to data analysis and interpretation, critically revised the manuscript; R.T.E., and A.P.K.; contributed to the conception and data acquisition, Y. P, contributed to methodology, data acquisition, and analysis, drafted the manuscript; and G.M.D, contributed to data interpretation, critically revised and drafting the manuscript, and S. D, contributed to data analysis. All authors have read and agreed to the published version of the manuscript.

## 7. Funding

This research was funded by the South African Medical Research Council (SAMRC), with funds from National Treasury under its Economic Competitiveness and Support Package (MRC-RFA-UFSP-01-2013/VMH Study) and South African National Research Foundation (SANRF) (Grant No. 115450).

## 8. Acknowledgments

Thank you to the Bellville South Ward 009 community for partaking in the study as well as the Bellville South Community Health Forum for supporting the engagement with the Bellville South Community.

## 9. References

Abusleme L, Dupuy AK, Dutzan N., et al 2013. The subgingival microbiome in health and periodontitis and its relationship with community biomass and inflammation.
ISME J. 7(5):1016–1025.

Alberti KG, Zimmet PZ. 1998. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. Diabet Med.15(7):539–553.

Andriankaja OM, Barros SP, Moss K., *et al*. 2009. Levels of serum interleukin (IL)-6 and gingival crevicular fluid of IL-1beta and prostaglandin E(2) among non-smoking subjects with gingivitis and type 2 diabetes. J Periodontol. 80(2):307–316.

Caton JG, Armitage G, Berglundh T., *et al*. 2018. A new classification scheme for periodontal and peri-implant diseases and conditions: introduction and key changes from the 1999 classification. J Clin Periodontol. 45 Suppl 20:S1–S8.

Chikte U, Pontes CC, Karangwa I, Kimmie-Dhansay F, Erasmus RT, Kengne AP, Matsha TE. 2019. Periodontal disease status among adults from South Africa—prevalence and effect of smoking. Int J Environ Res Public Health. 16(19):E3662.

Cutress TW, Hunter PB, Hoskins DI. 1986. Comparison of the Periodontal Index (PI) and Community Periodontal Index of Treatment Needs (CPITN). Community Dent Oral Epidemiol. 14(1):39–42.

D'Argenio V, Salvatore F. 2015. The role of the gut microbiome in the healthy adult status. Clin Chim Acta. 451(Pt A):97–102. Duarte PM, Bezerra JP, Miranda TS, Feres M, Chambrone L, Shaddox LM. 2014. Local levels of inflammatory mediators in uncontrolled type 2 diabetic subjects with chronic periodontitis. J Clin Periodontol. 41(1):11–18.

Erasmus RT, Soita DJ, Hassan MS, Blanco-Blanco E, Vergotine Z, Kengne
AP, Matsha TE. 2012. High prevalence of diabetes mellitus and metabolic syndrome in a South African coloured population: baseline data of a study in Bellville, Cape Town. S Afr Med J. 102(11):841–844. Forscher BK, Paulsen AG, Hess WC. 1954. The pH of the periodontal pocket and the glycogen content of the adjacent tissue. J Dent Res. 33(4):444–453.

Grice EA, Segre JA. 2012. The human microbiome: our second genome. Annu Rev Genomics Hum Genet. 13:151–170.

Griffen AL, Beall CJ, Campbell JH, Firestone ND, Kumar PS, Yang ZK, Podar M, Leys EJ. 2012. Distinct and complex bacterial profiles in human periodontitis and health revealed by 16S pyrosequencing. ISME J. 6(6):1176–1185. Haffajee AD, Socransky SS, Goodson JM. 1992. Subgingival temperature (I): relation to baseline clinical parameters. J Clin Periodontol. 19(6):401–408.

Hsu KL, Osgood RC, Cutter GR, Childers NK. 2010. Variability of two plaque sampling methods in quantitation of *Streptococcus mutans*. Caries Res. 44(2):160–164.

International Diabetes Federation. 2017. IDF Diabetes Atlas, 8th ed. Brussels (Belgium): International Diabetes Federation; [accessed 2019 October 27]. http://www.diabetesatlas.org.

Kengne AP, Erasmus RT, Levitt NS, Matsha TE. 2017. Alternative indices of glucose homeostasis as biochemical diagnostic tests for abnormal glucose tolerance in an African setting. Prim Care Diabetes. 11(12):119–131.

Li Y, Feng X, Xu L, Zhang L, Lu R, Shi D, Wang X, Chen F, Li J, Meng H. 2015. Oral microbiome in Chinese patients with aggressive periodontitis and their family members. J Clin Periodontol. 42(11):1015–1023.

Liu B, Faller LL, Klitgord N, Mazumdar V, Ghodsi M, Sommer DD, Gibbons TR, Treangen TJ, Chang YC, Li S, et al. 2012. Deep sequencing of the oral microbiome reveals signatures of periodontal disease. PLoS One. 7(6):e37919.

Loesche WJ, Gusberti F, Mettraux G, Higgins T, Syed S. 1983. Relationship between oxygen tension and subgingival bacterial flora in untreated human periodontal pockets. Infect Immun. 42(2):659–667.

Long J, Cai Q, Steinwandel M., *et al*. 2017. Association of oral microbiome with type 2 diabetes risk. J Periodontal Res. 52(3):636–643.

Mealey BL, Oates TW. 2006. Diabetes mellitus and periodontal diseases. J Periodontol. 77(8):1289–1303.

Mettraux GR, Gusberti FA, Graf H. 1984. Oxygen tension (pO2) in untreated human periodontal pockets. J Periodontol. 55(9):516–521.

Negrato CA, Tarzia O, Jovanovič L, Chinellato LE. 2013. Periodontal disease and diabetes mellitus. J Appl Oral Sci. 21(1):1–12.

Ogawa T, Honda-Ogawa M, Ikebe K, Notomi Y., et al. 2017. Characterizations of oral microbiota in elderly nursing home residents with diabetes. J Oral Sci. 59(4):549–555.

Okada A, Sogabe K, Takeuchi H, Okamoto M, Nomura Y, Hanada N. 2017. Characterization of specimens obtained by different sampling methods for evaluation of periodontal bacteria. J Oral Sci. 59(4):491–498.

Padmalatha GV, Bavle RM, Satyakiran GV, Paremala K, Sudhakara M, Makarla S. 2016. Quantification of *Porphyromonas gingivalis* in chronic periodontitis patients associated with diabetes mellitus using real-time polymerase chain reaction. J Oral Maxillofac Pathol. 20(3):413–418.

Park OJ, Yi H, Jeon JH, Kang SS, Koo KT, Kum KY, Chun J, Yun CH, Han

SH. 2015. Pyrosequencing analysis of subgingival microbiota in distinct periodontal conditions. J Dent Res. 94(7):921–927.

Preshaw PM, Alba AL, Herrera D, Jepsen S, Konstantinidis A, Makrilakis K, Taylor R. 2012. Periodontitis and diabetes: a two-way relationship. Diabetologia. 55(1):21–31.

Saeb ATM, Al-Rubeaan KA, Aldosary K, Udaya Raja GK, Mani B, Abouelhoda M, Tayeb HT. 2019. Relative reduction of biological and phylogenetic diversity of the oral microbiota of diabetes and pre-diabetes patients. Microb Pathog. 128:215–229.

Shi M, Wei Y, Hu W, Nie Y, Wu X, Lu R. 2018. The subgingival microbiome of periodontal pockets with different probing depths in chronic and aggressive periodontitis. Front Cell Infect Microbiol. 8:124.

Sonnenschein SK, Meyle J. 2015. Local inflammatory reactions in patients with diabetes and periodontitis. Periodontol 2000. 69(1):221–254.

Tam J, Hoffmann T, Fischer S, Bornstein S, Grasler J, Noack B. 2018. Obesity alters composition and diversity of the oral microbiota in patients with type 2 diabetes mellitus independently of glycemic control. PLoS One. 13(10):e0204724.

Taylor GW. 2001. Bidirectional interrelationships between diabetes, and periodontal diseases: an epidemiologic perspective. Ann Periodontol. 6(1):99–112.

Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI. 2007. The human microbiome project. Nature. 449(7164):804–810.

Wang J, Qi J, Zhao H, He S, Zhang Y, Wei S, Zhao F. 2013. Metagenomic sequencing reveals microbiota and its function potential associated with periodontal disease. Sci Rep. 3:1843.

World Health Organization. 2016. Oral health surveys: basic methods. 5th ed. São Paulo (Brazil): School of Dentistry, University of São Paulo.

Yang X, Zhang J, Ni J, Ouyang B, Wang D, Luo S, Xie B, Xuan D. 2014. Toll-like receptor 4-mediated hyper-responsiveness of gingival epithelial cells to lipopolysaccharide in high-glucose environments. J Periodontol. 85(11):1620–1628.

Young VB. 2017. The role of the microbiome in human health and disease: an

introduction for clinicians. BMJ. 356:j831.

Zekeridou A, Giannopoulou C, Cancela J, Courvoisier D, Mombelli A. 2017. Effect of initial periodontal therapy on gingival crevicular fluid cytokine profile in subjects with chronic periodontitis. Clin Exp Dent Res. 3(2):62–68.

## 10. Figure's legends

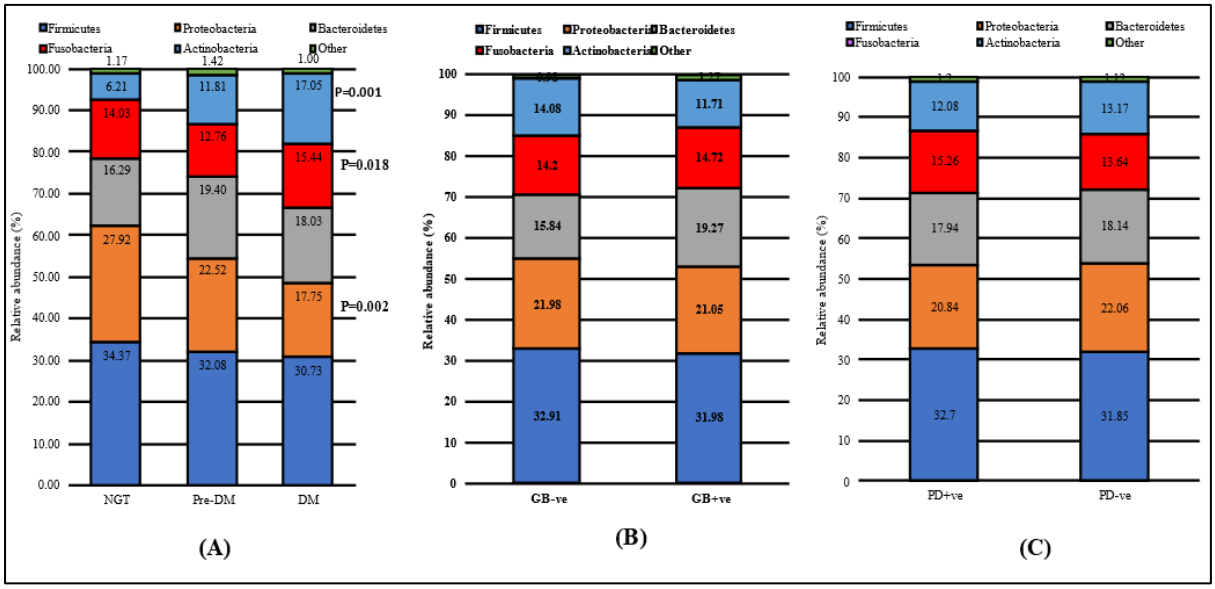



**Figure 1.** Composition of the oral microbial community at the phylum level as the percentage of relative abundance by subgroup: (**A**) normal glucose tolerance (NGT), prediabetes (Pre-DM), and diabetes (DM); (**B**) gingival bleeding on probing (GV+) and no gingival bleeding on probing (GV–); (**C**) pocket depth ≥4 mm (PD+) and <4 mm (PD–).

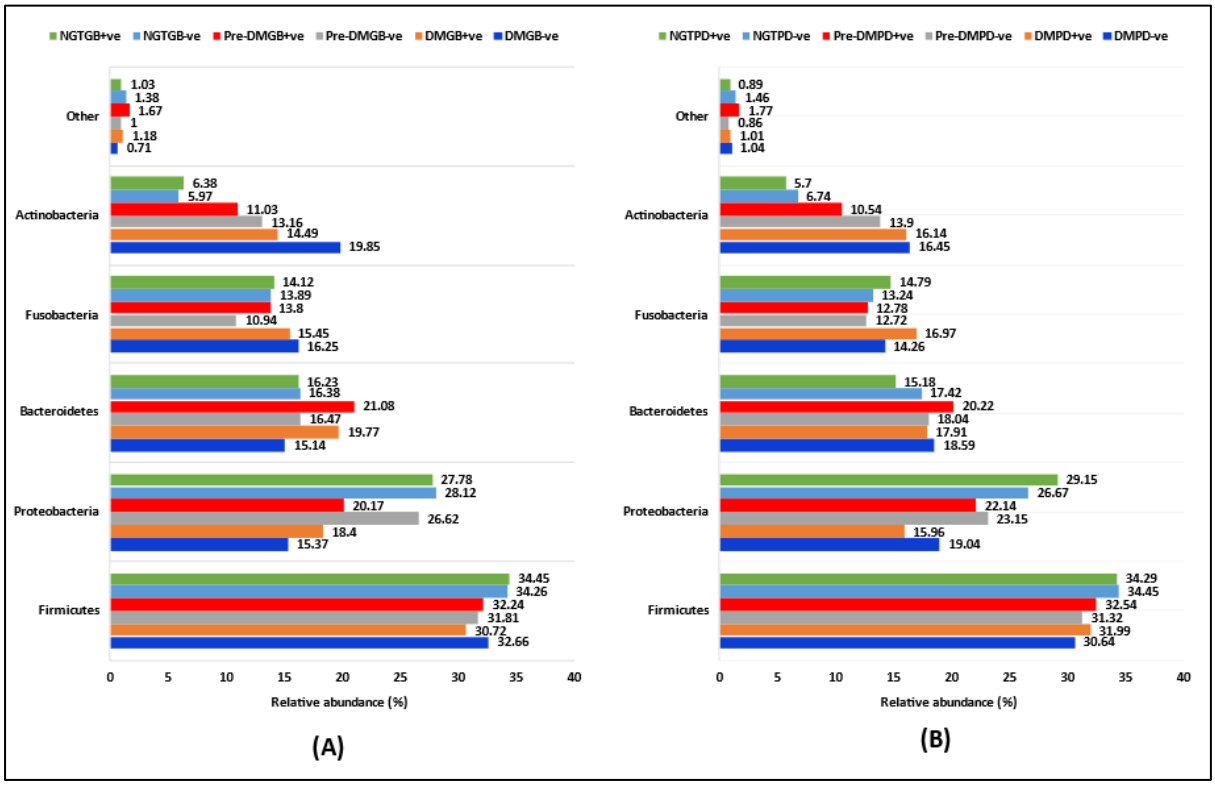



**Figure 2.** Composition of the oral microbial community at the phylum level as the percentage of relative abundance by subgroup: (**A**) normal glucose tolerance (NGT), prediabetes (Pre-DM), and diabetes mellitus (DM) with or without gingival bleeding on probing (GB+, GB–); (**B**) NGT, Pre-DM, and DM with or without pocket depth ≥4 mm (PD+, PD–).

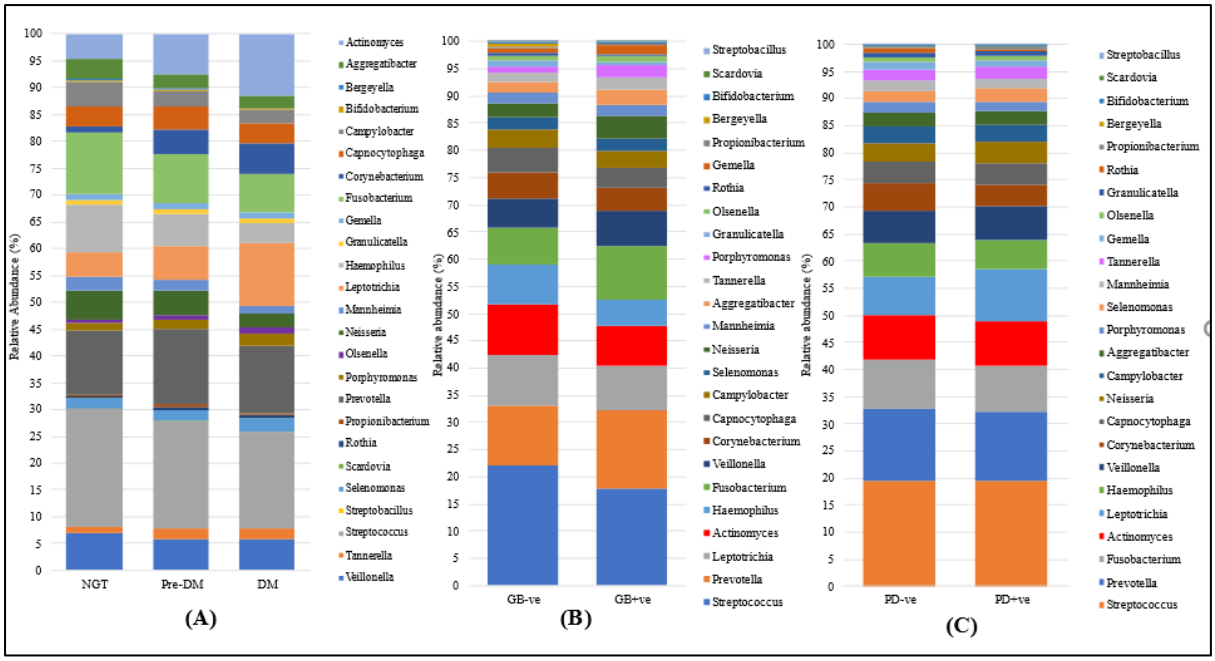



**Figure 3.** Composition of the oral microbial community at the genus level as the percentage of relative abundance by subgroup: (**A**) normotolerant (NGT), prediabetes (Pre-DM) and diabetes (DM); (**B**), gingival bleeding on probing (GV+) and no gingival bleeding on probing (GV–); (**C**) pocket depth ≥4 mm (PD+) and <4 mm (PD–).

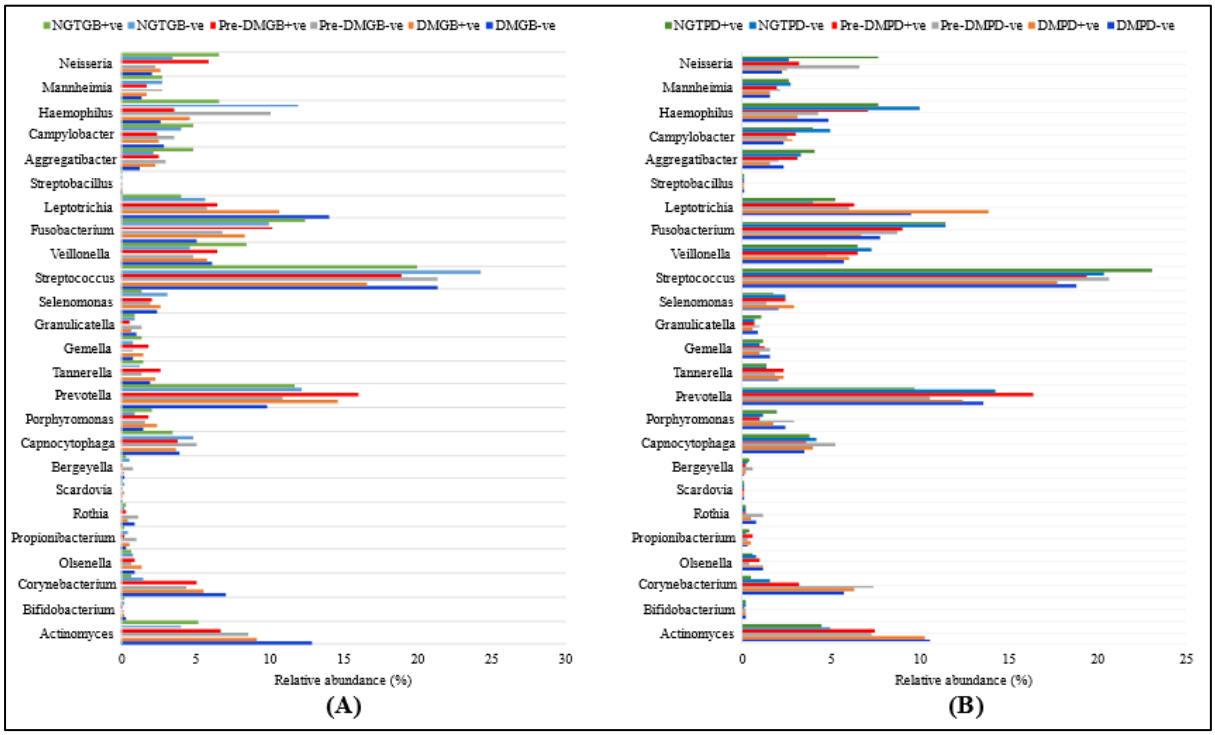



**Figure 4.** Composition of the oral microbial community at the genus level as the percentage of relative abundance by subgroup: (**A**) normal glucose tolerance (NGT), prediabetes (Pre-DM), and diabetes mellitus (DM) with or without gingival bleeding on probing (GB+, GB–); (**B**) NGT, Pre-DM, and DM with or without pocket depth ≥4 mm (NGT PD+, NGT PD–).

**Table 1.** General characteristics of participants according to glycaemic status

| Characteristics | Total group N128 | Normo-glycaemic N32 | Pre-DM N32 | Screen-detected DM, N32 | Known DM N32 | Sex P-value | Diagnosis P-value | Gender*Diagnosis interaction P |
|---|---|---|---|---|---|---|---|---|
| Age (years) | 47.0±13.0 | 40.0±14.2 | 47.6±12.5 | 52.5±9.0 | 47.9±13.0 | 0.5296 | 0.0090 | 0.9328 |
| BMI | 31.6±9.7 | 24.9±5.7 | 30.0±8.1 | 36.2±9.0 | 35.2±11.0 | <0.0001 | 0.0001 | 0.4341 |
| WaistC (cm) | 95±20.2 | 77±10.7 | 93±15.8 | 106±16.8 | 105±20.5 | 0.0069 | <0.0001 | 0.5510 |
| HipC (cm) | 105±20.7 | 88±10.7 | 104±16.8 | 116±18.8 | 114±22.3 | <0.0001 | <0.0001 | 0.4720 |
| WHR | 0.90±0.08 | 0.87±0.05 | 0.90±0.06 | 0.92±0.09 | 0.93±0.10 | 0.0011 | 0.0003 | 0.0523 |
| SBP (mmHg) | 130±23.2 | 117±20.3 | 131.±19.8 | 136±23.1 | 134±25.0 | 0.6541 | 0.0534 | 0.5153 |
| DBP (mmHg) | 84±13.8 | 79±11.9 | 87±12.6 | 88±14.6 | 84±14.4 | 0.0630 | 0.0780 | 0.3983 |
| FBG (mmol/L) | 7.13±3.88 | 4.64±0.46 | 5.41±0.86 | 8.52±4.73 | 9.96±4.33 | 0.6175 | <0.0001 | 0.3620 |
| Post 2 HRs BG (mmol/L) | 9.65±5.19 | 5.24±1.38 | 8.84±1.30 | 15.02±5.43 | NA | 0.5671 | <0.0001 | 0.8974 |
| HbA1c (%) | 6.86±2.07 | 5.38±0.31 | 5.84±0.70 | 7.34±1.85 | 8.87±2.40 | 0.6627 | <0.0001 | 0.5683 |
| FBI (mIU/L) | 15.0±27.7 | 6.3±5.4 | 12.3±15.3 | 25.9±50.0 | 15.2±11.8 | 0.8561 | 0.0670 | 0.9499 |
| Post 2 HRs BI (mIU/L) | 58.3±51.1 | 40.6±34.5 | 69.9±62.4 | 63.6±48.3 | NA | 0.0279 | 0.0539 | 0.5393 |
| FBG/FBI ratio | 1.09±1.21 | 1.04±0.68 | 1.21±1.21 | 0.76±0.86 | 1.31±1.76 | 0.1114 | 0.3076 | 0.7436 |
| Triglycerides (mmol/L) | 1.57±1.58 | 1.12±0.45 | 1.45±0.86 | 2.15±2.87 | 1.55±0.63 | 0.0340 | 0.0004 | 0.0042 |
| LDL C (mmol/L) | 3.12±1.05 | 2.56±0.73 | 2.95±0.87 | 3.80±0.99 | 3.20±1.18 | 0.0168 | 0.0065 | 0.5560 |
| HDL C (mmol/L) | 1.38±0.45 | 1.48±0.38 | 1.41±0.48 | 1.40±0.61 | 1.23±0.25 | 0.2759 | 0.2494 | 0.3670 |
| Chol (mmol/L) | 5.14±1.17 | 4.70±0.88 | 4.93±0.85 | 5.89±1.27 | 5.04±1.27 | 0.0230 | 0.0157 | 0.5930 |
| Chol/HDL-C ratio | 3.98±1.24 | 3.35±0.96 | 3.83±1.26 | 4.51±1.26 | 4.25±1.21 | 0.2113 | 0.0427 | 0.8813 |
| U-CRP (mg/L) | 7.9±9.5 | 5.7±11.6 | 7.4±9.5 | 8.9±6.9 | 9.4±9.3 | 0.2285 | 0.9475 | 0.1432 |
| Cotinine (ng/mL) | 121.1±152.5 | 210.6±152.7 | 131.5±150.6 | 74.3±130.0 | 68.2±137.5 | 0.4593 | 0.0047 | 0.9148 |
| GGT (IU/L) | 58.8±90.0 | 55.1±85.5 | 50.8±51.5 | 67.5±56.4 | 61.8±141 | 0.4384 | 0.5155 | 0.0999 |
| Gingival bleeding, Yes | 81 (63.3) | 19 (59.4) | 21 (65.6) | 17 (53.1) | 24 (75.0) | 0.9893 | 0.4997 | - |
| PD≥ 4mm n (%) | 68 (53.1) | 16 (50.0) | 20 (62.5) | 16 (50.1%) | 16 (50.0%) | 0.4371 | 0.7000 | - |
| PD ≥6 mm n (%) | 7 (5.5) | 3 (9.4) | 3 (9.4) | 0 | 1 (3.1) | 0.9039 | 0.2923 | - |

WaistC, waist circumference; HipC, hip circumference; SBP, systolic blood pressure; DBP, diastolic blood pressure; FBG, fasting blood glucose; Post 2 HRs BG, post-2-hour blood glucose, FBI, fasting blood insulin; Post 2 HRs BI, post 2-hour blood insulin; LDL-C, low-density lipoprotein cholesterol, HDL-C, high-density lipoprotein cholesterol, Chol, total cholesterol, U-CRP, ultrasensitive C-reactive protein, GGT, Gamma-glutamyl transferase, PD, pocket dept.

# CHAPTER 4: MANUSCRIPT 2

**The Effect of Cigarette Smoking on the Oral Microbiome in a South African Population.**

Yvonne Prince[1*], Saarah Davids[1], Rajiv T Erasmus[3], Andre P Kengne [4,5], Shanel Raghubeer[1], Glenda M Davison[1], Tandi E Matsha [1*]

[1]SAMRC/CPUT/Cardiometabolic Health Research Unit, Department of Biomedical Sciences, Faculty of Health and Wellness Sciences, Cape Peninsula University of Technology, Bellville, South Africa. [2]Department of Global Health, Stellenbosch University, Cape Town 8000, South Africa. [3]Department of Pathology, Faculty of Medicine and Health Sciences, Stellenbosch University, Cape Town, South Africa. [4]Non-Communicable Diseases Research Unit, South African Medical Research Council, Cape Town, South Africa. [5]Department of Medicine, University of Cape Town, Cape Town, South Africa.

*Correspondence should be addressed to: Tandi E. Matsha, and Yvonne Prince, Biomedical Sciences, Faculty of Health and Wellness Sciences, Cape Peninsula University of Technology, PO Box 1906, Bellville 7530, Cape Town, South Africa Tel: +27 21 959 6366, Fax: +27 21 959 6760;
Email: matshat@cput.ac.za and/or princey@cput.ac.za

Word count, 2495
Number of figures and Tables: 6

**Abstract**

Disturbances in the oral microbiome may be due to several mechanisms and factors, such as smoking. An imbalance in oral bacteria may result in changes to the innate immune system and the development of periodontal disease. This study aimed to investigate the distribution of oral microbiota in smokers and non-smokers residing in South Africa. Participants were recruited (128 individuals), and 57 out of 128 participants identified as smokers (serum cotinine: >15 ng/ml). Analysis of 16S rDNA gene sequencing demonstrated significant differences between the two groups with a reduced abundance of the phyla *Actinobacteria* in smokers. *Fusobacterium* and *Campylobacter* were found in higher abundance, while a decrease in the genera *Leptotrichia, Actinomyces, Corynebacterium,* and *Lautropia* were observed. The species *F. nucleatum*, *C. gracilis, V. rogosae*, *F. canifelinum,* and *A. odontolyticus* were increased among smokers, whereas *C. matruchotii*, *A. dentalis*, *A. naeslundii*, *C. sputigena*, and *S. sanguinis* were decreased. Taken together, this study highlighted significant differences in the oral microbiome of smokers, indicating an abundance of anaerobic gram-negative bacteria. These findings suggest that smoking may predispose individuals to periodontitis and other oral diseases.

**Keywords: Smoking, Oral microbiome, South Africa, Periodontitis**

## 1. Introduction

The human oral microbiome is host to thousands of microbes and is comprised of a core and variable portions. The core oral microbiome remains stable throughout life; however, studies have shown that each oral habitat or niche is colonised by distinct bacterial communities(Gao et al., 2018; Ursell et al., 2012). The human oral microbiome database (HOMD) has attempted to collect information and analyse the diversity and distribution of the bacteria occupying the oral cavity using a variety of techniques, including next-generation sequencing and 16S rDNA analysis. This collection of data has recorded the phenotypic, phylogenetic, clinical, and bibliographic, information of 150 genera, 700 species, and thousands of strains of oral microorganisms (Gao et al., 2018; Dewhirst et al., 2010). The interaction between these oral microorganisms, particularly within the variable oral biome, assists in maintaining oral health and protection from disease. However, it has been well established that disruptions to their balance, diversity, and interactions may result in certain species gaining dominance, and the development of oral diseases, such as dental caries and periodontitis (Huang & Shi, 2019).

The arrangement of the oral biome can be affected by numerous factors including the environment, antibiotic usage, diet, alcohol, and tobacco use (Karabudak et al., 2019). Studies regarding tobacco products in the European Union have revealed that cigarettes contain toxins and several microorganisms including both soil and human bacteria (Sapkota et al., 2010; Wu et al., 2016) while others have observed that tobacco can suppress the innate immune system including the activation of natural killer cells and neutrophils (Huang & Shi, 2019). These studies have consequently led to the hypothesis that tobacco use can either directly or indirectly affect microorganisms inhabiting the oral cavity. Although tobacco use has declined since 2000, the World Health Organisation (WHO) estimates that over 1.2 billion people will still smoke by 2025 and that many of these individuals will reside in low-and middle-income countries (World Health Organization, 2019). Similarly, in South Africa, government policies to reduce smoking in the population have been successful; however, recent reports suggest that 17.6% of adult South Africans still smoke tobacco (Reddy et al., 2015). In this regard, this study aimed to investigate the oral microbiome distribution in smokers and non-smokers.

## 2. Materials and Methods

### *2.1. Ethical considerations*

The study was conducted according to the Code of Ethics of the World Medical Association (Declaration of Helsinki) and formed part of the Vascular and Metabolic Health (VMH) study registered at the Cape Peninsula University of Technology, Bellville South, Cape Town, South Africa. Ethical approval for the study was obtained from the Research Ethics Committees of the Cape Peninsula University of Technology (CPUT) and Stellenbosch University (respectively, NHREC: REC - 230 408 – 014, CPUT/HW-REC 2015/H01 and N14/01/003). Further ethics approval was specifically granted for the microbiome study analysis (CPUT/HW-REC 2017/H31). A written consent form was signed by all participants after all the procedures had been fully explained in the language of their choice.

### *2.2. Study design and procedures*

Participants from this study were selected from the ongoing Vascular and Metabolic Health (VMH) study, in which detailed procedures are described (Kengne et al., 2017). In this case-control study, 128 participants were randomly selected from the 1998 VMH cohort. Anthropometric measures, blood pressure measurements, and oral glucose tolerance tests (OGTT) were performed on all participants. Plasma glucose, HBA1c, triglycerides, low-density lipoprotein cholesterol (LDL-chol), high-density cholesterol (HDL-chol), insulin, γ-Glutamyltransferase (GGT), ultra-sensitive C-reactive protein (us-CRP), and serum cotinine levels were measured in an ISO 15189 accredited laboratory (PathCare Reference Laboratory, Cape Town, South Africa). The plaque samples were collected using a wood toothpick as previously described (Matsha et al., 2020).

### *2.3. Smoking assessment*

The STEPwise questionnaire following guidelines established in 2011, was used to assess the smoking status of all participants (Global Adult Tobacco Survey Collaborative Group, 2011). In addition, serum cotinine levels >15 ng/ml were used to define smokers (Pirkle et al., 1996; Slattery et al., 1989).

### *2.4.16S rDNA metagenomic sequencing*

DNA was extracted from the two pooled plaque toothpicks using a DNA extraction kit from Zymo Quick-DNA Fungal Bacterial Miniprep KIT (Zymo Research, IRVIN, CA, USA) according to the manufacturer's instructions Metagenomic DNA (mgDNA) was quantified using the Qubit™ 1X dsDNA HS (High Sensitivity) assay Kit (MAN0017455 Rev. A.0). The

NanoDrop ND-1000 was employed to measure the purity of the mgDNA samples, and the genomic quality scores were analysed using the DNA Extended Range LabChip and Genomic DNA Reagent Kit (PerkinElmer) according to the protocol (CLS140166 Rev. C; Supplementary Report: Genomic DNA [gDNA] Quality Control).

*2.5. Statistical analysis*

The software SPSS v.26 (IBM Corp, 2019) was used for data analysis. The results were reported as mean ± standard deviation, median ($25^{th}$ and $75^{th}$ percentiles), and count (percentages). For comparison, analysis of variance (ANOVA) or Kruskal Wallis tests were used for numerical variables, while chi-square tests were used for categorical variables. The Cohens Kappa assessment was used to statistically evaluate the agreement between serum cotinine values and questionnaire responses. Microbiome data was presented in terms of relative abundance percent for phyla, genus, and species. Those comprising ≤ 1% of the total abundance were grouped as others. The independent *t*-test was used to determine statistically significant differences in the relative percent abundance between cases and controls for the phylum, genus, or species. Multivariate logistic regression models were used to assess the association between microorganisms present in smokers compared to non-smokers in crude or adjusted odds ratios (OR). A $p$-value < 0.05 was used to characterize statistically significant results. Chao1, Shannon, and Simpson indices were used to determine alpha diversity, and EMPeror (v0.9.60) was used for principal coordinate analysis (PCA) to visualise beta diversity.

## 3. Results

From a total of 128 participants, five participants were excluded due to missing data. The subjects either did not indicate their smoking status or serum cotinine levels were not obtained. Therefore, 123 participants (males, $n$ = 34) were included in this study. We observed good agreement between serum cotinine levels and participants' responses regarding smoking (kappa score = 0.903, $p$<0.001). Fifty-seven (46.3%) participants exhibited serum cotinine levels >15 ng/ml and were classified as smokers. The general participant characteristics are summarised in Table 1. No significant difference was observed in the demographics, anthropometric measurements, or biochemical parameters between the two groups. Ultrasensitive CRP was slightly elevated in smokers ($p$=0.088). Based on the Chao index, the alpha diversity appeared decreased in smokers compared to non-smokers (Table 2). Beta diversity indicated a 25% difference in species population between smokers and non-smokers (Figure 1).

The five most abundant phylas observed across all participants were *Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria,* and *Fusobacteria*, and made up more than 98% of the total number of phyla. *Actinobacteria* was the only phyla that was significantly lower in smokers ($p$<0.001; Figure 2). Nineteen different genera with percentage reads ≥1% were observed (Table 3). The most abundant genera across all subgingival plaque samples included *Streptococcus* and *Prevotella.* Among those participants who smoked, the genera *Fusobacterium* ($p$=0.002) and *Campylobacter* ($p$=0.010) were seen in abundance, while a significant decrease was observed in genera *Leptotrichia* ($p$=0.029), *Actinomyces* ($p$=0.001)*, Corynebacterium* ($p$=0.002), and *Lautropia* ($p$=0.023). A near significant difference was observed in *Haemophilus* ($p$=0.086) and *Porphyromonas* ($p$=0.083; Table 3). Several species, including *F. nucleatum* ($p$<0.001), *C. gracilis* ($p$=0.010), V. *rogosae* ($p$=0.001), *F. canifelinum (*$p$=0.009), and *A. odontolyticus* ($p$=0.003), were significantly increased among smokers, while *C. matruchotii* ($p$=0.002), *A. dentalis* ($p$=0.033), *A. naeslundii* ($p$=0.010), *C. sputigena* ($p$=*0*.042), and *S. sanguinis* ($p$=0.016) were decreased in smokers (Table 3).

In multivariable logistic regressions, the species *F. canifelinum, C. gracilis, F. nucleatum, A. odontolyticus*, and *V. rogosae* were associated with higher odds of being present in smokers (odds ratio (OR) ≥1.07, 95% confidence interval (CI): >1.03-1.12, $p$≤0.012), whilst *A. dentalis, C. matruchotii, A. naeslundii*, *S. sanguinis*, *or A sanguinis* were associated with lower odds in smokers (OR ≤0.74, 95% CI: ≤0.60–0.97, $p$≤0.040) in crude models (Table 4).

## 4. Discussion

This case-control study compared the oral microbiome of 57 smokers and 66 non-smokers. We observed significant differences in the distribution of bacteria within the oral microbiome of smokers. This is reflected across all taxonomic levels, including the phyla, genera, and species. The findings suggested that smoking induces an anaerobically rich environment that favours gram-negative bacteria.

In smokers, we observed a significant reduction in *Actinobacteria*, which are gram-positive (gpb) anaerobic bacteria, predominantly inhabiting the oral cavity and forming part of the commensals of the skin (Sharma, Bhatia, Sodhi, et al., 2018; Grice & Segre, 2013). However, this finding contradicts the results obtained by other investigators (Karabudak et al., 2019; Wu et al., 2016; Mason et al., 2015), who have all reported an abundance of this phyla. Mason *et al*. reported an increase of anaerobes and a decrease of aerobes in smokers (Mason et al., 2015). In support of this, we also observed depletion in aerobes, such as the genera *Corynebacterium* (aerobe) and *Actinomyces* (gram-positive bacilli), which are associated with biofilm and plaque formation. Both genera fall under the phyla *Actinobacteria.*

Although smoking produces an enriched anaerobic environment through oxygen deprivation (Mason et al., 2015b), our study noted that certain anaerobic bacteria were reduced, namely *Leptotrichia* and *Lautropia*. Both genera are gram-negative bacilli (gnb) facultative anaerobic microorganisms and are commensals of the oral cavity. *Leptotrichia* has been associated with tooth decay and can act as a pathogenic microorganism while *Lautrophia* can be found in both the healthy and the ill. This observation is supported by previous research (Wu et al., 2016), which reported that *Corynebacterium* and *Leptotrichia* appeared significantly lower in smokers, but others have reported enrichment of these genera (Wu et al., 2019; Kumar et al., 2011).

The genera *Fusobacterium* and *Campylobacter* were both enriched in smokers. Both are gnb anaerobic microorganisms that have been linked with periodontal disease progression. *Fusobacterium,* in particular, plays a vital role in dental biofilm formation and may explain why smoking has been shown to promote the formation of biofilm (Signat et al., 2011; Buduneli, 2020). This genera, especially the species *F. nucleatum,* has also been associated with systemic diseases, including cardiovascular diseases (CVD) (Han Yiping, 2015) and uncontrolled type-2 diabetes (Casarin et al., 2013). Similar to our findings, others (Lõivukene et al., 2005; Kumar et al., 2011; Shchipkova et al., 2010; Moon et al., 2015) have also reported

an abundance of both *Fusobacterium* and *Campylobacter* in smokers and an association with the development of the periodontal disease.

The species *F. nucleatum, C. gracalis, V. rogosae, F. canifelinum*, and *A. odontolyticus,* were increased amongst smoking subjects in our study. Apart from. *A. odintolyticus,* these are all gram-negative anaerobic microbiota and reside as commensals in the oral cavity. *F. nucleatum* (genus *Fusobacteruim*) is reportedly an important pathogen in severe periodontal disease, including gingivitis, whereas *F. canifelinum* plays a vital role in the subgingival biofilm function (Signat et al., 2011; Griffen et al., 2012). *C. gracalis* (genus *Campylobacter*) resides in deep sites of the oral cavity and is associated with different stages of periodontitis, including progression (Macuch & Tanner, 2000; Haririan et al., 2014). However, *V. rogosae* (genus *Veilonella*) are commensals of saliva and reside on the dorsal and lateral surfaces of the tongue, but their association with smoking and periodontitis remains controversial (Mager et al., 2003; Kato et al., 2016). Further, *A. odontolyticus* is a purple-complex bacteria (genus *Actinomyces*) known to be associated with the adherence and congregation of oral microorganisms, allowing bridging to both orange- and red-complex bacteria (Haffajee et al., 2008). Further studies have associated this species of bacteria with chronic periodontitis and a role in plaque composition alteration leading to disease (Vielkind et al., 2015).

The differences in the oral microbiome of smokers may be explained by the antibiotic toxicants produced by cigarettes or by bacteria competing for colonization and co-aggregating with smoking depleted microorganisms. It is therefore suggested that smoking can cause a variation in the bacterial communities through various mechanisms. Although the core oral microbiome is stable at the genus level in healthy individuals, diversity in the microbiome is still site-specific and is expected amongst individuals (Wu et al., 2016). This concept has been supported by researchers who had analysed the microbial communities of subgingival plaque samples from 200 systemic and periodontal healthy smokers and non-smokers (Mason et al., 2015). This work demonstrated variation across all taxonomic levels. In the principal coordinate analysis, we discovered distinct clustering of the microbial communities based on smoking status.

The variability in the oral microbiome of smokers is controversial, with many researchers recording different findings (Moon et al., 2015; Medikeri et al., 2015). These differences may be due to various factors, such as diet, pH changes, interaction among microorganisms, gene mutation, gene transfers, and different locations and methods of sampling (McLean, 2014;

Willis & Gabaldón, 2020). In our study, subgingival plaque swabs were used, while samples were obtained using oral washes and buccal swabs in other reports(Karabudak et al., 2019). Furthermore, the techniques utilised to perform next-generation sequencing may present another reason for the discordant findings. In our study the Ion Torrent S5 Gene Studio with the Ion S5 Sequencing was employed, while others have utilised the 454 Roche FLX Titanium pyrosequencing system (Karabudak et al., 2019). Variations in methodologies may have further contributed to discordant results.

This study is limited by the fact that it was a case-controlled study with a small sample size. Further longitudinal studies on larger sample populations are recommended to thoroughly investigate the effects of smoking on the oral microbiome and health. However, despite this limitation, we can conclude that the subgingival microbiome of smokers demonstrated a highly diverse pathogen-rich, gram-negative anaerobic microbiome, which is more closely aligned with a periodontal disease-associated community in clinically healthy individuals. These findings suggest that changes in the diversity and communities of the oral microbiome may create an environment associated with periodontal disease (Mason et al., 2015; Jiang et al., 2020). We further concluded that smoking increases populations of red- and orange-complex bacteria (*F. nucleatum, C. gracilis*) and lowers percentages of *Actinomyces* species (Hanioka et al., 2019).

## 5. Conflict of interest

*The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest*.

## 6. Author contributions

Conceptualization, T.E.M., contributed to conception and data acquisition and interpretation, methodology. R.T.E., A.P.K.; contributed to conception and data acquisition, Y. P, contributed to methodology, data acquisition, and analysis, drafted the manuscript; and G.M.D, contributed to data interpretation, critically revised the manuscript, and S. D, contributed to data analysis. S.R. revising manuscript. All authors have read and agreed to the published version of the manuscript.

## 7. Funding

This research was funded by the South African Medical Research Council (SAMRC), with funds from National Treasury under its Economic Competitiveness and Support Package (MRC-RFA-UFSP-01-2013/VMH Study) and South African National Research Foundation (SANRF) (Grant No. 115450).

## 8. Acknowledgments

Thank you to the Bellville South Ward 009 community for partaking in the study as well as the Bellville South Community Health Forum for supporting the engagement with the Bellville South Community.

## 9. References


Alberti, K.G.M.M., Eckel, R.H., Grundy, S.M., *et al.* 2009. Harmonizing the metabolic syndrome: A joint interim statement of the international diabetes federation task force on epidemiology and prevention; National heart, lung, and blood institute; American heart association; World heart federation; International. *Circulation*, 120(16): 1640–1645.

Andersen, C.J., Murphy, K.E. & Fernandez, M.L. 2016. Impact of obesity and metabolic syndrome on immunity. *Adv Nutr*, 7(1): 66–75.

Arimatsu, K., Yamada, H., Miyazawa, H., *et al* 2014. Oral pathobiont induces systemic inflammation and metabolic changes associated with alteration of gut microbiota. *Sci Rep*, 4: 1–9.

Beutler, J., Jentsch, H.F.R., Rodloff, A.C. & Stingu, C.S. 2019. Bacteremia after professional mechanical plaque removal in patients with chronic periodontitis. *Oral Dis*, 25(4): 1185–1194.

Buduneli, N. 2020. Biomarkers in Periodontal Health and Disease. *Biomarkers in Periodontal Health and Disease*: 25–36.

Casarin, R.C.V., Barbagallo, A., Meulman, T., *et al*. 2013. Subgingival biodiversity in subjects with uncontrolled type-2 diabetes and chronic periodontitis. *J Periodontal Res*, 48(1): 30–36.

Chistiakov, D.A., Orekhov, A.N. & Bobryshev, Y. V. 2016. Links between atherosclerotic and periodontal disease. *Exp Mol Pathol*, 100(1): 220–235. http://dx.doi.org/10.1016/j.yexmp.2016.01.006.

Chukkapalli, S.S., Velsko, I.M., Rivera-Kweh,. *et al*. 2015. Polymicrobial oral infection with four periodontal bacteria orchestrates a distinct inflammatory response and atherosclerosis in ApoEnull mice. *PLoS ONE*, 10(11): 1–17.

Dewhirst, F.E., Chen, T., Izard, J., Paster, B.J., Tanner, A.C.R., Yu, W.H., Lakshmanan, A. & Wade, W.G. 2010. The human oral microbiome. *J Bacteriol*, 192(19): 5002–5017.

Enberg, N., Alho, H., Loimaranta, V. & Lenander-Lumikari, M. 2001. Saliva flow rate, amylase activity, and protein and electrolyte concentrations in saliva after acute alcohol consumption. *Oral Surg, Oral Med Oral Pathol Oral Radiol Endod,* 92(3): 292–298.

Feres, F Teles, R. Teles, L. C Figueiredo, M.F. 2017. The subgingival periodontal microbiota in the aging mouth Magda. *Physiology & behavior*, 176(1): 139–148.

Ferroni, P., Roselli, M., Riondino, S., Cavaliere, F. & Guadagni, F. 2016. Insulin resistance as a predictor of venous thromboembolism in breast cancer. *Endocr Relat Cancer*, 23(6): L25–L28.

G. Caton, J., Armitage, G., Berglundh, T., *et al.* 2018. A new classification scheme for periodontal and peri-implant diseases and conditions – Introduction and key changes from the 1999 classification. *J Clin Periodontol* 45(March): S1–S8.

Gao, L., Xu, T., Huang, G., Jiang, S., Gu, Y. & Chen, F. 2018. Oral microbiomes : more and more importance in oral cavity and whole body. *Protein & Cell*, 9(5): 488–500. https://doi.org/10.1007/s13238-018-0548-1.

Global Adult Tobacco Survey Collaborative Group. 2011. Tobacco Questions for Surveys: A Subset of Key Questions from the Global Adult Tobacco Surveys. http://www.who.int/tobacco/surveillance/en_tfi_tqs.pdf.

Grice, E.A. & Segre, J.A. 2013. The skin microbiome. *Nat Rev Microbiol*, 9(4): 244–253.

Griffen, A.L., Beall, C.J., Campbell, J.H., *et al.* 2012. Distinct and complex bacterial profiles in human periodontitis and health revealed by 16S pyrosequencing. *ISME Journal*, 6(6): 1176–1185. http://dx.doi.org/10.1038/ismej.2011.191.

Haffajee, A.D., Socransky, S.S., Patel, M.R. & Song, X. 2008. Microbial complexes in supragingival plaque. *Oral Microbiol Immunol*, 23(3): 196–205.

Han Yiping. 2015. Fusobacterium nucleatum: a commensal truned pathogen. *Curr Opin Microbiol*, 1(23): 141–147.

Hanioka, T., Morita, M., Yamamoto, T., *et al*. 2019. Smoking and periodontal microorganisms. *Jpn Dent Sci Rev*, 55(1): 88–94. https://doi.org/10.1016/j.jdsr.2019.03.002.

Haririan, H., Andrukhov, O., Bertl, K., *et al*. 2014. Microbial Analysis of Subgingival Plaque Samples Compared to That of Whole Saliva in Patients With Periodontitis. *J Periodontol*, 85(6): 819–828.

Henne, K., Fuchs, F., Kruth, S., Horz, H.P. & Conrads, G. 2014. Shifts in Campylobacter species abundance may reflect general microbial community shifts in periodontitis progression. *J Oral Microbiol*, 6(1): 6–11.

Homann, N., Tillonen, J., Meurman, J.H., *et al*. 2000. Increased salivary acetaldehyde levels in heavy drinkers and smokers: A microbiological approach to oral cavity cancer.

*Carcinogenesis*, 21(4): 663–668.

Huang, C. & Shi, G. 2019a. Smoking and microbiome in oral, airway, gut and some systemic diseases. *J Transl Med*, 17(1): 1–15. https://doi.org/10.1186/s12967-019-1971-7.

Janket, S.J., Baird, A.E., Chuang, S.K. & Jones, J.A. 2003. Meta-analysis of periodontal disease and risk of coronary heart disease and stroke. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*, 95(5): 559–569.

Jansson, L. 2008. Association between alcohol consumption and dental health. *J Clin Periodontal,* 35(5): 379–384.

Ji, S., Kim, Y., Min, B.M., Han, S.H. & Choi, Y. 2007. Innate immune responses of gingival epithelial cells to nonperiodontopathic and periodontopathic bacteria. *J Periodontal Res*, 42(6): 503–510.

Jiang, Y., Zhou, X., Cheng, L. & Li, M. 2020. The Impact of Smoking on Subgingival Microflora: From Periodontal Health to Disease. *Front Microbiol*, 11(January): 1–13.

Kantorski, K.Z., de Souza, D.M., Yujra, V.Q., Junqueira, J.C., Jorge, A.O.C. & da Rocha, R.F. 2007. Effect of an alcoholic diet on dental caries and on Streptococcus of the mutans group: Study in rats. *Braz Oral Res* 21(2): 101–105.

Karabudak, S., Ari, O., Durmaz, B., Dal, T., Basyigit, T., Kalcioglu, M.T. & Durmaz, R. 2019. Analysis of the effect of smoking on the buccal microbiome using next-generation sequencing technology. *J Med Microbiol*, 68(8): 1148–1158.

Kato, I., Vasquez, A.A., Moyerbrailean, G., Land, S., Sun, J., Lin, H.-S. & Ram, J.L. 2016. Oral microbiome and history of smoking and colorectal cancer. *J Epidemiol Res*, 2(2): 92–101.

Keijser, B.J.F., Zaura, E., Huse, S.M., van der Vossen, J.M.B.M., Schuren, F.H.J., Montijn, R.C., ten Cate, J.M. & Crielaard, W. 2008. Pyrosequencing analysis of the oral microflora of healthy adults. *J Dent Res*, 87(11): 1016–1020.

Kengne, A.P., Erasmus, R.T., Levitt, N.S. & Matsha, T.E. 2017. Alternative indices of glucose homeostasis as biochemical diagnostic tests for abnormal glucose tolerance in an African setting. *Prim Care* Diabetes, 11(2): 119–131. http://dx.doi.org/10.1016/j.pcd.2017.01.004.

Khader, Y.S., Albashaireh, Z.S.M. & Alomari, M.A. 2004. Periodontal Diseases and the Risk of Coronary Heart and Cerebrovascular Diseases: A Meta-Analysis. *J Periodontol*, 75(8): 1046–1053.

Kocher, T., König, J., Borgnakke, W.S., Pink, C. & Meisel, P. 2018. Periodontal complications of hyperglycemia/diabetes mellitus: Epidemiologic complexity and clinical challenge. *Periodontol 2000*, 78(1): 59–97.

Krisanaprakornkit, S., Kimball, J.R., Weinberg, A., et al. 2000. Inducible expression of human β-defensin 2 by Fusobacterium nucleatum in oral epithelial cells: Multiple signaling pathways and role of commensal bacteria in innate immunity and the epithelial barrier. *Infect Immun*, 68(5): 2907–2915.

Kumar, P.S., Matthews, C.R., Joshi, V., de Jager, M. & Aspiras, M. 2011b. Tobacco smoking affects bacterial acquisition and colonization in oral biofilms. *Infect Immun*, 79(11): 4730–4738.

Lamster, I.B. & Pagan, M. 2017. Periodontal disease and the metabolic syndrome. *Int Dent J*, 67(2): 67–77.

Linden, G.J., Lyons, A. & Scannapieco, F.A. 2013. Periodontal systemic associations : review of the evidence. , 84: 8–19.

Lõivukene, K., Pähkla, E.R., Koppel, T., Saag, M. & Naaber, P. 2005. The microbiological status of patients with periodontitis in southern Estonia after non-surgical periodontal therapy. *Stomatologija ]*, 7(2): 45–47.

Long, J., Cai, Q., Steinwandel, M., Hargreaves, M.K., Bordenstein, S.R., Blot, W.J., Zheng, W. & Shu, X.O. 2017. Association of oral microbiome with type 2 diabetes risk. *J Period Res*, 52(3): 636–643.

Lu, M., Xuan, S. & Wang, Z. 2019. Oral microbiota: A new view of body health. *Food Science and Human Wellness*, 8(1): 8–15. https://doi.org/10.1016/j.fshw.2018.12.001.

Kilian, M., Chapple, I.L., Hannig, M., et al. 2016. The oral microbiome - An update for oral healthcare professionals. *Bri Dent J* 221(10): 657–666.

Macuch, P.J. & Tanner, A.C.R. 2000. Campylobacter species in health, gingivitis, and periodontitis. *J Dent Res*, 79(2): 785–792.

Mager, D.L., Ximenez-Fyvie, L.A., Haffajee, A.D. & Socransky, S.S. 2003. Distribution of selected bacterial species on intraoral surfaces. *J Clin Periodontol*, 30(7): 644–654.

Mason, M.R., Preshaw, P.M., Nagaraja, H.N., Dabdoub, S.M., Rahman, A. & Kumar, P.S. 2015a. The subgingival microbiome of clinically healthy current and never smokers. *ISME*

*Journal*, 9(1): 268–272.

Mason, M.R., Preshaw, P.M., Nagaraja, H.N., *et al*. 2015b. The subgingival microbiome of clinically healthy current and never smokers. *ISME Journal*, 9(1): 268–272. http://dx.doi.org/10.1038/ismej.2014.114.

Matsha, T.E., Hassan, M.S., Hon, G.M., Soita, D.J., Kengne, A.P. & Erasmus, R.T. 2013. Derivation and validation of a waist circumference optimal cutoff for diagnosing metabolic syndrome in a South African mixed ancestry population. *Int J Cardiol*, 168(3): 2954–2955. http://dx.doi.org/10.1016/j.ijcard.2013.03.150.

Matsha, T.E., Prince, Y., Davids, S., *et al*. 2020. Oral Microbiome Signatures in Diabetes Mellitus and Periodontal Disease. *J Dent Res*, 99(6): 658–665. https://doi.org/10.1177/0022034520913818.

McCracken, E., Monaghan, M. & Sreenivasan, S. 2018. Pathophysiology of the metabolic syndrome. *Clin Dermatol*, 36(1): 14–20. http://dx.doi.org/10.1016/j.clindermatol.2017.09.004.

McLean, J.S. 2014. Advancements toward a systems level understanding of the human oral microbiome. *Front Cell Infect Microbiol*, 4(JUL): 1–13.

Medikeri, R.S., Lele, S.V., Jain, P.M., Mali, P. & Medikeri, M.R. 2015. Quantification of selenomonas sputigena in chronic periodontitis in smokers using 16S rDNA based PCR analysis. *J Clin Diag Res*, 9(4): ZC13–ZC17.

Moon, J.H., Lee, J.H. & Lee, J.Y. 2015a. Subgingival microbiome in smokers and non-smokers in Korean chronic periodontitis patients. *Mol Oral Microbiol*, 30(3): 227–241.

Ogawa, T., Honda-Ogawa, M., Ikebe, K., *et al*. 2017. Erratum to 'Characterizations of oral microbiota in elderly nursing home residents with diabetes'. *J Oral Sci*, 60(1): 163.

Ohlrich, E.J., Cullinan, M.P. & Leichter, J.W. 2010. Diabetes, periodontitis, and the subgingival microbiota. *J Oral Microbiol*, 2(2010): 1–8.

Pirkle, J.L., Flegal, K.M., Bernert, J.T., *et al*. 1996. Exposure of the US population to environmental tobacco smoke: The Third National Health and Nutrition Examination Survey, 1988 to 1991. *JAMA*, 275(16): 1233–1240.

Preza, D., Olsen, I., Willumsen, T., *et al*. 2009. Microarray analysis of the microflora of root caries in elderly. *Eur J Clin Microbiol Infect Dis*, 28(5): 509–517.

Reddy, P., Zuma, K., Shisana, O., *et al*. 2015. Prevalence of tobacco use among adults in South Africa : Results from the first South African National Health and Nutrition Examination Survey. , 105(8).

Saeb, A.T.M., Al-Rubeaan, K.A., Aldosary, K., *et al.* 19. Relative reduction of biological and phylogenetic diversity of the oral microbiota of diabetes and pre-diabetes patients. *Microb Pathog*, 128(January): 215–229. https://doi.org/10.1016/j.micpath.2019.01.009.

Sapkota, A.R., Berger, S., Vogel, T.M., Reynolds, C.R.J. & Co, T. 2010. Human Pathogens Abundant in the Bacterial Metagenome of Cigarettes. *Environ Health Perspect,* 118(3): 351–356.

Sato, T., Watanabe, K., Kumada, H., Toyama, T., Tani-Ishii, N. & Hamada, N. 2012. Peptidoglycan of Actinomyces naeslundii induces inflammatory cytokine production and stimulates osteoclastogenesis in alveolar bone resorption. *Arch Oral Biol*, 57(11): 1522–1528. http://dx.doi.org/10.1016/j.archoralbio.2012.07.012.

Sharma, N., Bhatia, S., Singh Sodhi, A. & Batra, N. 2018. Oral microbiome and health. *AIMS Microbiology*, 4(1): 42–66. http://www.aimspress.com/article/10.3934/microbiol.2018.1.42.

Shchipkova, A.Y., Nagaraja, H.N. & Kumar, P.S. 2010. Subgingival microbial profiles of smokers with periodontitis. *J Dent Res* 89(11): 1247–1253.

Shepherd, S. 2011. Alcohol consumption a risk factor for periodontal disease. *Evid Based Dent*, 12(3): 76. http://dx.doi.org/10.1038/sj.ebd.6400808.

Si, J., Lee, C. & Ko, G. 2017. Oral Microbiota: Microbial Biomarkers of Metabolic Syndrome Independent of Host Genetic Factors. *Front Cell Infect Microbiol*, 7(December): 1–11. http://journal.frontiersin.org/article/10.3389/fcimb.2017.00516/full.

Si, J., Lee, C. & Ko, G.P. 2017. Oral microbiota: Microbial biomarkers of metabolic syndrome independent of host genetic factors. *Front Cell Infect Microbiol*, 7(DEC): 1–11.

Signat, B., Roques, C., Poulet, P. & Duffaut, D. 2011. Role of Fusobacterium nucleatum in periodontal health and disease. *Curr Isues Mol Biol*, 13(2): 25–36.

Signoretto, C., Bianchi, F., Burlacchini, G., Sivieri, F., Spratt, D. & Canepari, P. 2010. Drinking habits are associated with changes in the dental plaque microbial community. *J Clin Microbiol* 48(2): 347–356.

Slattery, M.L., Hunt, S.C., French, T.K., Ford, M.H. & Williams, R.R. 1989. Validity of

cigarette smoking habits in three epidemiologic studies in Utah. *Prev Med*, 18(1): 11–19.

Tefiku, U., Popovska, M., Cana, A., *et al.* 2020. Determination of the Role of Fusobacterium Nucleatum in the Pathogenesis in and Out the Mouth. *Prilozi*, 41(1): 87–99.

Ursell, L.K., Metcalf, J.L., Parfrey, L.W. & Knight, R. 2012. Defining the human microbiome. *Nutr Rev*, 70(SUPPL. 1).

Valour, F., Sénéchal, A., Dupieux, C., *et al*. 2014. Actinomycosis: Etiology, clinical features, diagnosis, treatment, and management. *Infect Drug Resist*, 7: 183–197.

Vielkind, P., Jentsch, H., Eschrich, K., Rodloff, A.C. & Stingu, C.S. 2015. Prevalence of Actinomyces spp. in patients with chronic periodontitis. *Int J Med Microbiol*, 305(7): 682–688. http://dx.doi.org/10.1016/j.ijmm.2015.08.018.

Wade, W.G. 2013. The oral microbiome in health and disease. *Pharmacol Res*, 69(1): 137–143. http://dx.doi.org/10.1016/j.phrs.2012.11.006.

Willis, J.R. & Gabaldón, T. 2020. The human oral microbiome in health and disease: From sequences to ecosystems. *Microorganisms*, 8(2): 1–28.

World Health Organization. 2019. *Report on Global Tobacco Epidemic*. https://apps.who.int/iris/bitstream/handle/10665/326043/9789241516204-eng.pdf?ua=1&ua=1.

Wu, J., Li, M. & Huang, R. 2019. The effect of smoking on caries-related microorganisms. *Tob Induc Dis*, 17(April).

Wu, J., Peters, B.A., Dominianni, C., *et al* 2016. Cigarette smoking and the oral microbiome in a large study of American adults. *The ISME Journal*, 10: 2435–2446. www.nature.com/ismej 27 February 2020.

Yost, S., Duran-Pinedo, A.E., Teles, R., Krishnan, K. & Frias-Lopez, J. 2015. Functional signatures of oral dysbiosis during periodontitis progression revealed by microbial metatranscriptome analysis. *Genome Med*, 7(1): 1–19.

Yu, Q., Huang, S., Xu, T.T., Wang, Y.C. & Ju, S. 2020. Measuring Brown Fat Using MRI and Implications in the Metabolic Syndrome. *J Mag Reson Imaging*.

Zhong, E.F., Chang, A., Stucky, A., *et al*. 2020. Genomic analysis of oral lichen planus and related oral microbiome pathogens. *Pathogens*, 9(11): 1–13.

## 10. Figures and Legends

**Table 1.** General characteristics of participants according to smoking status

| | **Non-smoker n=66** | **Smoker n=57** | |
|---|---|---|---|
| | **Mean ± SD** | **Mean ± SD** | **p-value** |
| Men, n (%) | 21 (32) | 13 (23) | 0.265 |
| Age, (years) | 47.71 ± 14.02 | 46.70 ± 12.01 | 0.671 |
| Body mass index, (kg/m$^2$ ) | 31.66 ± 10.03 | 30.48 ± 8.47 | 0.489 |
| Waist circumference, (cm) | 95.41 ± 20.41 | 93.24 ± 18.64 | 0.543 |
| Hip circumference, (cm) | 105.44 ± 21.62 | 103.34 ± 18.55 | 0.569 |
| Systolic blood pressure, (mmHg) | 130.36 ± 23.60 | 127.82 ± 22.97 | 0.548 |
| Diastolic blood pressure, (mmHg) | 84.42 ± 14.73 | 83.82 ± 13.05) | 0.785 |
| Fasting plasma glucose, (mmol/L)* | 5.70 (4.90;7.63) | 5.60 (4.70;7.30) | 0.544 |
| Post 2-hour glucose, (mmol/L)* | 8.35 (4.90;7.63) | 8.90 (5.68;11.40) | 0.866 |
| HbA1c, (%) | 6.05 (5.40;7.50) | 6.30 (5.50;7.45) | 0.603 |
| Triglycerides, (mmol/L)* | 1.28 (0.90;1.77) | 1.26 (0.96;1.85) | 0.534 |
| LDL-Cholesterol, (mmol/L) | 3.05 ± 1.00 | 3.16 ± 1.10 | 0.538 |
| HDL-Cholesterol, (mmol/L)* | 1.20 (1.10;1.60) | 1.30 (1.10;1.60) | 0.703 |
| us-C-reactive Protein, (mg/L)* | 3.75 (1.50;8.59) | 5.90 (2.07;11.38) | 0.088 |
| Cotinine, (ng/mL)* | 10 (10;10) | 259 (183;348) | <0.001 |
| γ-Glutamyltransferase, IU/L* | 31 (22;57) | 38 (26;72) | 0.164 |

* median (25$^{th}$ and 75$^{th}$ percentiles)

**Table 2:** Alpha diversity in species indices according to smoking status

| | **Smoking status** | |
|---|---|---|
| | **No** | **Yes** |
| Number of taxa | 273 | 250 |
| Shannon | 4.232 | 4.228 |
| Chao1 | 273 | 250 |
| Simpson | 0.0297 | 0.0283 |

**Table 3.** Relative percent abundance of genus and species in smokers and non-smokers.

| **Genus** | **Non-Smoker** | **Smoker** | **P-value** | **Species** | **Non-smoker** | **Smoker** | **P-value** |
|---|---|---|---|---|---|---|---|
| *Streptococcus* | 16.88 | 17.74 | 0.798 | *H. Parainfluenzae* | 4.58 | 9.01 | 0.054 |
| *Prevotella* | 10.94 | 12.04 | 0.938 | *F. Nucleatum* | 4.35 | 6.70 | <0.001 |
| *Fusobacterium* | 6.53 | 9.32 | 0.002 | *V. Alcalescens* | 3.71 | 3.82 | 0.668 |
| *Haemophilus* | 3.57 | 6.95 | 0.086 | *C. Gracilis* | 2.08 | 3.57 | 0.010 |
| *Leptotrichia* | 8.35 | 6.54 | 0.029 | *P. Melaninogenica* | 3.65 | 3.37 | 0.483 |
| *Veillonella* | 5.15 | 5.75 | 0.855 | *C. Matruchotii* | 6.24 | 2.90 | 0.002 |
| *Actinomyces* | 9.92 | 5.08 | 0.001 | *M. Varigena* | 2.34 | 2.81 | 0.772 |
| *Campylobacter* | 2.27 | 3.49 | 0.010 | *H. Segnis* | 1.81 | 2.19 | 0.801 |
| *Capnocytophaga* | 3.66 | 3.4 | 0.233 | *A. Genomospecies* | 1.66 | 1.84 | 0.964 |
| *Neisseria* | 3.54 | 3.16 | 0.527 | *A. Dentalis* | 2.19 | 1.68 | 0.033 |
| *Aggregatibacter* | 2.1 | 2.63 | 0.444 | *P.Pallens* | 0.73 | 1.48 | 0.242 |
| *Corynebacterium* | 5.01 | 2.3 | 0.002 | *V. Rogosae* | 0.78 | 1.38 | 0.001 |
| *Selenomonas* | 2.06 | 2.02 | 0.444 | *L.Buccalis* | 2.09 | 1.33 | 0.067 |
| *Mannheimia* | 1.59 | 1.93 | 0.716 | *P.Oulorum* | 0.70 | 1.28 | 0.137 |
| *Tannerella* | 1.74 | 1.74 | 0.189 | *F. Periodonticum* | 0.76 | 1.21 | 0.556 |
| *Porphyromonas* | 1.89 | 1.38 | 0.083 | *A. Naeslundii* | 3.35 | 1.21 | 0.010 |
| *Lachnoanaerobaculum* | 1.13 | 1.25 | 0.928 | *V. Parvula* | 0.74 | 1.16 | 0.065 |
| *Gemella* | 1.09 | 1.09 | 0.526 | *L. Wadei* | 1.23 | 1.15 | 0.266 |
| *Lautropia* | 1.27 | 0.16 | 0.023 | *F. Canifelinum* | 0.71 | 1.12 | 0.009 |
| *Other* | 11.31 | 12.03 | 0.897 | *A. Odontolyticus* | 0.57 | 1.07 | 0.003 |
| | | | | *C. Leadbetteri* | 1.07 | 1.04 | 0.343 |
| | | | | *S. Gordonii* | 0.61 | 1.00 | 0.051 |
| | | | | *S. Noxia* | 1.01 | 1.00 | 0.500 |
| | | | | *P*Oris, L. Oris* | 1.39 | 0.99 | 0.082 |
| | | | | *P. Veroralis* | 1.13 | 0.85 | 0.087 |
| | | | | ***S. Sputigena or C. Sputigena* | 1.19 | 0.85 | 0.042 |
| | | | | *S. Sanguinis* | 1.28 | 0.33 | 0.016 |
| | | | | *A. Viscosus* | 1.21 | 0.28 | 0.287 |
| | | | | *Other* | 35.60 | 36.01 | 0.220 |

*A. Oris [Non -smoker (NS)= 0.34 ; Smoker (S)= 0.24; p-value=0.160] ; P.Oris[ NS=1.05; S=0.75; p-value=0.260]

**C.Sputigena [NS=0.52; S=0.25; p-value=0.153] ;S.Sputigena[NS=0.66; S=0.60; p-value:0.341

**Table 4.** Multivariable regression analysis for the presence of oral microbiome species in smokers compared to non-smokers.

| | Model 1 | | Model 2 | | Model 3 | | Model 4 | |
|---|---|---|---|---|---|---|---|---|
| | **OR (95% CI)** | **p-value** | **OR (95% CI)** | **p-value** | **OR (95% CI)** | **p-value** | **OR (95% CI)** | **p-value** |
| *V.Alcalescens* | 0.93 (0.66 ;1.30) | 0.666 | 0.92 (0.64 ;1.31) | 0.633 | 0.96 (0.63 ;1.47) | 0.856 | 0.95 (0.62 ;1.45) | 0.802 |
| *B.Buccalis* | 0.65 (0.40 ;1.06) | 0.086 | 0.68 (0.41 ;1.12) | 0.127 | 0.74 (0.46 ;1.19) | 0.210 | 0.72 (0.45 ;1.15) | 0.169 |
| *F. Canifelinum** | 1.22 (1.04 ;1.43) | 0.012 | 1.19 (1.02 ;1.41) | 0.032 | 1.13 (0.95 ;1.34) | 0.174 | 1.17 (0.97 ;1.40) | 0.101 |
| *A. Dentalis* | 0.50 (0.26 ;0.97) | 0.040 | 0.57 (0.29 ;1.13) | 0.106 | 0.77 (0.39 ;1.51) | 0.443 | 0.78 (0.40 ;1.52) | 0.467 |
| *A. Genomosp.* | 1.01 (0.65 ;1.56) | 0.964 | 1.18 (0.75 ;1.87) | 0.475 | 1.20 (0.75 ;1.93) | 0.445 | 1.18 (0.74 ;1.90) | 0.482 |
| *S.Gordonii** | 1.13 (1.00 ;1.28) | 0.056 | 1.09 (0.96 ;1.25) | 0.179 | 1.11 (0.97 ;1.28) | 0.132 | 1.11 (0.96 ;1.27) | 0.155 |
| *C. Gracilis* | 1.7 (1.11 ;2.61) | 0.015 | 1.69 (1.08 ;2.63) | 0.021 | 1.67 (1.06 ;2.64) | 0.027 | 1.67 (1.05 ;2.66) | 0.031 |
| *C. Leadbetteri* | 0.62 (0.24 ;1.65) | 0.343 | 0.58 (0.22 ;1.55) | 0.274 | 0.51 (0.17 ;1.49) | 0.215 | 0.52 (0.17 ;1.59) | 0.253 |
| *C. Matruchotii* | 0.74 (0.60 ;0.91) | 0.004 | 0.77 (0.62 ;0.95) | 0.017 | 0.84 (0.67 ;1.04) | 0.116 | 0.83 (0.67 ;1.04) | 0.099 |
| *P.Melaninogenica* | 0.93 (0.75 ;1.15) | 0.482 | 0.93 (0.74 ;1.16) | 0.513 | 0.9 (0.72 ;1.14) | 0.399 | 0.89 (0.70 ;1.13) | 0.334 |
| *A. Naeslundii* | 0.32 (0.15 ;0.69) | 0.003 | 0.35 (0.16 ;0.76) | 0.008 | 0.48 (0.22 ;1.07) | 0.072 | 0.45 (0.20 ;1.02) | 0.056 |
| *S. Noxia** | 1.03 (0.72 ;1.47) | 0.499 | 0.99 (0.9 ;1.09) | 0.878 | 1.00 (0.9 ;1.11) | 0.995 | 0.99 (0.89 ;1.09) | 0.793 |
| *F. Nucleatum** | 1.07 (1.03 ;1.12) | 0.001 | 1.06 (1.02 ;1.11) | 0.004 | 1.04 (0.99 ;1.09) | 0.084 | 1.04 (1.00 ;1.09) | 0.075 |
| *A. Odontolyticus** | 1.26 (1.07 ;1.47) | 0.005 | 1.29 (1.09 ;1.54) | 0.004 | 1.28 (1.06 ;1.54) | 0.009 | 1.27 (1.06 ;1.54) | 0.011 |
| *P. Oris* | 0.50 (0.22 ;1.14) | 0.098 | 0.53 (0.24 ;1.17) | 0.115 | 0.70 (0.32 ;1.5) | 0.358 | 0.66 (0.30 ;1.45) | 0.299 |
| *P. Oulorum* | 1.26 (1.07 ;1.47) | 0.174 | 1.26 (1.07 ;1.47) | 0.112 | 1.26 (1.07 ;1.47) | 0.095 | 1.26 (1.07 ;1.47) | 0.100 |
| *E. Pallens** | 1.05 (0.97 ;1.13) | 0.244 | 1.07 (0.99 ;1.16) | 0.104 | 1.06 (0.97 ;1.15) | 0.171 | 1.05 (0.97 ;1.15) | 0.235 |
| *H. Parainfluenzae* | 1.10 (0.99 ;1.22) | 0.083 | 1.09 (0.99 ;1.2) | 0.078 | 1.06 (0.96 ;1.17) | 0.259 | 1.05 (0.96 ;1.16) | 0.285 |
| *V. Parvula** | 1.12 (0.99 ;1.26) | 0.072 | 1.10 (0.97 ;1.25) | 0.137 | 1.09 (0.95 ;1.25) | 0.244 | 1.08 (0.94 ;1.24) | 0.261 |
| *F.Periodonticum* | 1.16 (0.71 ;1.91) | 0.557 | 1.17 (0.70 ;1.94) | 0.556 | 0.95 (0.55 ;1.62) | 0.839 | 0.92 (0.54 ;1.58) | 0.774 |
| *V.Rogosae** | 1.26 (1.09 ;1.46) | 0.002 | 1.24 (1.07 ;1.44) | 0.005 | 1.24 (1.05 ;1.46) | 0.011 | 1.25 (1.05 ;1.48) | 0.011 |
| *S. Sanguinis* | 0.18 (0.04 ;0.87) | 0.033 | 0.26 (0.06 ;1.19) | 0.081 | 0.39 (0.10 ;1.54) | 0.177 | 0.39 (0.10 ;1.49) | 0.167 |
| *H.Segnis* | 1.05 (0.70 ;1.59) | 0.799 | 1.00 (0.66 ;1.54) | 0.986 | 0.91 (0.57 ;1.47) | 0.712 | 0.93 (0.56 ;1.55) | 0.794 |
| *S. Sputigena* | 0.30 (0.09 ;1.00) | 0.050 | 0.31 (0.09 ;1.04) | 0.057 | 0.39 (0.11 ;1.35) | 0.137 | 0.43 (0.11 ;1.65) | 0.221 |
| *M.Varigena* | 1.05 (0.78 ;1.41) | 0.770 | 1.02 (0.75 ;1.4) | 0.883 | 0.89 (0.64 ;1.25) | 0.517 | 0.90 (0.64 ;1.25) | 0.521 |
| *P.Veroralis* | 0.47 (0.19 ;1.16) | 0.103 | 0.43 (0.16 ;1.18) | 0.102 | 0.51 (0.17 ;1.49) | 0.217 | 0.48 (0.16 ;1.44) | 0.192 |
| *A.Viscosus** | 0.91 (0.76 ;1.10) | 0.337 | 0.93 (0.79 ;1.08) | 0.325 | 0.97 (0.86 ;1.08) | 0.546 | 0.96 (0.85 ;1.09) | 0.545 |
| *L.Wadei** | 0.96 (0.9 ;1.03) | 0.270 | 0.97 (0.91 ;1.04) | 0.456 | 0.99 (0.92 ;1.07) | 0.796 | 0.99 (0.92 ;1.06) | 0.731 |
| *Other* | 0.94 (0.85 ;1.04) | 0.224 | 0.96 (0.86 ;1.06) | 0.380 | 0.99 (0.89 ;1.1) | 0.874 | 0.97 (0.87 ;1.09) | 0.658 |

Model 1: Crude; Model 2: included age and sex; Model 3: included age, sex, and BMI ; Model 4: included age, sex, BMI and bleeding; *calculated for 0.1 unit increase

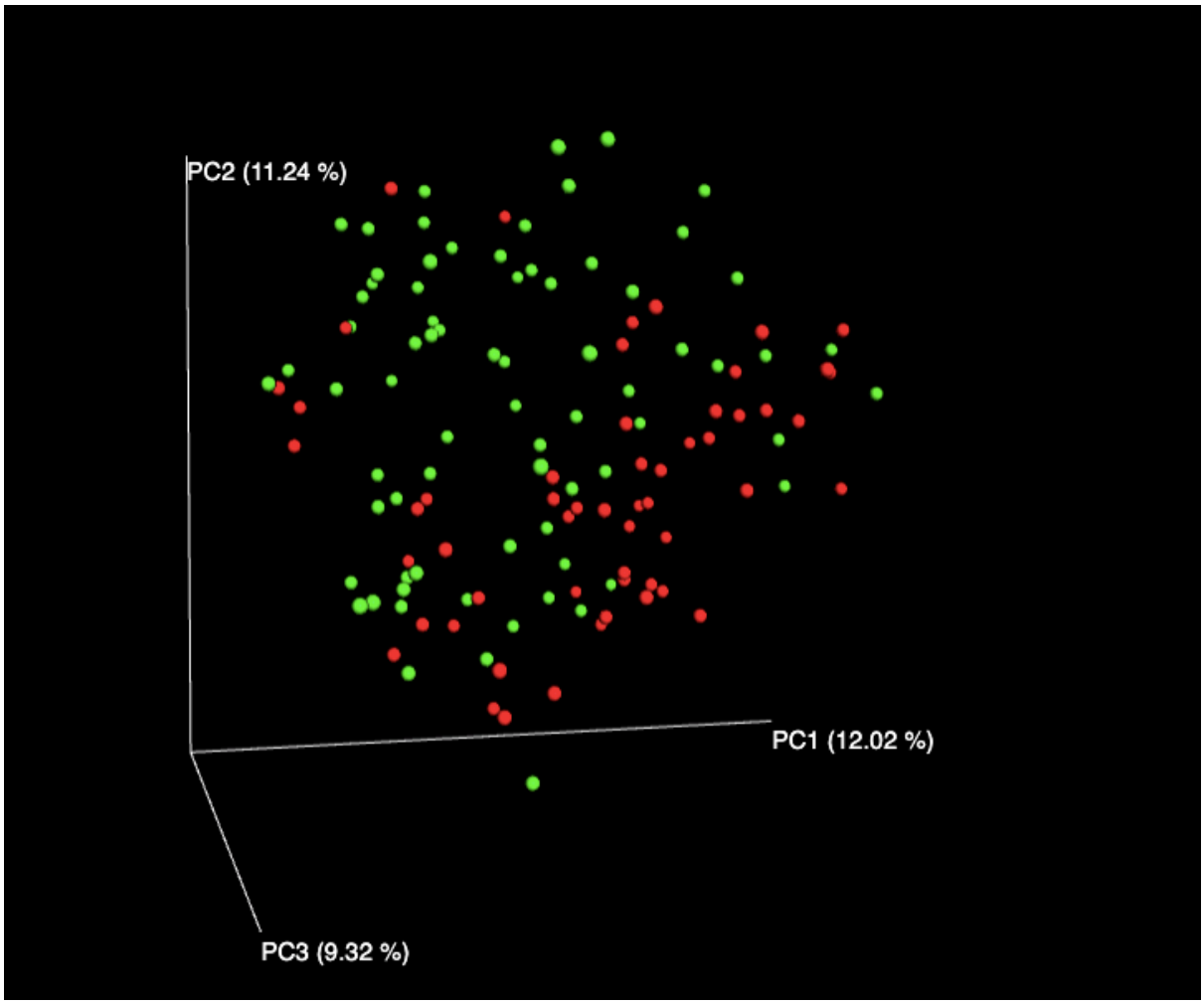



**Figure 1.** Beta diversity comparisons of microbial communities in smokers and non-smokers for smokers (red), and non-smokers (green) are shown to determine Bray–Curtis distances.

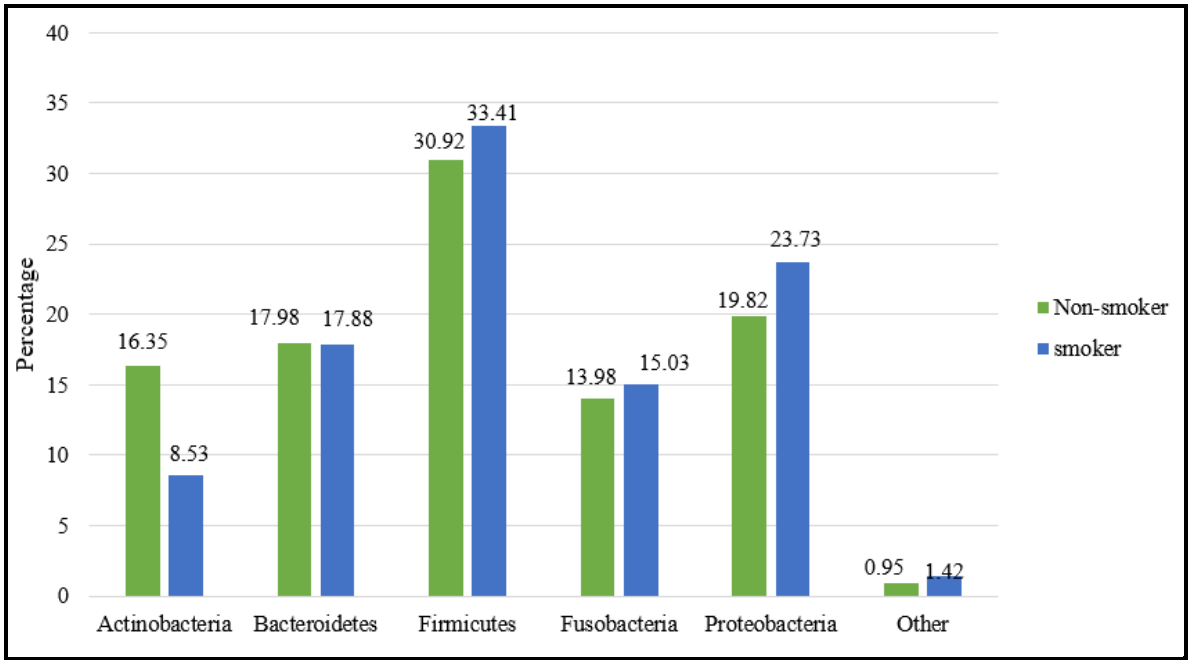



**Figure 2.** Relative percentage abundance of phyla in smokers and non-smokers

# CHAPTER 5: MANUSCRIPT 3

# The Relationship between the Oral Microbiome and Metabolic Syndrome.

Yvonne Prince[1], Saarah Davids[1], Rajiv T Erasmus[3], Andre P Kengne [4,5], Glenda M Davison[1], Tandi E Matsha [1*]

[1]SAMRC/CPUT/Cardiometabolic Health Research Unit, Department of Biomedical Sciences, Faculty of Health and Wellness Sciences, Cape Peninsula University of Technology, Bellville, South Africa. [2]Department of Global Health, Stellenbosch University, Cape Town 8000, South Africa. [3]Department of Pathology, Faculty of Medicine and Health Sciences, Stellenbosch University, Cape Town, South Africa. [4]NonCommunicable Diseases Research Unit, South African Medical Research Council, Cape Town, South Africa. [5]Department of Medicine, University of Cape Town, Cape Town, South Africa.

*Correspondence should be addressed to: Tandi E. Matsha, and Yvonne Prince, Biomedical Sciences, Faculty of Health and Wellness Sciences, Cape Peninsula University of Technology, PO Box 1906, Bellville 7530, Cape Town, South Africa Tel: +27 21 959 6366, Fax: +27 21 959 6760 ;
Email: matshat@cput.ac.za and/or tandimatsha@gmail.com

Word count, 4045

Number of figures and Tables: 6

**Abstract**

The oral microbiota plays a crucial role in both systemic inflammation and metabolic syndrome (MetS) which is characterised by low-grade inflammation. Studies have analysed the gut microbiota using stool specimens from subjects with MetS, however, the etiological role of the oral microbiota in the development of MetS is still uncertain. We, therefore, investigated the oral microbiota of 128 subgingival plaque samples from a South African cohort with and without MetS. After a comprehensive analysis of the oral microbiome, a significant increase in gram-positive aerobic and anaerobic microbiota was observed in those with MetS . In the MetS group an abundance in the genera Actinomyces, Corynebacterium, and Fusobacterium were observed, which was significantly different from previous studies which have found *Granulicatella* to be enriched in MetS. To further assess the impact of the metabolic parameters (FBG, Waist C, HDL, TGs, and BP) on the oral microbiome, we calculated the odds ratio (ORs) for significant oral bacteria identified between the MetS group and we found that different species were associated with at least four of the MetS risk factors.

In conclusion, this study has shown that the oral microbiota is disrupted in MetS and may promote inflammation providing a gateway to other systemic diseases which include diabetes, and cardiovascular disease.

**Keywords: Metabolic Syndrome, Oral Microbiome, Subgingival plaque, rDNA**

## 1. Introduction

Metabolic syndrome (MetS), known to cause low-grade inflammation, consists of a cluster of risk factors that can predispose individuals to cardiovascular disease (CVD) and type 2 diabetes mellitus (T2DM) (McCracken et al., 2018). The definition of MetS is based on the 2009 JIS (The Joint Interim Statement) criteria and includes any three of the following five risk factors: central obesity, dyslipidaemia (raised triglycerides (TGs), and decreased levels of high-density lipoprotein (HDL) cholesterol), hyperglycaemia and hypertension (Alberti et al., 2009). MetS has become a public health concern globally and is largely attributed to increasing obesity and lifestyle changes (Yu et al., 2020).

Previous studies have confirmed that the oral microbiome plays a vital role in both local and systemic inflammation (Lu et al., 2019) and harbours over 1000 different species of microorganisms (Wade, 2013; Sharma, Bhatia, Singh Sodhi, et al., 2018). These microorganisms play a role in human physiological status, including the properties of the innate and adaptive immune system, host metabolism, and genotype (Kilian et al., 2016). The oral microbiota has further been associated with a variety of oral diseases of which the most common are gingivitis and periodontitis. Recently periodontitis has been associated with systemic diseases such as coronary heart disease and types 2 diabetes (Si eta l., 2017; Janket et al., 2003; Khader et al., 2004)

The oral pathogen *Porphyromonas gingivalis* has been shown to induce systemic inflammation and metabolic disorders in mice (Arimatsu et al., 2014). These findings suggest that oral microbiota may cause low-grade inflammation in humans leading to the development of MetS. Given that the oral microbiome may be an important etiological agent in the development of systemic disease its role in the development of MetS is important. Most previous studies have been performed on the gut microbiota using stool samples from mice (Si et al., 2017) and therefore, in this study, we aimed to investigate and analyse the oral microbiome, using plaque samples from individuals with risk factors for Mets to characterise and identify microbiome signatures that are associated with MetS.

## 2. Materials and Methods

### *2.1. Study subjects and sample collection*

This was a case-controlled study conducted on a South African population in the Western Cape, Capetown, A total of 128 subjects, were recruited from the ongoing Vascular and Metabolic Health (VMH) study which had obtained ethical approval from Stellenbosch University and the Cape Peninsula University of Technology (respectively, NHREC: REC - 230 408 – 014, CPUT/HW-REC 2015/H01 and N14/01/003). The oral microbiome analysis received further ethical clearance from the Ethics Committee of the Cape Peninsula University of Technology (CPUT/HW-REC 2017/H31) and the study was performed according to the guidelines of the Helsinki Declaration.

### *2.2. Sample collection*

Blood samples were collected after overnight fasting. The Oral glucose tolerance test (OGTT) was performed on all subjects except subjects with known diabetes on treatment. All other blood tests were conducted in an ISO 15189 accredited laboratory (PathCare Reference Laboratory, Cape Town, South Africa) and included plasma glucose, HBA1c, triglycerides, low-density lipoprotein cholesterol (LDL-chol), high-density cholesterol (HDL-chol), insulinγ-Glutamyl transferase (GGT) and ultra-sensitive (us-CRP). Lifestyle and clinical conditions were recorded using a questionnaire, and a written consent form was signed by all participants after all the procedures had been fully explained in the language of their choice.

Anthropometrical measurements which included waist circumference (WC) and blood pressure (BP) were obtained as follows: The Waist (WaistC) and hip circumference (hipC) measurements were taken while subjects were in a relaxed position and were rounded off to the nearest 0.5cm. The Systolic (SBP) and Diastolic (DBP) (mmHg) measurements were taken at three-time points within a 5-minute interval. The lowest SBP and its matching measurement were recorded and were used for statistical analysis in this study. This procedure was performed according to (1999) World Health Organization (WHO) recommendations Bodyweight (in kilograms) was measured using a calibrated Omron body fat meter HBF 511 digital bathroom scale and the Body Mass Index (BMI) ($kg/m^2$) was calculated as the body weight (in kilograms) divided by the square of his or her height (in meters) and rounded off to the nearest 0.1kg.

*2.3. Metabolic syndrome classification*

For this study, the JIS MetS classification was used to diagnose MetS (Alberti et al., 2009). The diagnosis was made if the participant presented with 3 or more risk factors which included central obesity, hyperglycaemia, hyperglyceridaemia, low HDL-cholesterol (dyslipidaemia), and hypertension. Central obesity was defined as a waist circumference of >90cm (Matsha et al., 2013) while fasting blood glucose (FBG) of >5.6 indicated hyperglycaemia. Hypertension was identified when the systolic blood pressure (SBP): ≥ 130 mmHg, and diastolic (DBP) ≥ 85 mmHg, Low HDL (men<1mmol and women <1.3 mmol), and triglycerides of >1.7 mmol was indicative of dyslipidaemia.

*2.4. Dental examination*

The examination was conducted according to guidelines from the World Health Organization (WHO., 2016) and the Community Periodontal Index. Each tooth was probed for bleeding on probing (BOP) and was recorded as presence or absence of bleeding after gentle periodontal probing around each tooth circumference. For pocket depth (PD), each tooth was probed in its whole circumference, and the highest score was recorded.

*2.5. Collection of plaque samples*

After dental examination and assessment, four subgingival plaque samples were collected from all the subjects after 12 h of fasting and without tooth brushing, food intake, or smoking. The plaque samples were collected using the wood toothpick method from both sides of the oral cavity and marked as right side and left side similar to a study performed by (Keijser et al., 2008). This was done by inserting the device in the subgingival crevice between the first premolar and last upper premolar according to the guidelines from the World Health Organization (WHO, 2016). The samples were stored at −80° C immediately upon collection until further analysis was performed.

*2.6. Smoking assessment*

Serum cotinine levels of >15 ng/ml were used to define smokers (Pirkle et al., 1996; Slattery et al., 1989). In addition, participants were required to complete the STEP questionnaire following guidelines established in 2011, and this together with the serum cotinine was used to assess the smoking status of all participants (Global Adult Tobacco Survey Collaborative Group, 2011).

*2.7. DNA extraction and Quality control*

Microbial Plaque DNA was extracted from the plaque samples using the Zymo Quick-DNA Fungal Bacterial Miniprep KIT (Zymo Research) and extraction was done according to the manufacturer's protocol. DNA quality and quantity were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). The Qubit 4.0 Fluorometer was used to quantify the metagenomic DNA (mgDNA) using the Qubit dsDNA HS assay kit according to the (MAN0017455 Rev. A.0) protocol. The mgDNA purity was established using the NanoDrop ND-1000 Spectrophotometer, while the LapChip GXII was used to determine the genomic quality score following the DNA Reagent Kit (PerkinElmer) and (CLS140166 Rev. C; Supplementary Report: Genomic DNA [gDNA] Quality Control manufacturers protocol. A genomic score between 0 and 5, with 5 indicating high-quality gDNA was used.

*2.8. Metagenomics 16S rDNA*

Amplification of hypervariable regions from the polybactarial DNA samples was conducted using the Ion 16S Metagenomic Kit and the (MAN0010799 REV C.0) protocol. 2 µL of mgDNA was used to amplify the target regions across 25 cycles with 2 primers pools using the SimpliAmp Thermal Cycler (ThermoFisher Scientific). After verification of amplification (polymerase chain reaction), the 2 primers primer 1 (V2-4-8) and primer 2 (V3-6-7-9) pools were combined for each sample. Purification was done and eluted with Agencourt AMPure XP reagent, and 15 µL of nuclease-free water. The Qubit 1x dsDNA HS assay kit was used to quantify the purified amplicons on the Qubit 4.0 Fluorometer following the (MAN001 7455 Rev. A.0) protocol.

*2.9. Library preparation*

NEXTflex DNA Sequencing Kit was used to prepare the library of which 100 ng of the pooled amplification product was used for each sample following the (v 15.12; Bio Scientific Corporation) protocol. The LabChip GXII Touch (PerkinElmer) was used for library fragmentation size distribution, with the X-mark chip and HT DNA NGS 3K reagent kit according to the (CLS145098 Rev. E) manufacturer's protocol (CLS145098 Rev. E).

*2.10.Template Preparation, Enrichment, Sequencing, and Analysis*

Library dilution was done targeting a 10pM concentration and thereafter, the diluted 16S barcoded libraries were combined in equimolar amounts for template preparation with the Ion

510, Ion 520, and Ion 530 Chef Kit. In short, 25 µL of the pooled library was loaded on the Ion Chef liquid handler with reagents, solutions, and supplies according to the (MAN001 6854, REV.C.0) protocol. The Ion 530 Chip was used to load the Enriched template-positive ion sphere particles onto the chip. The Ion S5 Gene Studio with the Ion S5 Sequencing Solutions and Sequencing Reagents Kits was used to run massive parallel sequencing according to the protocol (MAN0016854, REVC.0). The Torrent Suite software (v 5.12.0 was then used for flow space calibration, and BaseCaller analyses were performed with default analysis parameters. Raw sequence data and taxonomy assignment were performed as previously described (Saeb et al., 2019).

*2.11.Statistical Analysis*

The software SPSS v.26 (IBM Corp, 2019) was used for data analysis. The results were reported as mean (standard deviation), median ($25^{th}$ and $75^{th}$ percentiles), and count (percentages). For comparison, analysis of variance test (ANOVA) or Kruskal Wallis was used for numerical variables while for categorical variables the *chi-square test* of association was used. The Cohens Kappa assessment was used to statistically evaluate the agreement between serum cotinine values and questionnaire responses. Microbiome data was presented in terms of relative abundance percent for genus and species comprising ≤ 1% of the total abundance were grouped as "other". The independent *t-test* was used to determine statistically significant differences in the relative percent abundance between cases and controls for the genus or species. Multivariate logistic regression models were used to assess the association between microorganisms present in MetS, periodontitis, and bleeding and the adjusted odds ratios (OR). A *P*-value <0.05 was used to characterize statistically significant results. To determine species richness and how much species was present in our oral microbial samples alpha diversity was performed using Chao1, Shannon, and Simpson indices. Emperor (v0.9.60) was used to plot the principal coordinate plot (PCO) using transformed OUT counts to determine beta analysis. Bray-Curtis calculation was used to determine the compositional dissimilarities between MetS and subjects without MetS.

## 3. Results

We performed 16S rDNA gene sequencing using subgingival plaque samples from 128 participants of which 62 (48%) subjects were diagnosed with MetS. This was done to determine and characterise the oral microbiome of subjects with and without MetS. As expected, those with MetS had significantly higher BMI, Waist and Hip circumferences, Diastolic Blood pressure as well as increased glycaemic and triglyceride parameters. The inflammatory marker ultra-sensitive C-reactive protein (usCRP) was also significantly higher in subjects with MetS (Table 1). Based on the Chao index, the alpha diversity was increased in subjects with MetS as compared with subjects without MetS (Table 2). As for Simpson diversity, low diversity was seen in the species communities between the two groups (Table 2.). Beta diversity indicated that there was a 24% dissimilarity in species population between subjects with MetS and subjects without MetS (Figure 1).

A significantly higher percentage of *Actinomyces dentalis* (p=<0.001), *Actinomyces naeslundii* (p=<0.001), *Actinomyces viscosus* (p=0.021), *Corynebacterium matruchotii* (p=<0.001), *Leptotrichia buccalis* (p=0.007), *Streptococcus sanguinis* (p=<0.001), were seen in MetS subjects while lower percentages of *Actinomyces odontolyticus* (p=0.005), *Campylobacter gracilis* (p=0.002), *Fusobacterium canifelinum* (p=0.001), *Fusobacterium nucleatum* (p=<0.001), *Fusobacterium periodonticum* (p=0.022), *Haemophilus parainfluenzae* (p=<0.001) and *Veillonella rogosae* (p=0.007) were observed in subjects with MetS (Table 3)

Although there was no significant difference in the incidence of periodontal disease or bleeding between those with and without MetS, *Fusobacterium nucleatum* (p=0.001), *Neisseria flavescens* (p=0.036), and *Veillonella rogosae* (0.037) were increased in those with gingival bleeding, while *Granulicatella adiacens* (p=0.016), *Selenomonas noxia* (p=0.01), *Streptococcus sanguinis* (p=0.007) were decreased in Mets ( Table 3).

To assess the impact of metabolic parameters (FBG, Waist-C, HDL, TGs, and BP) on the oral microbiome correlations between the genus, species, and the oral microbiota was performed (Table 4). The results revealed that *Actinomyces dentalis, Actinomyces naeslundii, Actinomyces odontolyticus*, *Actinomyces viscosus*, *Campylobacter gracilis Corynebacterium matruchotii Fusobacterium canifelinum Fusobacterium nucleatum Fusobacterium periodonticum*, *Haemophilus parinfluenzae*, *Leptotrichia bucallis*, *Prevotella pellens*, *Streptococcus sanguinis*, and *Veilonella ragosae* were all positively correlated with an

increased waist circumference of >90cm, all p ≤0.042, while *Actinomyces dentalis*, *Actinomyces naeslundii*, *Actinomyces odontolyticus, Campylobacter gracilis, , Corynebacterium matruchotii, Fusobacterium canifelinum*, *Fusobacterium nucleatum*, *Granulicatella adiacens*, *Haemophilus parinflenazae*, *Leptotrichia bucallis*, *Leptotrichia genomosp*, *Mannheima varigena*, *Selenomas noxia*, *Streptococcus sanguinis*, *Veilonella parvula*, and *Veilonella ragosae*, all p≤ 0.045 correlated with a fasting blood glucose of >5.6mmol/L.

Furthermore, a significant positive correlation was observed between triglycerides (>1.7 mmol/L) and *Actinomyces naeslundii*, *Actinomyces odontolyticus*, *Aggregatibacter segnis*, *Corynebacterium matruchotii*, *Fusobacterium canifelinum*, *Fusobacterium nucleatum*, *Fusobacterium periodonticum* and *Streptococcus sanguinis* all p ≤0.015. Positive associations between HDL (men ≤1 mmol/L and women ≤ 1.3 mmol/L) and *Actinomyces naeslundii*, *Actinomyces odontolyticus*, *Aggregatibacter segnis*, *Corynebacterium matruchotii*, *Fusobacterium periodonticum* and *Haemophilus parinfluenzae* all p≤0.038 were also recorded (Table 4).

*Actinomyces dentalis*, *Actinomyces naeslundii*, *Corynebacterium matruchotii*, *Fusobacterium canifelinum* , *Fusobacterium nucleatum*, *Leptotrichia genomosp*, *Leptotrichia wadei*, *Prevotella oris*, *Streptococcus gordonii*, *Streptococcus sanguinis*, *Veilonella parvula* all p≤ 0.045 were positively correlated with age while the inflammatory marker CRP was significantly associated with *Actinomyces odontolyticus*, *Fusobacterium periodonticum*, *Haemophilus parinfluenzae*, *Mannheima varigena*, *Prevotella histicola* and *Prevotella oulorum* all p ≤0.047 (Table 4).

To further assess the impact of MetS on the oral microbiome we performed a multivariate logistic regression, on genus and species, (Table 5) and the species that remained significant throughout the odds ratio was *Campylobacter gracilis* (OR 0.29, 95% CI 0.12;0.68, p=0.005) although reduced abundance was observed.

## 4. Discussion

Literature states that the presence of periodontal pathogens and their metabolic by-products may modify the immune response beyond the oral cavity, thus promoting the development of systemic conditions. As MetS are characterised by low-grade inflammation, this study aimed to investigate and report on oral pathogens associated with subjects with and without Mets. In this study of 128 participants, 62 subjects met the criteria of MetS, and when 16S rDNA analysis was performed a significant difference was observed in the oral microbiota between the two groups. Those who met the criteria for MetS had a significantly enriched abundance of gram-positive aerobic and anaerobic microbiota (20%) while gram-negative bacteria (gnb) (18%) were less abundant.

In the MetS subjects, the most dominant gram-positive genera were the Actinomyces and Corynebacterium, while the genera *Haemophilus species parainfluenzae* was more abundant among the healthy controls. This finding is in contrast to other published research. For instance, in a comprehensive analysis of the oral microbiota genera, Granulicatella and Neisseria were found to be abundant in subjects with MetS, while in those without MetS, Peptococcus was abundant (Si et al ., 2017). *Actinomyces* spp. are gram-positive pleomorphic bacteria (gpb) that are part of the normal flora of the oral cavity and do not normally cause disease as long as they are confined to the surface of the mucosa. However, when perturbation occurs to the mucosal integrity and defense mechanisms are perturbed, they can settle on deep periodontal tissues and may cause pathologic reactions and progress into periodontal disease (Vielkind et al., 2015). Although the presence of bleeding did not reach significance, the most predominant Actinomyces species present in both Mets and those with gingival bleeding was *Actinomyces naeslundii* which has previously been reported to be abundant in individuals with gingival bleeding (Beutler et al., 2019). *Actinomyces naeslundii* forms part of the “early colonizers “and forms the basis for colonisation of the sulcus with other periodontitis-associated microbiota. This was confirmed recently when *Actinomyces naeslundii* was reported to induce horizontal alveolar bone loss similar to that caused by periodontal pathogenic *Porphoromonas gingivalis,* which is the main etiological factor in *periodontal diseases* (Sato et al., 2012).

Periodontal disease is considered an inflammatory disease and has been linked with systemic diseases such as diabetes and metabolic syndrome (Linden et al., 2013). It has been hypothesised that the presence of these oral pathogens may cause systemic oxidative stress and

may serve as a potential marker for both periodontitis and Mets (Lamster & Pagan, 2017). In literature, it has been suggested that the Actinomyces species are present as polymicrobial flora and therefore co-aggregates with *Eikenella corrodens* from the green-complex have been observed (Valour et al., 2014).

The second most dominant gpb present in our Mets subjects was *Corynebacterium matruchotii.* This oral microbiota is present in human biofilm formation and has been associated with oral lichen planus (OLP) which is a common chronic inflammatory disease affecting the oral mucosa. In a previous study comparing host cell gene expression profiles with oral microbial profiles within patients with OLP and healthy individuals, researchers *found that Corynebacterium matruchotii*, *Fusobacterium periodonticum*, and other species were capable of activating the Hepatocyte nuclear factor-alpha (HNF4A) gene network which mediates mucosal inflammatory processes (Zhong et al., 2020). As we also observed an abundance of this bacteria, it strongly suggests that these changes could be associated with inflammation.

Although in less abundance than those without MetS, the third most dominant genera in the MetS participants, were Fusobacteria. The dominant species *Fusobacterium nucleatum,* which is normally present as a commensal of the human oral activity, is an opportunistic orange complex anaerobic gram-negative bacterium and is the most dominant species present in periodontal disease. In this present study, although the presence of this bacteria was lower in those with MetS, it was significantly increased in those who had gingival bleeding. Previous studies have confirmed its role in other chronic inflammatory conditions, and have suggested that its presence could adversely influence the clinical outcome (Tefiku et al., 2020). Although *Fusobacterium nucleatum* normally presents as an oral flora it may be of benefit due to its ability to encourage the production of antimicrobial peptides in gingival epithelial cells (Krisanaprakornkit et al., 2000; Ji et al., 2007). Therefore, a reduction in this bacterium has been observed in our study could lead to a disruption in the overall balance of the oral biome leading to dysbiosis and abnormal immune responses.

The relationship of *Fusobacterium nucleatum* with the adaptive immune response is unclear and it is thought that in health, the immune system and the normal oral microbiota exist in synergy. However, when the distribution of the oral microbiome is disrupted, T-cells, B- cells and innate immune cells become activated, which can lead to systemic inflammation. In an attempt to study this, the response of T-cells and the antibody response to FaDA and Td92

(*Fusobacterium nucleatum* specific antigens) were investigated in both healthy and individuals with chronic periodontitis (CP). The results demonstrated that in both groups there was an increase in antibodies specific to the FadA antigen together with an increase in CD4+ specific T-cells. The production of the cytokines IFNγ and IL-10 were also elevated. Although there was no difference between the healthy and CP groups, the numbers in this study were small and those with CP had an increased response (Shin et al, 2013). This work clearly demonstrated that the relationship between the immune response and oral bacteria is important and could therefore potentially lead to systemic inflammation and play a role in the development of Metabolic syndrome.

Systemic oxidative stress may also be a potential link between periodontitis and MetS. The increase of inflammatory markers such as cytokines and oxidative stress markers due to periodontitis could lead to reduced or inactive insulin sensitivity which is considered a significant event in the development of MetS. Mets may have various definitions or classifications in literature but the consistent pathophysiology of MetS is insulin resistance and obesity (Andersen et al., 2016). Studies have found that insulin resistance and inflammation increases with age, and therefore, older people are at risk of developing CVD (Ferroni et al., 2016). This was confirmed by our findings as those with MetS were significantly older and had an increased CRP which is a marker of inflammation.

In multivariate analysis when the presence of oral microbiota was correlated with age and insulin resistance *Actinomyces dentalis, Actinomyces naeslundii, Corynebacterium matruchotii, Fusobacterium canifelinum, Fusobacterium nucleatum, Prevotella oris, Streptococcus sanguinis.* had a significant positive correlation. These findings support previous studies in which the supragingival biofilms of the healthy periodontium in subjects over 60 years were investigated. Researchers found a predominance of gram-positive, aerobic bacteria and relatively less gram-negative anaerobes (Feres et al., 2017). The results demonstrated that the Actinomyces species (blue complex) especially *Actinomyces naeslundii* and *Actinomyces oris* were significantly higher in older individuals supporting our research. Our findings have further been supported by a publication in which the diversity of the oral microbiota within the different oral niches, from older adults without root caries or periodontitis, was reported. These included species such as *Streptococcus oralis*, *Veillonella atypica*, *Streptococcus parasanguinis,* and *Fusobacterium nucleatum*. (Preza et al., 2009) Similar to our study an

abundance of *Fusobacterium nucleatum* was reported. This study also highlighted the importance of differing techniques, sample sites, and geographical areas in contributing to the controversies amongst researchers.

After performing multivariate analysis, the only bacteria which remained significant throughout was *Campylobacter gracilis.* Even though the odds of having *Campylobacter gracilis* remained low in Mets its presence in the oral microbiome may signify different stages of progression of periodontal disease (Macuch & Tanner, 2000, Haririan et al., 2014). Other authors have determined that the percentage or proportion of species within the genus Campylobacter is an important marker for periodontitis progression. Henne et al., 2014 hypothesised that the progression of periodontal disease can be predicted by the presence of three different species. These included *Campylobacter rectus* (higher abundance), *Campylobacter concisus* (lower abundance) while the presence of *Campylobacter gracilis* is associated with intermediate progression (Henne et al., 2014). Although oral microbiota from the yellow, green, purple, and blue complex are normally associated with normal periodontal health, compelling evidence suggests that these bacteria may be associated with periodontal disease and systemic diseases (Chukkapalli et al., 2015; Yost et al., 2015; Chistiakov et al., 2016).

This present study had several limitations which included low sample size and significantly fewer males in comparison to females. Another limitation is that plaque samples were collected at only two sites within the oral cavity and thus may not adequately represent the microbial profiles of the entire microbiota Furthermore this was a cross-sectional study and therefore further longitudinal studies are required to analyse how the oral biome changes may lead to the progression of the disease.

Despite these limitations, the findings of this study strongly suggest that the variation and distribution of the oral pathogens in MetS are associated with chronic inflammation and may provide a gateway to systemic disorders. The most abundant genera observed in this cohort of MetS individuals were *Actinomyces, Corynebacterium,* and *Fusobacterium* which is different from some other reports (Si, et al., 2017) which could be due to the population group and both sampling and analytical techniques. Despite variation in the literature, our findings support the theory that disruptions in the oral biome are associated with both local and systemic

inflammation. To confirm these findings, future longitudinal studies should include larger sample sizes that aim to correlate changes to the oral biome with the progression of MetS.

## 5. Conflict of interest

*The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.*

## 6. Author contributions

Conceptualization, T.E.M., contributed to conception and data acquisition and interpretation, methodology. R.T.E, and A.P.K.; contributed to the conception and data acquisition, Y. P, contributed to methodology, data acquisition, and analysis, drafted the manuscript; and G.M.D, contributed to data interpretation, critically revised the manuscript, and S. D, contributed to data analysis. All authors have read and agreed to the published version of the manuscript.

## 7. Funding

This research was funded by the South African Medical Research Council (SAMRC), with funds from National Treasury under its Economic Competitiveness and Support Package (MRC-RFA-UFSP-01-2013/VMH Study) and South African National Research Foundation (SANRF) (Grant No. 115450).

## 8. Acknowledgments

Thank you to the Bellville South Ward 009 community for partaking in the study as well as the Bellville South Community Health Forum for supporting the engagement with the Bellville South Community.

## 9. References


Alberti, K.G.M., Eckel, R.H., Grundy, S.M., *et al*. 2009. Harmonizing the metabolic syndrome: A joint interim statement of the international diabetes federation task force on epidemiology and prevention; National heart, lung, and blood institute; American heart association; World heart federation; International . *Circulation*, 120(16): 1640–1645.

Andersen, C.J., Murphy, K.E. & Fernandez, M.L. 2016. Impact of obesity and metabolic syndrome on immunity. *Adv Nurt* 7(1): 66–75.

Arimatsu, K., Yamada, H., Miyazawa, H., Minagawa, T., Nakajima, M., Ryder, M.I., Gotoh, K., Motooka, D., Nakamura, S., Iida, T. & Yamazaki, K. 2014. Oral pathobiont induces systemic inflammation and metabolic changes associated with alteration of gut microbiota. *Sci Rep*, 4: 1–9.

Beutler, J., Jentsch, H.F.R., Rodloff, A.C. & Stingu, C.S. 2019. Bacteremia after professional mechanical plaque removal in patients with chronic periodontitis. *Oral Dis*, 25(4): 1185–1194.

Buduneli, N. 2020. Biomarkers in Periodontal Health and Disease. *Biomarkers in Periodontal Health and Disease*: 25–36.

Casarin, R.C.V., Barbagallo, A., Meulman, T., Santos, V.R., Sallum, E.A., Nociti, F.H., Duarte, P.M., Casati, M.Z. & Gonçalves, R.B. 2013. Subgingival biodiversity in subjects with uncontrolled type-2 diabetes and chronic periodontitis. *J Periodontal Res*, 48(1): 30–36.

Chistiakov, D.A., Orekhov, A.N. & Bobryshev, Y. V. 2016. Links between atherosclerotic and periodontal disease. *Exp Mol Pathol* 100(1): 220–235. http://dx.doi.org/10.1016/j.yexmp.2016.01.006.

Chukkapalli, S.S., Velsko, I.M., Rivera-Kweh, *et al*. 2015. Polymicrobial oral infection with four periodontal bacteria orchestrates a distinct inflammatory response and atherosclerosis in ApoEnull mice. *PLoS ONE*, 10(11): 1–17.

Dewhirst, F.E., Chen, T., Izard, J., Paster, B.J., Tanner, A.C.R., Yu, W.H., Lakshmanan, A. & Wade, W.G. 2010. The human oral microbiome. *J Bacteriol*, 192(19): 5002–5017.

Enberg, N., Alho, H., Loimaranta, V. & Lenander-Lumikari, M. 2001. Saliva flow rate, amylase activity, and protein and electrolyte concentrations in saliva after acute alcohol consumption. *Oral Surg, Oral Med, Oral Pathol, Oral Radiol, and Endod*, 92(3): 292–298.

Feres, F Teles, R. Teles, L. C Figueiredo, M.F. 2017. The subgingival periodontal microbiota

in the aging mouth Magda. *Physiology & behavior*, 176(1): 139–148.

Ferroni, P., Roselli, M., Riondino, S., Cavaliere, F. & Guadagni, F. 2016. Insulin resistance as a predictor of venous thromboembolism in breast cancer. *Endocr-Relat Cancer*, 23(6): L25–L28.

Caton, G ., Armitage, J., Berglundh, G.; *et al* . 2018. A new classification scheme for periodontal and peri-implant diseases and conditions – Introduction and key changes from the 1999 classification. *J Clin Periodontol*, 45(March): S1–S8.

Gao, L., Xu, T., Huang, G., Jiang, S., Gu, Y. & Chen, F. 2018. Oral microbiomes : more and more importance in oral cavity and whole body. *Protein & Cell*, 9(5): 488–500. https://doi.org/10.1007/s13238-018-0548-1.

Global Adult Tobacco Survey Collaborative Group. 2011. Tobacco Questions for Surveys: A Subset of Key Questions from the Global Adult Tobacco Surveys. http://www.who.int/tobacco/surveillance/en_tfi_tqs.pdf.

Grice, E.A. & Segre, J.A. 2013. The skin microbiome. *Nat Rev Microbiol*, 9(4): 244–253.

Griffen, A.L., Beall, C.J., Campbell, J.H., Firestone, N.D., Kumar, P.S., Yang, Z.K., Podar, M. & Leys, E.J. 2012. Distinct and complex bacterial profiles in human periodontitis and health revealed by 16S pyrosequencing. *ISME Journal*, 6(6): 1176–1185. http://dx.doi.org/10.1038/ismej.2011.191.

Haffajee, A.D., Socransky, S.S., Patel, M.R. & Song, X. 2008. Microbial complexes in supragingival plaque. *Oral Microbiol Immunol*, 23(3): 196–205.

Han Yiping. 2015. Fusobacterium nucleatum: a commensal truned pathogen. *Curr Opin Microbiol*, 1(23): 141–147.

Hanioka, T., Morita, M., Yamamoto, T., *et al*. 2019. Smoking and periodontal microorganisms. *Jpn Dent Sci Rev*, 55(1): 88–94. https://doi.org/10.1016/j.jdsr.2019.03.002.

Haririan, H., Andrukhov, O., Bertl, K., *et al*. 2014. Microbial Analysis of Subgingival Plaque Samples Compared to That of Whole Saliva in Patients With Periodontitis. *J Periodontol*, 85(6): 819–828.

Henne, K., Fuchs, F., Kruth, S., Horz, H.P. & Conrads, G. 2014. Shifts in Campylobacter species abundance may reflect general microbial community shifts in periodontitis progression. *J Oral Microbiol*, 6(1): 6–11.

Homann, N., Tillonen, J., Meurman, J.H., Rintamäki, H., Lindqvist, C., Rautio, M., Jousimies-Somer, H. & Salaspuro, M. 2000. Increased salivary acetaldehyde levels in heavy drinkers and smokers: A microbiological approach to oral cavity cancer. *Carcinogenesis*, 21(4): 663–668.

Huang, C. & Shi, G. 2019a. Smoking and microbiome in oral, airway, gut and some systemic diseases. *J Transl Med*, 17(1): 1–15. https://doi.org/10.1186/s12967-019-1971-7.

Janket, S.J., Baird, A.E., Chuang, S.K. & Jones, J.A. 2003. Meta-analysis of periodontal disease and risk of coronary heart disease and stroke. *Oral Surg, Oral Med, Oral Pathol, Oral Radiol, Endod*, 95(5): 559–569.

Jansson, L. 2008. Association between alcohol consumption and dental health. *J Clin Periodontol*, 35(5): 379–384.

Ji, S., Kim, Y., Min, B.M., Han, S.H. & Choi, Y. 2007. Innate immune responses of gingival epithelial cells to nonperiodontopathic and periodontopathic bacteria. *J Periodontal Res*, 42(6): 503–510.

Jiang, Y., Zhou, X., Cheng, L. & Li, M. 2020. The Impact of Smoking on Subgingival Microflora: From Periodontal Health to Disease. *Front Microbiol*, 11(January): 1–13.

Kantorski, K.Z., de Souza, D.M., Yujra, V.Q., Junqueira, J.C., Jorge, A.O.C. & da Rocha, R.F. 2007. Effect of an alcoholic diet on dental caries and on Streptococcus of the mutans group: Study in rats. *Braz Oral Res*, 21(2): 101–105.

Karabudak, S., Ari, O., Durmaz, B., Dal, T., Basyigit, T., Kalcioglu, M.T. & Durmaz, R. 2019. Analysis of the effect of smoking on the buccal microbiome using next-generation sequencing technology. *J Med Microbiol*, 68(8): 1148–1158.

Kato, I., Vasquez, A.A., Moyerbrailean, G., *et al*. 2016. Oral microbiome and history of smoking and colorectal cancer. *J Epidemiol Res*, 2(2): 92–101.

Keijser, B.J.F., Zaura, E., Huse, S.M., et al. 2008. Pyrosequencing analysis of the oral microflora of healthy adults. *J Dent Res*, 87(11): 1016–1020.

Kengne, A.P., Erasmus, R.T., Levitt, N.S. & Matsha, T.E. 2017. Alternative indices of glucose homeostasis as biochemical diagnostic tests for abnormal glucose tolerance in an African setting. *Prim Care Diabetes*, 11(2): 119–131. http://dx.doi.org/10.1016/j.pcd.2017.01.004.

Khader, Y.S., Albashaireh, Z.S.M. & Alomari, M.A. 2004. Periodontal Diseases and the Risk of Coronary Heart and Cerebrovascular Diseases: A Meta-Analysis. *J Periodontol*, 75(8): 1046–1053.

Kocher, T., König, J., Borgnakke, W.S., Pink, C. & Meisel, P. 2018. Periodontal complications of hyperglycemia/diabetes mellitus: Epidemiologic complexity and clinical challenge. *Periodontol 2000*, 78(1): 59–97.

Krisanaprakornkit, S., Kimball, J.R., Weinberg, A., et al. 2000. Inducible expression of human β-defensin 2 by Fusobacterium nucleatum in oral epithelial cells: Multiple signaling pathways and role of commensal bacteria in innate immunity and the epithelial barrier. *Infect Immun*, 68(5): 2907–2915.

Kumar, P.S., Matthews, C.R., Joshi, V., de Jager, M. & Aspiras, M. 2011a. Tobacco smoking affects bacterial acquisition and colonization in oral biofilms. *Infect Immun* 79(11): 4730–4738.

Lamster, I.B. & Pagan, M. 2017. Periodontal disease and the metabolic syndrome. *Int Dent J*. 67(2): 67–77.

Linden, G.J., Lyons, A. & Scannapieco, F.A. 2013. Periodontal systemic associations : review of the evidence. , 84: 8–19.

Lõivukene, K., Pähkla, E.R., Koppel, T., Saag, M. & Naaber, P. 2005. The microbiological status of patients with periodontitis in southern Estonia after non-surgical periodontal therapy. 7(2): 45–47.

Long, J., Cai, Q., Steinwandel, M., Hargreaves, M.K., Bordenstein, S.R., Blot, W.J., Zheng, W. & Shu, X.O. 2017. Association of oral microbiome with type 2 diabetes risk. *J Periodontal Res*, 52(3): 636–643.

Lu, M., Xuan, S. & Wang, Z. 2019. Oral microbiota: A new view of body health. *Food Science and Human Wellness*, 8(1): 8–15. https://doi.org/10.1016/j.fshw.2018.12.001.

M. Kilian, Chapple, I.L., Hannig, M., et al. 2016. The oral microbiome - An update for oral healthcare professionals. *Br Dent J*, 221(10): 657–666.

Macuch, P.J. & Tanner, A.C.R. 2000. Campylobacter species in health, gingivitis, and periodontitis. *J Dent Res* 79(2): 785–792.

Mager, D.L., Ximenez-Fyvie, L.A., Haffajee, A.D. & Socransky, S.S. 2003. Distribution of selected bacterial species on intraoral surfaces. *J Clin Periodontol*, 30(7): 644–654.

Mason, M.R., Preshaw, P.M., Nagaraja, H.N., Dabdoub, S.M., Rahman, A. & Kumar, P.S. 2015a. The subgingival microbiome of clinically healthy current and never smokers. *ISME Journal*, 9(1): 268–272.

Mason, M.R., Preshaw, P.M., Nagaraja, H.N., Dabdoub, S.M., Rahman, A. & Kumar, P.S. 2015b. The subgingival microbiome of clinically healthy current and never smokers. *ISME Journal*, 9(1): 268–272. http://dx.doi.org/10.1038/ismej.2014.114.

Matsha, T.E., Hassan, M.S., Hon, G.M., Soita, D.J., Kengne, A.P. & Erasmus, R.T. 2013. Derivation and validation of a waist circumference optimal cutoff for diagnosing metabolic syndrome in a South African mixed ancestry population. *Int J Cardiol*, 168(3): 2954–2955. http://dx.doi.org/10.1016/j.ijcard.2013.03.150.

Matsha, T.E., Prince, Y., Davids, S., *et al.* 2020. Oral Microbiome Signatures in Diabetes Mellitus and Periodontal Disease. *J Dent Res*, 99(6): 658–665. https://doi.org/10.1177/0022034520913818.

McCracken, E., Monaghan, M. & Sreenivasan, S. 2018. Pathophysiology of the metabolic syndrome. *Clin Dermatol*, 36(1): 14–20. http://dx.doi.org/10.1016/j.clindermatol.2017.09.004.

McLean, J.S. 2014. Advancements toward a systems level understanding of the human oral microbiome. *Front Cell Infect Microbiol*, 4(JUL): 1–13.

Medikeri, R.S., Lele, S.V., Jain, P.M., Mali, P. & Medikeri, M.R. 2015. Quantification of selenomonas sputigena in chronic periodontitis in smokers using 16S rDNA based PCR analysis. *J Clin Diagn Res*, 9(4): ZC13–ZC17.

Moon, J.H., Lee, J.H. & Lee, J.Y. 2015a. Subgingival microbiome in smokers and non-smokers in Korean chronic periodontitis patients. *Mol Oral Microbiol*, 30(3): 227–241.

Ogawa, T., Honda-Ogawa, M., Ikebe, K., *et al.* 2017. Erratum to 'Characterizations of oral microbiota in elderly nursing home residents with diabetes'. J *Oral Sci*, 60(1): 163.

Ohlrich, E.J., Cullinan, M.P. & Leichter, J.W. 2010. Diabetes, periodontitis, and the subgingival microbiota. *J Oral Microbiol*, 2(2010): 1–8.

Pirkle, J.L., Flegal, K.M., Bernert, J.T., Brody, D.J., Etzel, R.A. & Maurer, K.R. 1996. Exposure of the US population to environmental tobacco smoke: The Third National Health and Nutrition Examination Survey, 1988 to 1991. *JAMA*, 275(16): 1233–1240.

Preza, D., Olsen, I., Willumsen, T., *et al.* 2009. Microarray analysis of the microflora of root caries in elderly. *Eur J Clin Microbiol Infect Dis*, 28(5): 509–517.

Reddy, P., Zuma, K., Shisana, O.,et al. 2015. Prevalence of tobacco use among adults in South Africa : Results from the first South African National Health and Nutrition Examination Survey. , 105(8).

Saeb, A.T.M., Al-Rubeaan, K.A., Aldosary, K., *et al*. 2019. Relative reduction of biological and phylogenetic diversity of the oral microbiota of diabetes and pre-diabetes patients. *Microb Pathog*, 128(January): 215–229. https://doi.org/10.1016/j.micpath.2019.01.009.

Sapkota, A.R., Berger, S., Vogel, T.M., Reynolds, C.R.J. & Co, T. 2010. Human Pathogens Abundant in the Bacterial Metagenome of Cigarettes. *Environ Health Perspect*, 118(3): 351–356.

Sato, T., Watanabe, K., Kumada, H., *et al.* 2012. Peptidoglycan of Actinomyces naeslundii induces inflammatory cytokine production and stimulates osteoclastogenesis in alveolar bone resorption. *Arch Oral Biol*, 57(11): 1522–1528. http://dx.doi.org/10.1016/j.archoralbio.2012.07.012.

Sharma, N., Bhatia, S., Singh Sodhi, A. & Batra, N. 2018. Oral microbiome and health. *AIMS Microbiology*, 4(1): 42–66. http://www.aimspress.com/article/10.3934/microbiol.2018.1.42.

Sharma, N., Bhatia, S., Sodhi, A.S. & Batra, N. 2018. Oral microbiome and health. *AIMS Microbiol,* 4(1): 42–66. http://www.aimspress.com/journal/microbiology.

Shchipkova, A.Y., Nagaraja, H.N. & Kumar, P.S. 2010. Subgingival microbial profiles of smokers with periodontitis. *J Dent Res*, 89(11): 1247–1253.

Shepherd, S. 2011. Alcohol consumption a risk factor for periodontal disease. *Evid Based Dent*, 12(3): 76. http://dx.doi.org/10.1038/sj.ebd.6400808.

Si, J., Lee, C. & Ko, G. 2017. Oral Microbiota: Microbial Biomarkers of Metabolic Syndrome Independent of Host Genetic Factors. *Front Cell Infect Microbiol*, 7(December): 1–11. http://journal.frontiersin.org/article/10.3389/fcimb.2017.00516/full.

Signat, B., Roques, C., Poulet, P. & Duffaut, D. 2011. Role of Fusobacterium nucleatum in periodontal health and disease. *Curr Issues Mol Biol*, 13(2): 25–36.

Signoretto, C., Bianchi, F., Burlacchini, G., Sivieri, F., Spratt, D. & Canepari, P. 2010. Drinking habits are associated with changes in the dental plaque microbial community. *J Clin Microbiol*, 48(2): 347–356.

Slattery, M.L., Hunt, S.C., French, T.K., Ford, M.H. & Williams, R.R. 1989. Validity of cigarette smoking habits in three epidemiologic studies in Utah. *Prev Med*, 18(1): 11–19.

Tefiku, U., Popovska, M., Cana, A., *et al*. 2020. Determination of the Role of Fusobacterium Nucleatum in the Pathogenesis in and Out the Mouth. *Prilozi*, 41(1): 87–99.

Ursell, L.K., Metcalf, J.L., Parfrey, L.W. & Knight, R. 2012. Defining the human microbiome. *Nut Rev*, 70(SUPPL. 1).

Valour, F., Sénéchal, A., Dupieux, C., *et al.* 2014. Actinomycosis: Etiology, clinical features, diagnosis, treatment, and management. *Infect Drug Resist*, 7: 183–197.

Vielkind, P., Jentsch, H., Eschrich, K., Rodloff, A.C. & Stingu, C.S. 2015. Prevalence of Actinomyces spp. in patients with chronic periodontitis. *Int J Med Microbiol*, 305(7): 682–688. http://dx.doi.org/10.1016/j.ijmm.2015.08.018.

Wade, W.G. 2013. The oral microbiome in health and disease. *Pharmacol Res*, 69(1): 137–143. http://dx.doi.org/10.1016/j.phrs.2012.11.006.

Willis, J.R. & Gabaldón, T. 2020. The human oral microbiome in health and disease: From sequences to ecosystems. *Microorganisms*, 8(2): 1–28.

World Health Organization. 2019. *Report on Global Tobacco Epidemic*. https://apps.who.int/iris/bitstream/handle/10665/326043/9789241516204-eng.pdf?ua=1&ua=1.

Wu, J., Li, M. & Huang, R. 2019. The effect of smoking on caries-related microorganisms. *Tob Induc Dis*, 17(April).

Wu, J., Peters, B.A., Dominianni, C., *et al.* 2016. Cigarette smoking and the oral microbiome in a large study of American adults. *The ISME Journal*, 10: 2435–2446. www.nature.com/ismej 27 February 2020.

Yost, S., Duran-Pinedo, A.E., Teles, R., Krishnan, K. & Frias-Lopez, J. 2015. Functional signatures of oral dysbiosis during periodontitis progression revealed by microbial metatranscriptome analysis. *Genome Med*, 7(1): 1–19.

Yu, Q., Huang, S., Xu, T.T., Wang, Y.C. & Ju, S. 2020. Measuring Brown Fat Using MRI and Implications in the Metabolic Syndrome. *J Magn Reson Imaging*.

Zhong, E.F., Chang, A., Stucky, A., Chen, X., Mundluru, T., Khalifeh, M. & Sedghizadeh, P.P. 2020. Genomic analysis of oral lichen planus and related oral microbiome pathogens. *Pathogens*, 9(11): 1–13.

## 10. Figures and Legends

**Table1.** General classification of participants according to the JIS classification.

| | JIS=No (n=66) | JIS=Yes (n=62) | P-Value |
|---|---|---|---|
| Age (years) | 44.24(14.55) | 49.98(10.45) | 0.012 |
| BMI ((kg/m2) | 25.55(6.12) | 37.82(8.72) | <0.001 |
| Waist-C (cm) | 80.70(12.63) | 110.5(14.69) | <0.001 |
| Hip (cm) | 92.07(13.00) | 119.3(18.03) | <0.001 |
| SBP (mmHg) | 126.1(23.46) | 133.3(22.41) | 0.079 |
| DBP (mmHg) | 81.80(14.60) | 87.24(12.32) | 0.025 |
| Glucose Fasting Blood (mmolL) | 4.90(4.50;5.40) | 7.30(6.15;11.05) | <0.001 |
| Glucose2HRsPostPrandial (mmolL) | 6.95(5.20;8.80) | 11.80(9.90;15.50) | <0.001 |
| GlycatedHBHbA1cTrial (%) | 5.50(5.28;5.93) | 7.30(6.58;9.30) | <0.001 |
| Insulin Fasting (mIUL) | 5.00(3.40;8.40) | 15.90(8.15;22.40) | <0.001 |
| Insulin120Minutes (mIUL) | 32.10(19.18;64.15) | 67.50(39.10;110.30) | 0.003 |
| TriglyceridesS (mmolL) | 1.03(0.77;1.32) | 1.63(1.25;2.19) | <0.001 |
| LDLCholesterol (mmolL) | 2.65(2.20;3.23) | 3.40(3.00;4.15) | <0.001 |
| CholesterolHDLS (mmolL) | 1.50(1.20;1.80) | 5.20(4.70;6.13) | <0.001 |
| CholesterolS (mmolL) | 4.90(4.10;5.53) | 4.90(4.10;5.53) | 0.059 |
| CRP (mgL) | 2.24(1.38;5.72) | 7.19(3.70;15.18) | <0.001 |
| Gamma GTS (IUL) | 32.50(21.00;54.00) | 34.00(25.75;65.25) | 0.228 |
| | | | |
| **Gender** | | | 0.049 |
| Female (n=93) | 43(65%) | 50(81%) | |
| Male (n=35) | 23(35%) | 12(19%) | |
| | | | |
| **Gingival bleeding** | | | 0.964 |
| No (n=44) | 23(35%) | 21 (35%) | |
| Yes (n=81) | 42(65%) | 39 (65) | |
| | | | |
| **Periodontitis** | | | 0.499 |
| No (n=56) | 31(48%) | 25(42%) | |
| Yes (69) | 34(52%) | 35(58%) | |

**Table2.** Alpha diversity in species indices according to Metabolic Syndrome Status.

| | Metabolic Syndrome | |
|---|---|---|
| | **No** | **Yes** |
| Number of taxa | 253 | 275 |
| Shannon | 4.207 | 4.239 |
| Chao1 | 253 | 275 |
| Simpson | 0.0296 | 0.0297 |

**Table 3.** Genus and Species associated with MetS and Periodontal status.

| | MetS | | | Periodontitis | | | Bleeding | | |
|---|---|---|---|---|---|---|---|---|---|
| | **No** | **Yes** | **p-value** | **No** | **Yes** | **p-value** | **No** | **Yes** | **p-value** |
| *Actinomyces dentalis* | 1.59 | 2.80 | <0.001 | 1.79 | 2.54 | 0.117 | 2.07 | 2.28 | 0.513 |
| *Actinomyces naeslundii* | 1.48 | 4.03 | <0.001 | 2.58 | 2.15 | 0.166 | 2.93 | 1.99 | 0.056 |
| *Actinomyces odontolyticus* | 1.04 | 0.67 | 0.005 | | | | | | |
| *Actinomyces viscosus* | 0.27 | 1.58 | 0.021 | | | | | | |
| *Aggregatibacter segnis* | 2.52 | 1.81 | 0.174 | 2.13 | 2.08 | 0.438 | 1.33 | 2.56 | 0.152 |
| *Campylobacter gracilis* | 3.71 | 2.22 | 0.002 | 2.94 | 3.07 | 0.984 | 3.35 | 2.81 | 0.123 |
| *Capnocytophaga leadbetteri* | 1.34 | 1.01 | 0.219 | 1.02 | 1.23 | 0.607 | 1.12 | 1.14 | 0.802 |
| *Corynebacterium matruchotii* | 2.97 | 7.91 | <0.001 | 5.94 | 5.07 | 0.281 | 5.72 | 5.31 | 0.706 |
| *Fusobacterium canifelinum* | 1.22 | 0.72 | 0.001 | 1.13 | 0.81 | 0.187 | | | |
| *Fusobacterium nucleatum* | 7.09 | 4.62 | <0.001 | 6.23 | 5.68 | 0.447 | 4.49 | 6.78 | 0.001 |
| *Fusobacterium periodonticum* | 1.68 | 0.38 | 0.022 | 0.95 | 1.17 | 0.328 | 0.72 | 1.27 | 0.249 |
| *Granulicatella adiacens* | | | | | | | 1.13 | 0.69 | 0.016 |
| *Haemophilus parainfluenzae* | 10.10 | 3.94 | <0.001 | 7.78 | 6.75 | 0.712 | 9.08 | 6.11 | 0.051 |
| *Leptotrichia buccalis* | 1.45 | 2.51 | 0.007 | 1.52 | 2.38 | 0.356 | 2.41 | 1.75 | 0.592 |
| *Leptotrichia genomosp.* | 0.97 | 1.84 | 0.183 | 1.12 | 1.63 | 0.538 | 1.39 | 1.41 | 0.329 |
| *Leptotrichia hofstadii* | 0.49 | 1.01 | 0.184 | | | | 1.06 | 0.58 | 0.343 |
| *Leptotrichia hongkongensis* | | | | 1.33 | 0.47 | 0.261 | 1.47 | 0.49 | 0.236 |
| *Leptotrichia wadei* | 1.23 | 1.43 | 0.327 | 1.18 | 1.49 | 0.180 | 1.24 | 1.42 | 0.495 |
| *Mannheimia varigena* | 3.54 | 2.03 | 0.075 | 2.86 | 2.80 | 0.150 | 2.99 | 2.74 | 0.462 |
| *Neisseria flavescens* | 1.19 | 0.57 | 0.835 | | | | 0.57 | 1.02 | 0.036 |
| *Other** | 38.22 | 40.17 | 0.118 | 41.21 | 41.56 | 0.646 | 38.50 | 40.62 | 0.528 |
| *Prevotella histicola* | 0.39 | 1.00 | 0.225 | | | | | | |
| *Prevotella maculosa* | | | | 1.00 | 0.91 | 0.763 | 1.03 | 0.90 | 0.197 |
| *Prevotella melaninogenica* | 3.71 | 4.13 | 0.405 | 4.09 | 3.92 | 0.514 | 2.94 | 4.62 | 0.124 |
| *Prevotella oris* | 0.73 | 1.34 | 0.160 | 1.09 | 1.02 | 0.227 | 1.37 | 0.86 | 0.059 |
| *Prevotella oulorum* | 1.32 | 0.75 | 0.069 | 1.06 | 1.06 | 0.255 | 1.50 | 0.80 | 0.326 |
| *Prevotella pallens* | 1.39 | 0.90 | 0.078 | 1.11 | 1.18 | 0.983 | 0.99 | 1.24 | 0.722 |
| *Prevotella veroralis* | 1.17 | 1.09 | 0.818 | 1.23 | 1.08 | 0.787 | 0.68 | 1.43 | 0.084 |
| *Selenomonas noxia* | 1.19 | 1.04 | 0.534 | 0.92 | 1.30 | 0.273 | 1.55 | 0.88 | 0.001 |
| *Streptococcus gordonii* | 1.03 | 0.67 | 0.339 | | | | | | |
| *Streptococcus mutans* | | | | 0.33 | 1.39 | 0.269 | 1.08 | 0.81 | 0.836 |
| *Streptococcus sanguinis* | 0.43 | 1.56 | <0.001 | 1.35 | 0.65 | 0.209 | 1.76 | 0.50 | 0.007 |
| *Veillonella alcalescens* | 3.96 | 4.37 | 0.954 | 3.94 | 4.43 | 0.414 | 3.62 | 4.56 | 0.541 |
| *Veillonella parvula* | 1.08 | 0.94 | 0.140 | 1.03 | 1.02 | 0.933 | 0.92 | 1.08 | 0.919 |
| *Veillonella rogosae* | 1.37 | 0.89 | 0.007 | 1.13 | 1.16 | 0.757 | 0.87 | 1.31 | 0.037 |

*The number of others depends on the genus/species count and/or relative abundance

**Table 4.** Correlation table of Genus and species of and impact of metabolic parameters

| | Age | | Weight | | BMI | | Waist | | Hip | | WHR | | SBP | | DBP | | FBG | | 2hr BG | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | r | p-value | r | p-value | r | p-value | r | p-value | r | p-value | r | p-value | r | p-value | r | p-value | r | p-value | r | p-value |
| *Actinomyces dentalis* | 0.218 | 0.014 | 0.347 | <0.001 | 0.305 | 0.001 | 0.448 | <0.001 | 0.397 | <0.001 | 0.206 | 0.021 | 0.093 | 0.298 | 0.064 | 0.479 | 0.293 | 0.001 | 0.426 | <0.001 |
| *Actinomyces naeslundii* | 0.200 | 0.024 | 0.465 | <0.001 | 0.445 | <0.001 | 0.456 | <0.001 | 0.426 | <0.001 | 0.140 | 0.120 | -0.042 | 0.639 | -0.104 | 0.246 | 0.300 | 0.001 | 0.345 | 0.001 |
| *Actinomyces odontolyticus* | -0.023 | 0.803 | -0.250 | 0.006 | -0.244 | 0.008 | -0.291 | 0.001 | -0.266 | 0.003 | -0.121 | 0.191 | -0.021 | 0.817 | -0.073 | 0.431 | -0.240 | 0.008 | -0.312 | 0.003 |
| *Actinomyces viscosus* | -0.049 | 0.618 | 0.304 | 0.002 | 0.301 | 0.002 | 0.284 | 0.003 | 0.307 | 0.001 | 0.075 | 0.445 | -0.131 | 0.179 | -0.026 | 0.789 | 0.113 | 0.248 | 0.175 | 0.122 |
| *Aggregatibacter segnis* | -0.095 | 0.313 | -0.180 | 0.057 | -0.118 | 0.213 | -0.169 | 0.073 | -0.100 | 0.290 | -0.253 | 0.007 | 0.128 | 0.172 | 0.075 | 0.423 | -0.096 | 0.307 | -0.157 | 0.152 |
| *Campylobacter gracilis* | -0.143 | 0.109 | -0.194 | 0.030 | -0.072 | 0.424 | -0.243 | 0.006 | -0.230 | 0.009 | -0.085 | 0.346 | -0.161 | 0.071 | -0.164 | 0.065 | -0.328 | <0.001 | -0.249 | 0.016 |
| *Capnocytophaga leadbetteri* | 0.013 | 0.886 | -0.078 | 0.397 | -0.091 | 0.323 | -0.079 | 0.389 | -0.096 | 0.297 | -0.007 | 0.936 | 0.141 | 0.123 | 0.146 | 0.111 | -0.005 | 0.960 | 0.033 | 0.754 |
| *Corynebacterium matruchotii* | 0.339 | <0.001 | 0.431 | <0.001 | 0.372 | <0.001 | 0.495 | <0.001 | 0.445 | <0.001 | 0.195 | 0.035 | 0.144 | 0.120 | 0.117 | 0.206 | 0.390 | <0.001 | 0.445 | <0.001 |
| *Fusobacterium canifelinum* | -0.234 | 0.009 | -0.257 | 0.004 | -0.226 | 0.012 | -0.305 | 0.001 | -0.286 | 0.001 | -0.168 | 0.062 | -0.056 | 0.536 | 0.046 | 0.609 | -0.300 | 0.001 | -0.174 | 0.093 |
| *Fusobacterium nucleatum* | -0.336 | <0.001 | -0.404 | <0.001 | -0.410 | <0.001 | -0.476 | <0.001 | -0.434 | <0.001 | -0.269 | 0.002 | -0.061 | 0.491 | 0.036 | 0.685 | -0.299 | 0.001 | -0.345 | 0.001 |
| *Fusobacterium periodonticum* | -0.067 | 0.500 | -0.263 | 0.008 | -0.290 | 0.003 | -0.279 | 0.005 | -0.255 | 0.010 | -0.187 | 0.060 | 0.078 | 0.435 | 0.046 | 0.644 | -0.080 | 0.423 | -0.230 | 0.043 |
| *Granulicatella adiacens* | -0.107 | 0.242 | -0.023 | 0.800 | -0.031 | 0.736 | -0.118 | 0.199 | -0.128 | 0.163 | 0.031 | 0.736 | 0.031 | 0.738 | -0.026 | 0.776 | -0.212 | 0.019 | -0.148 | 0.160 |
| *Haemophilus parainfluenzae* | -0.132 | 0.161 | -0.209 | 0.027 | -0.192 | 0.043 | -0.301 | 0.001 | -0.237 | 0.011 | -0.151 | 0.111 | 0.009 | 0.924 | 0.039 | 0.684 | -0.336 | <0.001 | -0.287 | 0.008 |
| *Leptotrichia buccalis* | 0.086 | 0.363 | 0.266 | 0.005 | 0.212 | 0.025 | 0.323 | <0.001 | 0.292 | 0.002 | 0.110 | 0.248 | 0.134 | 0.155 | 0.146 | 0.120 | 0.348 | <0.001 | 0.230 | 0.035 |
| *Leptotrichia genomosp.* | 0.196 | 0.045 | 0.090 | 0.364 | 0.120 | 0.227 | 0.175 | 0.075 | 0.163 | 0.097 | 0.122 | 0.216 | 0.065 | 0.512 | 0.062 | 0.532 | 0.200 | 0.041 | 0.296 | 0.009 |
| *Leptotrichia hofstadii* | 0.035 | 0.724 | 0.171 | 0.086 | 0.121 | 0.224 | 0.154 | 0.120 | 0.119 | 0.231 | 0.098 | 0.324 | 0.036 | 0.716 | 0.037 | 0.710 | 0.109 | 0.269 | 0.241 | 0.037 |
| *Leptotrichia hongkongensis* | 0.046 | 0.654 | 0.142 | 0.165 | 0.166 | 0.104 | 0.133 | 0.193 | 0.077 | 0.451 | 0.118 | 0.248 | -0.084 | 0.411 | -0.102 | 0.317 | -0.148 | 0.145 | 0.014 | 0.905 |
| *Leptotrichia wadei* | 0.221 | 0.017 | 0.128 | 0.176 | 0.153 | 0.106 | 0.181 | 0.055 | 0.150 | 0.111 | 0.106 | 0.263 | 0.078 | 0.408 | -0.011 | 0.906 | 0.032 | 0.732 | 0.285 | 0.008 |
| *Mannheimia varigena* | -0.088 | 0.340 | -0.054 | 0.562 | -0.025 | 0.789 | -0.112 | 0.223 | -0.075 | 0.415 | -0.082 | 0.376 | 0.048 | 0.602 | 0.068 | 0.459 | -0.187 | 0.040 | -0.144 | 0.170 |
| *Neisseria flavescens* | 0.075 | 0.459 | -0.015 | 0.881 | -0.043 | 0.672 | -0.001 | 0.995 | -0.013 | 0.902 | -0.020 | 0.841 | 0.016 | 0.873 | -0.044 | 0.661 | 0.006 | 0.951 | 0.111 | 0.339 |
| *Prevotella histicola* | -0.047 | 0.650 | 0.165 | 0.111 | 0.198 | 0.055 | 0.173 | 0.094 | 0.165 | 0.109 | 0.016 | 0.876 | -0.079 | 0.448 | 0.075 | 0.473 | 0.058 | 0.575 | 0.015 | 0.902 |
| *Prevotella maculosa* | 0.002 | 0.982 | 0.083 | 0.364 | 0.108 | 0.236 | 0.054 | 0.552 | 0.061 | 0.503 | -0.031 | 0.731 | -0.137 | 0.129 | -0.120 | 0.183 | -0.057 | 0.526 | 0.086 | 0.410 |
| *Prevotella melaninogenica* | 0.030 | 0.747 | -0.132 | 0.151 | -0.135 | 0.141 | -0.101 | 0.270 | -0.066 | 0.470 | -0.036 | 0.698 | -0.049 | 0.594 | -0.003 | 0.972 | 0.031 | 0.736 | -0.072 | 0.502 |
| *Prevotella oris* | 0.234 | 0.012 | 0.170 | 0.073 | 0.161 | 0.090 | 0.170 | 0.072 | 0.167 | 0.078 | -0.007 | 0.941 | 0.062 | 0.515 | 0.000 | 0.996 | 0.042 | 0.655 | 0.267 | 0.015 |
| *Prevotella oulorum* | -0.058 | 0.539 | 0.014 | 0.884 | 0.058 | 0.538 | -0.029 | 0.759 | -0.013 | 0.894 | -0.064 | 0.497 | -0.181 | 0.052 | -0.240 | 0.009 | -0.108 | 0.248 | -0.068 | 0.535 |
| *Prevotella pallens* | 0.030 | 0.777 | -0.171 | 0.113 | -0.178 | 0.099 | -0.217 | 0.042 | -0.152 | 0.158 | -0.218 | 0.041 | 0.056 | 0.605 | 0.014 | 0.897 | -0.145 | 0.175 | -0.019 | 0.875 |
| *Prevotella veroralis* | -0.036 | 0.702 | 0.059 | 0.534 | 0.081 | 0.396 | 0.036 | 0.706 | 0.059 | 0.534 | 0.003 | 0.972 | -0.113 | 0.228 | -0.162 | 0.083 | 0.053 | 0.576 | -0.037 | 0.736 |
| *Selenomonas noxia* | 0.173 | 0.056 | 0.142 | 0.123 | 0.187 | 0.040 | 0.147 | 0.108 | 0.099 | 0.279 | 0.154 | 0.092 | -0.015 | 0.873 | -0.056 | 0.539 | -0.043 | 0.635 | 0.035 | 0.744 |
| *Streptococcus gordonii* | -0.230 | 0.010 | -0.006 | 0.948 | 0.022 | 0.809 | -0.083 | 0.366 | -0.023 | 0.798 | -0.132 | 0.147 | -0.029 | 0.746 | -0.017 | 0.849 | -0.218 | 0.016 | -0.118 | 0.264 |
| *Streptococcus mutans* | -0.126 | 0.345 | -0.069 | 0.606 | -0.109 | 0.416 | -0.149 | 0.263 | -0.105 | 0.434 | -0.066 | 0.622 | 0.001 | 0.994 | 0.104 | 0.439 | -0.154 | 0.250 | -0.216 | 0.193 |
| *Streptococcus sanguinis* | 0.252 | 0.007 | 0.462 | <0.001 | 0.436 | <0.001 | 0.376 | <0.001 | 0.362 | <0.001 | 0.144 | 0.130 | 0.112 | 0.238 | 0.093 | 0.327 | 0.189 | 0.045 | 0.367 | 0.001 |
| *Veillonella alcalescens* | -0.057 | 0.522 | 0.044 | 0.629 | 0.118 | 0.191 | -0.017 | 0.853 | 0.016 | 0.856 | -0.053 | 0.554 | -0.072 | 0.418 | -0.071 | 0.425 | -0.164 | 0.065 | -0.039 | 0.712 |
| *Veillonella parvula* | -0.201 | 0.025 | -0.109 | 0.231 | -0.093 | 0.307 | -0.148 | 0.101 | -0.140 | 0.121 | -0.110 | 0.222 | -0.279 | 0.002 | -0.224 | 0.012 | -0.249 | 0.005 | -0.313 | 0.002 |
| *Veillonella rogosae* | -0.147 | 0.099 | -0.122 | 0.176 | -0.100 | 0.266 | -0.212 | 0.017 | -0.163 | 0.068 | -0.260 | 0.003 | -0.100 | 0.263 | -0.101 | 0.260 | -0.244 | 0.006 | -0.164 | 0.114 |
| Mets Other | 0.144 | 0.105 | 0.198 | 0.026 | 0.176 | 0.049 | 0.172 | 0.054 | 0.161 | 0.071 | 0.150 | 0.093 | 0.105 | 0.238 | 0.151 | 0.090 | 0.000 | 0.999 | 0.220 | 0.032 |
| Perid_Other | 0.011 | 0.904 | 0.182 | 0.041 | 0.145 | 0.105 | 0.094 | 0.293 | 0.123 | 0.170 | 0.019 | 0.829 | 0.005 | 0.956 | 0.117 | 0.189 | 0.000 | 0.996 | 0.247 | 0.016 |
| Bleeding Other | 0.013 | 0.888 | 0.164 | 0.067 | 0.131 | 0.142 | 0.082 | 0.357 | 0.106 | 0.234 | 0.025 | 0.779 | 0.018 | 0.842 | 0.129 | 0.147 | 0.017 | 0.850 | 0.222 | 0.031 |

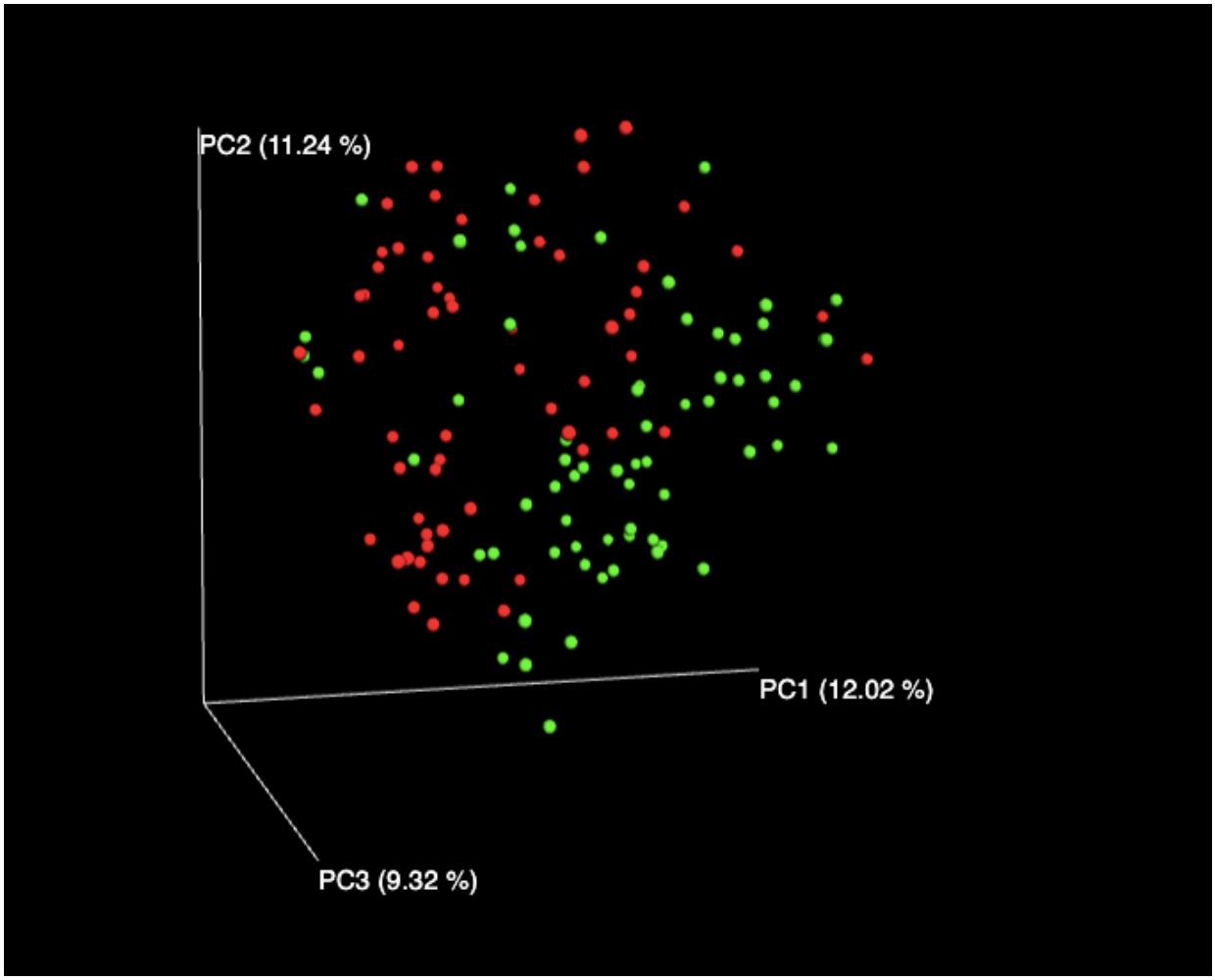
| | HBA1c | | Insulin fasting | | insulin 120 | | Trigs | | LDL | | HDL | | Total Chol | | CRP | | Cotinine | | Gamma GT | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | r | p-value | r | p-value | r | p-value | r | p-value | r | p-value | r | p-value | r | p-value | r | p-value | r | p-value | r | p-value |
| *Actinomyces dentalis* | 0.316 | <0.001 | 0.264 | 0.003 | 0.041 | 0.698 | 0.096 | 0.284 | 0.264 | 0.003 | -0.144 | 0.112 | 0.176 | 0.049 | 0.080 | 0.372 | -0.152 | 0.089 | -0.091 | 0.313 |
| *Actinomyces naeslundii* | 0.261 | 0.003 | 0.410 | <0.001 | 0.282 | 0.007 | 0.245 | 0.006 | 0.217 | 0.015 | -0.234 | 0.009 | 0.100 | 0.264 | 0.131 | 0.143 | -0.365 | <0.001 | -0.033 | 0.712 |
| *Actinomyces odontolyticus* | -0.292 | 0.001 | -0.331 | <0.001 | -0.276 | 0.010 | -0.190 | 0.038 | -0.310 | 0.001 | 0.250 | 0.006 | -0.162 | 0.077 | -0.187 | 0.041 | 0.262 | 0.004 | -0.137 | 0.137 |
| *Actinomyces viscosus* | 0.127 | 0.191 | 0.306 | 0.001 | 0.227 | 0.048 | 0.089 | 0.360 | 0.184 | 0.059 | -0.169 | 0.086 | 0.106 | 0.277 | 0.077 | 0.428 | -0.100 | 0.307 | -0.002 | 0.984 |
| *Aggregatibacter segnis* | -0.165 | 0.078 | -0.246 | 0.008 | -0.279 | 0.011 | -0.205 | 0.029 | -0.163 | 0.084 | 0.215 | 0.022 | -0.108 | 0.252 | -0.059 | 0.534 | 0.052 | 0.579 | 0.135 | 0.152 |
| *Campylobacter gracilis* | -0.194 | 0.029 | -0.123 | 0.170 | 0.040 | 0.706 | -0.172 | 0.055 | -0.162 | 0.070 | 0.018 | 0.841 | -0.161 | 0.071 | 0.128 | 0.151 | 0.314 | <0.001 | -0.068 | 0.449 |
| *Capnocytophaga leadbetteri* | -0.015 | 0.867 | -0.190 | 0.037 | -0.082 | 0.444 | -0.079 | 0.389 | 0.028 | 0.760 | 0.122 | 0.185 | 0.027 | 0.767 | -0.052 | 0.573 | -0.049 | 0.597 | -0.159 | 0.082 |
| *Corynebacterium matruchotii* | 0.394 | <0.001 | 0.420 | <0.001 | 0.256 | 0.017 | 0.236 | 0.010 | 0.341 | <0.001 | -0.192 | 0.038 | 0.207 | 0.024 | 0.116 | 0.210 | -0.389 | <0.001 | -0.103 | 0.266 |
| *Fusobacterium canifelinum* | -0.289 | 0.001 | -0.243 | 0.007 | -0.022 | 0.832 | -0.219 | 0.014 | -0.062 | 0.497 | 0.104 | 0.253 | -0.006 | 0.947 | -0.091 | 0.310 | 0.215 | 0.016 | -0.122 | 0.176 |
| *Fusobacterium nucleatum* | -0.350 | <0.001 | -0.319 | <0.001 | -0.241 | 0.020 | -0.297 | 0.001 | -0.143 | 0.109 | 0.116 | 0.196 | -0.084 | 0.347 | -0.171 | 0.054 | 0.307 | <0.001 | -0.074 | 0.409 |
| *Fusobacterium periodonticum* | -0.186 | 0.060 | -0.395 | <0.001 | -0.392 | <0.001 | -0.239 | 0.015 | -0.214 | 0.031 | 0.286 | 0.004 | -0.119 | 0.233 | -0.196 | 0.047 | 0.158 | 0.112 | 0.128 | 0.197 |
| *Granulicatella adiacens* | -0.212 | 0.019 | -0.022 | 0.814 | 0.128 | 0.231 | -0.006 | 0.945 | -0.053 | 0.564 | -0.009 | 0.920 | -0.081 | 0.377 | -0.076 | 0.408 | -0.006 | 0.946 | -0.057 | 0.532 |
| *Haemophilus parainfluenzae* | -0.393 | <0.001 | -0.224 | 0.017 | 0.191 | 0.083 | -0.128 | 0.178 | -0.134 | 0.157 | 0.250 | 0.008 | -0.045 | 0.633 | -0.227 | 0.015 | 0.123 | 0.194 | 0.136 | 0.150 |
| *Leptotrichia buccalis* | 0.290 | 0.002 | 0.215 | 0.022 | 0.041 | 0.714 | 0.156 | 0.099 | 0.176 | 0.063 | -0.010 | 0.915 | 0.150 | 0.111 | 0.079 | 0.402 | -0.172 | 0.068 | 0.010 | 0.919 |
| *Leptotrichia genomosp.* | 0.275 | 0.004 | 0.095 | 0.337 | -0.153 | 0.191 | 0.069 | 0.482 | 0.158 | 0.110 | -0.020 | 0.845 | 0.164 | 0.094 | 0.008 | 0.938 | -0.037 | 0.708 | -0.154 | 0.117 |
| *Leptotrichia hofstadii* | 0.119 | 0.228 | 0.248 | 0.012 | 0.370 | 0.001 | 0.060 | 0.546 | 0.150 | 0.130 | -0.127 | 0.203 | 0.034 | 0.729 | 0.102 | 0.303 | -0.087 | 0.381 | -0.114 | 0.251 |
| *Leptotrichia hongkongensis* | -0.037 | 0.715 | 0.135 | 0.187 | 0.379 | 0.001 | 0.182 | 0.073 | 0.119 | 0.241 | -0.079 | 0.441 | 0.104 | 0.310 | -0.046 | 0.652 | 0.104 | 0.306 | -0.023 | 0.825 |
| *Leptotrichia wadei* | 0.210 | 0.024 | 0.155 | 0.099 | 0.151 | 0.172 | 0.065 | 0.489 | 0.359 | <0.001 | -0.055 | 0.564 | 0.274 | 0.003 | 0.049 | 0.604 | -0.032 | 0.733 | -0.245 | 0.008 |
| *Mannheimia varigena* | -0.255 | 0.005 | -0.084 | 0.359 | 0.247 | 0.018 | -0.055 | 0.551 | -0.053 | 0.568 | 0.029 | 0.751 | -0.044 | 0.633 | -0.226 | 0.013 | -0.066 | 0.469 | 0.071 | 0.442 |
| *Neisseria flavescens* | -0.103 | 0.309 | -0.025 | 0.805 | 0.052 | 0.655 | -0.071 | 0.485 | 0.021 | 0.839 | 0.029 | 0.775 | 0.043 | 0.673 | -0.120 | 0.234 | -0.188 | 0.061 | 0.070 | 0.487 |
| *Prevotella histicola* | 0.212 | 0.040 | 0.183 | 0.078 | 0.100 | 0.419 | -0.059 | 0.572 | 0.074 | 0.479 | -0.071 | 0.502 | 0.008 | 0.936 | 0.212 | 0.039 | -0.041 | 0.696 | 0.149 | 0.148 |
| *Prevotella maculosa* | 0.049 | 0.589 | 0.133 | 0.144 | 0.117 | 0.268 | 0.062 | 0.498 | -0.034 | 0.705 | -0.115 | 0.208 | -0.069 | 0.443 | 0.048 | 0.595 | 0.111 | 0.218 | -0.097 | 0.285 |
| *Prevotella melaninogenica* | -0.120 | 0.187 | -0.134 | 0.143 | -0.179 | 0.098 | 0.047 | 0.610 | -0.073 | 0.424 | 0.067 | 0.467 | -0.005 | 0.957 | 0.005 | 0.952 | 0.044 | 0.634 | -0.012 | 0.899 |
| *Prevotella oris* | 0.059 | 0.530 | 0.208 | 0.027 | 0.286 | 0.010 | 0.116 | 0.219 | 0.093 | 0.327 | -0.154 | 0.105 | -0.003 | 0.978 | 0.036 | 0.705 | -0.215 | 0.021 | -0.112 | 0.234 |
| *Prevotella oulorum* | 0.012 | 0.902 | -0.024 | 0.803 | -0.067 | 0.545 | -0.143 | 0.127 | -0.009 | 0.925 | -0.011 | 0.905 | -0.009 | 0.925 | 0.191 | 0.040 | 0.203 | 0.029 | -0.115 | 0.220 |
| *Prevotella pallens* | -0.173 | 0.105 | -0.249 | 0.019 | -0.252 | 0.041 | -0.042 | 0.694 | -0.002 | 0.985 | 0.177 | 0.098 | 0.139 | 0.195 | 0.012 | 0.908 | 0.119 | 0.265 | -0.125 | 0.243 |
| *Prevotella veroralis* | 0.088 | 0.352 | 0.120 | 0.202 | -0.043 | 0.700 | 0.094 | 0.316 | -0.016 | 0.865 | -0.150 | 0.112 | -0.039 | 0.678 | -0.029 | 0.757 | -0.120 | 0.202 | -0.061 | 0.518 |
| *Selenomonas noxia* | 0.051 | 0.578 | 0.164 | 0.072 | 0.063 | 0.564 | -0.006 | 0.950 | 0.029 | 0.749 | 0.040 | 0.661 | 0.003 | 0.976 | 0.080 | 0.381 | 0.016 | 0.862 | -0.099 | 0.279 |
| *Streptococcus gordonii* | -0.155 | 0.087 | -0.099 | 0.280 | -0.038 | 0.723 | -0.099 | 0.276 | 0.029 | 0.749 | 0.140 | 0.127 | 0.080 | 0.379 | -0.054 | 0.553 | 0.203 | 0.024 | -0.215 | 0.017 |
| *Streptococcus mutans* | -0.144 | 0.282 | -0.255 | 0.055 | -0.070 | 0.680 | -0.029 | 0.827 | 0.284 | 0.032 | 0.084 | 0.533 | 0.243 | 0.066 | -0.006 | 0.967 | 0.158 | 0.238 | -0.282 | 0.032 |
| *Streptococcus sanguinis* | 0.109 | 0.250 | 0.409 | <0.001 | 0.361 | 0.001 | 0.269 | 0.004 | 0.213 | 0.024 | -0.123 | 0.198 | 0.116 | 0.222 | 0.094 | 0.321 | -0.318 | 0.001 | 0.056 | 0.556 |

| | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Veillonella alcalescens* | -0.129 | 0.148 | 0.004 | 0.964 | 0.119 | 0.258 | -0.051 | 0.567 | -0.051 | 0.570 | 0.025 | 0.783 | -0.030 | 0.736 | 0.001 | 0.993 | -0.003 | 0.976 | -0.092 | 0.305 |
| *Veillonella parvula* | -0.222 | 0.013 | -0.171 | 0.058 | -0.072 | 0.498 | -0.080 | 0.380 | -0.171 | 0.057 | 0.044 | 0.632 | -0.136 | 0.130 | -0.033 | 0.715 | 0.155 | 0.084 | -0.025 | 0.784 |
| *Veillonella rogosae* | -0.150 | 0.093 | -0.192 | 0.031 | -0.054 | 0.607 | -0.162 | 0.070 | -0.013 | 0.886 | 0.087 | 0.337 | 0.060 | 0.501 | -0.041 | 0.644 | 0.292 | 0.001 | -0.125 | 0.162 |
| Mets Other | 0.086 | 0.337 | 0.166 | 0.062 | 0.106 | 0.310 | 0.035 | 0.695 | 0.118 | 0.186 | -0.026 | 0.772 | 0.113 | 0.205 | 0.158 | 0.075 | -0.084 | 0.347 | 0.058 | 0.518 |
| Perid_Other | 0.012 | 0.897 | 0.139 | 0.118 | 0.049 | 0.638 | -0.005 | 0.954 | 0.092 | 0.305 | 0.006 | 0.950 | 0.118 | 0.183 | 0.173 | 0.051 | -0.113 | 0.205 | 0.190 | 0.031 |
| Bleeding Other | 0.032 | 0.719 | 0.137 | 0.125 | 0.033 | 0.754 | 0.010 | 0.910 | 0.100 | 0.263 | 0.001 | 0.994 | 0.123 | 0.165 | 0.156 | 0.079 | -0.082 | 0.355 | 0.140 | 0.114 |

**Table 5.** Odd ratio Genus's species vs MetS

| | Model 1 | | Model 2 | | Model 3 | | Model 4 | | Model 5 | | Model 6 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | OR (95% CI) | P-value | OR (95% CI) | P-value | OR (95% CI) | P-value | OR (95% CI) | P-value | OR (95% CI) | P-value | OR (95% CI) | P-value |
| *Actinomyces dentalis* | 3.33 (1.58; 7.03) | 0.002 | 2.14 (0.88; 5.22) | 0.094 | 2.07 (0.85; 5.04) | 0.112 | 2.07 (0.83; 5.13) | 0.117 | 2.02 (0.76; 5.34) | 0.156 | 2.00 (0.74; 5.45) | 0.174 |
| *Actinomyces naeslundii* | 2.21 (1.24; 3.95) | 0.007 | 1.10 (0.78; 1.55) | 0.584 | 0.96 (0.46; 1.99) | 0.906 | 0.89 (0.45; 1.77) | 0.744 | 0.72 (0.35; 1.45) | 0.356 | 0.72 (0.34; 1.51) | 0.382 |
| *Actinomyces odontolyticus* | 0.17 (0.03; 0.82) | 0.027 | 0.31 (0.04; 2.31) | 0.252 | 0.28 (0.04; 2.17) | 0.224 | 0.40 (0.05; 3.23) | 0.392 | 0.89 (0.08; 10.08) | 0.927 | 1.29 (0.1; 16.28) | 0.843 |
| *Actinomyces viscosus* | 5.10 (0.54; 48.32) | 0.155 | 1.18 (0.64; 2.17) | 0.594 | 0.98 (0.28; 3.46) | 0.979 | 0.92 (0.26; 3.29) | 0.897 | 1.04 (0.31; 3.51) | 0.944 | 1.11 (0.31; 4.00) | 0.876 |
| *Aggregatibacter segnis* | 0.82 (0.53; 1.25) | 0.354 | 0.93 (0.49; 1.77) | 0.820 | 0.85 (0.44; 1.66) | 0.639 | 0.92 (0.49; 1.75) | 0.808 | 1.20 (0.50; 2.87) | 0.679 | 1.22 (0.53; 2.81) | 0.638 |
| *Campylobacter gracilis* | 0.52 (0.32; 0.85) | 0.008 | 0.30 (0.15; 0.60) | 0.001 | 0.30 (0.14; 0.62) | 0.001 | 0.29 (0.14; 0.60) | 0.001 | 0.29 (0.12; 0.70) | 0.006 | 0.29 (0.12; 0.68) | 0.005 |
| *Capnocytophaga leadbetteri* | 0.58 (0.22; 1.53) | 0.270 | 0.64 (0.15; 2.66) | 0.540 | 0.72 (0.17; 3.03) | 0.654 | 0.65 (0.15; 2.79) | 0.562 | 0.76 (0.17; 3.44) | 0.721 | 0.66 (0.14; 3.07) | 0.594 |
| *Corynebacterium matruchotii* | 1.46 (1.18; 1.80) | 0.001 | 1.30 (1.01; 1.67) | 0.042 | 1.29 (1.00; 1.66) | 0.053 | 1.31 (1.01; 1.69) | 0.039 | 1.24 (0.95; 1.63) | 0.120 | 1.24 (0.94; 1.63) | 0.131 |
| *Fusobacterium canifelinum* | 0.06 (0.01; 0.34) | 0.002 | 0.12 (0.01; 1.22) | 0.074 | 0.07 (0.01; 0.90) | 0.041 | 0.07 (0.00; 0.92) | 0.043 | 0.12 (0.01; 1.99) | 0.139 | 0.14 (0.01; 2.38) | 0.172 |
| *Fusobacterium nucleatum* | 0.42 (0.27; 0.66) | <0.001 | 0.67 (0.37; 1.19) | 0.171 | 0.58 (0.31; 1.07) | 0.081 | 0.68 (0.38; 1.22) | 0.192 | 0.88 (0.45; 1.74) | 0.723 | 0.91 (0.49; 1.71) | 0.772 |
| *Fusobacterium periodonticum* | 0.16 (0.04; 0.60) | 0.006 | 0.21 (0.04; 1.20) | 0.080 | 0.15 (0.02; 0.98) | 0.047 | 0.19 (0.03; 1.07) | 0.060 | 0.27 (0.04; 1.79) | 0.175 | 0.26 (0.04; 1.67) | 0.154 |
| *Granulicatella adiacens* | 0.88 (0.23; 3.32) | 0.849 | 0.57 (0.10; 3.28) | 0.530 | 0.81 (0.13; 5.05) | 0.823 | 0.64 (0.11; 3.67) | 0.615 | 0.86 (0.09; 8.15) | 0.893 | 0.76 (0.09; 6.27) | 0.798 |
| *Haemophilus parainfluenzae* | 0.84 (0.72; 0.97) | 0.019 | 0.87 (0.74; 1.03) | 0.109 | 0.89 (0.76; 1.04) | 0.149 | 0.87 (0.73; 1.03) | 0.096 | 0.91 (0.81; 1.02) | 0.107 | 0.90 (0.79; 1.01) | 0.082 |
| *Leptotrichia buccalis* | 1.62 (1.01; 2.60) | 0.045 | 1.44 (0.88; 2.36) | 0.142 | 1.60 (0.96; 2.68) | 0.073 | 1.46 (0.88; 2.40) | 0.143 | 1.36 (0.76; 2.44) | 0.295 | 1.31 (0.74; 2.31) | 0.357 |
| *Leptotrichia genomosp.* | 2.04 (1.00; 4.14) | 0.049 | 1.68 (0.68; 4.15) | 0.262 | 1.58 (0.65; 3.84) | 0.314 | 1.59 (0.65; 3.90) | 0.313 | 1.22 (0.51; 2.95) | 0.652 | 1.31 (0.54; 3.18) | 0.554 |
| *Leptotrichia hofstadii* | 2.12 (0.67; 6.69) | 0.202 | 2.75 (0.42; 18.15) | 0.292 | 3.01 (0.45; 20.15) | 0.255 | 2.61 (0.40; 16.99) | 0.315 | 1.62 (0.40; 6.50) | 0.497 | 1.57 (0.40; 6.14) | 0.515 |
| *Leptotrichia hongkongensis* | 1.02 (0.63; 1.67) | 0.922 | 0.69 (0.39; 1.24) | 0.215 | 0.73 (0.40; 1.33) | 0.304 | 0.73 (0.4; 1.33) | 0.303 | 0.96 (0.45; 2.03) | 0.916 | 0.95 (0.44; 2.03) | 0.890 |
| *Leptotrichia wadei* | 1.30 (0.69; 2.45) | 0.420 | 0.69 (0.27; 1.76) | 0.437 | 0.66 (0.25; 1.71) | 0.391 | 0.67 (0.27; 1.66) | 0.386 | 0.67 (0.25; 1.83) | 0.437 | 0.62 (0.24; 1.65) | 0.340 |
| *Mannheimia varigena* | 0.74 (0.53; 1.04) | 0.084 | 0.85 (0.57; 1.28) | 0.446 | 0.85 (0.56; 1.29) | 0.442 | 0.86 (0.57; 1.29) | 0.457 | 0.88 (0.57; 1.37) | 0.581 | 0.86 (0.55; 1.34) | 0.510 |
| *Other* | 1.09 (0.98; 1.22) | 0.129 | 0.96 (0.82; 1.13) | 0.644 | 0.99 (0.84; 1.16) | 0.905 | 0.97 (0.83; 1.14) | 0.721 | 0.96 (0.79; 1.16) | 0.657 | 0.97 (0.80; 1.17) | 0.730 |
| *Neisseria flavescens* | 0.50 (0.21; 1.20) | 0.121 | 0.29 (0.06; 1.44) | 0.128 | 0.26 (0.05; 1.35) | 0.109 | 0.39 (0.08; 1.94) | 0.248 | 0.17 (0.02; 1.54) | 0.115 | 0.27 (0.03; 2.48) | 0.248 |
| *Prevotella histicola* | 1.99 (0.84; 4.70) | 0.117 | 1.35 (0.43; 4.26) | 0.606 | 1.47 (0.46; 4.69) | 0.516 | 1.38 (0.42; 4.54) | 0.595 | 0.99 (0.27; 3.63) | 0.986 | 0.92 (0.26; 3.29) | 0.899 |
| *Prevotella maculosa* | 0.93 (0.19; 4.47) | 0.931 | 0.24 (0.02; 2.93) | 0.266 | 0.29 (0.02; 3.70) | 0.343 | 0.24 (0.02; 3.04) | 0.268 | 0.41 (0.02; 7.27) | 0.543 | 0.46 (0.03; 8.09) | 0.596 |
| *Prevotella melaninogenica* | 1.06 (0.86; 1.30) | 0.597 | 1.13 (0.87; 1.46) | 0.372 | 1.11 (0.86; 1.44) | 0.424 | 1.14 (0.88; 1.47) | 0.333 | 1.14 (0.83; 1.56) | 0.422 | 1.14 (0.84; 1.56) | 0.403 |
| *Prevotella oris* | 1.64 (0.75; 3.57) | 0.216 | 1.13 (0.47; 2.74) | 0.789 | 1.43 (0.55; 3.74) | 0.463 | 1.17 (0.48; 2.85) | 0.732 | 1.45 (0.47; 4.45) | 0.518 | 1.57 (0.52; 4.68) | 0.423 |
| *Prevotella oulorum* | 0.39 (0.13; 1.12) | 0.081 | 0.12 (0.03; 0.54) | 0.006 | 0.12 (0.03; 0.55) | 0.007 | 0.11 (0.02; 0.54) | 0.006 | 0.12 (0.02; 0.62) | 0.012 | 0.15 (0.03; 0.76) | 0.023 |
| *Prevotella pallens* | 0.79 (0.36; 1.73) | 0.557 | 0.79 (0.25; 2.49) | 0.684 | 0.89 (0.28; 2.81) | 0.847 | 0.89 (0.29; 2.77) | 0.843 | 1.42 (0.37; 5.44) | 0.605 | 1.50 (0.38; 5.82) | 0.562 |
| *Prevotella veroralis* | 1.02 (0.51; 2.04) | 0.959 | 0.45 (0.17; 1.20) | 0.110 | 0.37 (0.13; 1.05) | 0.062 | 0.46 (0.17; 1.23) | 0.123 | 0.30 (0.10; 0.92) | 0.035 | 0.32 (0.11; 0.94) | 0.038 |
| *Selenomonas noxia* | 0.74 (0.31; 1.78) | 0.508 | 0.20 (0.06; 0.70) | 0.012 | 0.22 (0.06; 0.77) | 0.018 | 0.19 (0.05; 0.67) | 0.010 | 0.23 (0.06; 0.88) | 0.032 | 0.24 (0.06; 0.93) | 0.039 |
| *Streptococcus gordonii* | 0.31 (0.09; 1.15) | 0.081 | 0.19 (0.03; 1.07) | 0.060 | 0.22 (0.04; 1.30) | 0.095 | 0.20 (0.03; 1.13) | 0.069 | 0.27 (0.03; 2.11) | 0.211 | 0.30 (0.04; 2.25) | 0.243 |
| *Streptococcus mutans* | 1.00 (0.76; 1.33) | 0.978 | 0.84 (0.38; 1.85) | 0.659 | 0.84 (0.39; 1.81) | 0.653 | 0.84 (0.38; 1.86) | 0.664 | 0.68 (0.25; 1.83) | 0.447 | 0.63 (0.18; 2.18) | 0.470 |
| *Streptococcus sanguinis* | 4.58 (1.27; 16.55) | 0.020 | 1.9 (0.70; 5.16) | 0.207 | 2.15 (0.78; 5.90) | 0.138 | 1.86 (0.68; 5.10) | 0.228 | 2.66 (0.87; 8.19) | 0.088 | 2.73 (0.82; 9.11) | 0.102 |
| *Veillonella alcalescens* | 1.13 (0.81; 1.59) | 0.462 | 0.93 (0.55; 1.58) | 0.800 | 0.91 (0.55; 1.53) | 0.732 | 0.93 (0.55; 1.57) | 0.787 | 1.18 (0.63; 2.20) | 0.613 | 1.23 (0.65; 2.34) | 0.521 |
| *Veillonella parvula* | 0.65 (0.21; 2.04) | 0.461 | 1.26 (0.25; 6.34) | 0.782 | 1.32 (0.27; 6.48) | 0.734 | 1.30 (0.26; 6.47) | 0.752 | 2.53 (0.38; 16.74) | 0.335 | 2.78 (0.41; 18.82) | 0.296 |
| *Veillonella rogosae* | 0.12 (0.03; 0.53) | 0.005 | 0.12 (0.02; 0.88) | 0.037 | 0.08 (0.01; 0.69) | 0.022 | 0.11 (0.01; 0.91) | 0.041 | 0.15 (0.01; 1.65) | 0.121 | 0.16 (0.01; 1.78) | 0.137 |

Model 1: crude; Model 2: age, sex, and BMI; Model 3: age, sex, BMI and bleeding; Model 4: age, sex, BMI and periodontitis; Model 5: age, sex, BMI, HbA1c, insulin fasting, CRP and bleeding; Model 6: age, sex, BMI, HbA1c, insulin fasting, CRP and periodontitis.




**Appendix Figure 1.** Beta diversity comparisons of microbial communities in subjects with MetS and subjects without MetS. For MetS Yes (red), and MetS No (green) are shown to determine Bray–Curtis distances.

# CHAPTER 6: GENERAL DISCUSSION AND CONCLUSION

## 6.1 Summary of findings

The following section reflects and discusses the analyses performed on the oral microbiome to achieve our study objectives which were to investigate the composition of the oral microbiome and the role it plays in the development of chronic inflammatory disorders. In addition, the influence of lifestyle factors such as smoking was explored. To achieve this, we investigated the oral microbiota in a cohort of participants who had diabetes, MetS, and who smoked. Furthermore, recommendations for future studies are made to address the limitations mentioned in this study. The findings of this work are novel as it is the first investigation of the oral microbiome in individuals with diabetes and MetS in South Africa. In the complete analysis of this study, we found that the diversity of the oral microbiome was indeed disrupted on all taxonomic levels and warrants further investigation.

## 6.2 Summary of Results

### Manuscript 1: Chapter 3

When comparing the subgingival oral microbiota in DM patients with controls using 16S rDNA gene sequencing, 9 dominant phyla were observed of which *Bacteroidetes, Fusobacteria, and Actinobacteria*, *Firmicutes,* and *Proteobacteria* accounted for >98% of the oral microbiota across all glycaemic status. In the pre-diabetes and DM group, the most significantly abundant phyla were *Fusobacteria* and *Actinobacteria,* while *Proteobacteria* were less abundant. Controversial reports have been published regarding the representation of oral microbiota and DM status. In support of our findings, however, others have found similar phyla but the hierarchal order of taxa was different (Ogawa et al., 2017). In contradiction, however, there have been reports of decreased abundance of *Actinobacteria* and an increase of Firmicutes which was associated with an increased risk of DM (Long et al., 2017). There was no significant difference in the incidence of periodontal disease between those with and without DM, however, those with gingival bleeding had an abundance of *Bacteroides* genera.

**Manuscript 2: Chapter 4**

The effects of lifestyle and particularly smoking was investigated and demonstrated that smoking resulted in significant differences and alterations of the oral biome. The five most abundant phyla observed across both smokers and non-smokers were Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria, and Fusobacteria and they made up more than 98% of the total number of phyla. Smokers however had a reduced abundance of the phyla *Actinobacteria* while *Fusobacterium* and *Campylobacter* were found in higher abundance. Furthermore, an increased abundance of gram-negative anaerobic bacterium was observed in smokers.

**Manuscript 3: Chapter 5**

In this manuscript, the composition of the oral microbiome was investigated in those with and without metabolic syndrome. The study findings again demonstrated that there was a significant abundance of genera *Actinomyces, Corynebacterium, and Fusobacterium* present in our MetS group which was different from those without MetS and to other reports in the literature in which an abundance of *Granulicatella* has been observed (Si, Lee & G. Ko, 2017).

## 6.3 Limitations and strengths

This thesis was a cross-sectional study that did not allow a full investigation into how the predominant phyla would change with progression into periodontal disease. An additional limitation was the small sample size which limited the analysis of small sample groups even though other studies of the oral microbiome have similar numbers of participants. Furthermore, we omitted the measurement of the pocket depth (PD) of each subgingival plaque sample due to small representative numbers in the group of participants with PD ≥6 mm ($n$ = 7). Caton et al 2018 suggested the use of a new classification scheme for periodontal and peri-implant diseases and conditions to assist researchers to properly investigate etiology, pathogenesis, and natural history, and treatment of disease and conditions(Caton et al., 2018). However, due to missing data collected between 2014 and 2016, we were unable to do this. Another limitation that could have affected the outcome was the method of sampling. In this study, analysis was conducted on subgingival plaque samples using the toothpick method. Although this is a well-described method others have made use of samples such as oral washes and buccal swabs (Karabudak et al., 2019). Therefore, the difference and variation of species observed in our study could be due to the different techniques, sample sites, and the difference in geographical areas.

## 6.4 Conclusion

Despite these limitations, our study has made a major contribution to the investigation of the oral microbiome and how it contributes to the development of chronic inflammatory disease in South Africa. Very few studies have been performed in this region and this study has demonstrated that the oral microbiome in those who smoke, have DM or MetS is different on all taxonomic levels including genus and species level. These significant changes may result in periodontal disease which is associated with inflammation and predisposes subjects to systemic disease such as diabetes and metabolic syndrome. It is believed that the alterations may be due to increased levels of glucose in the subgingival microenvironment that may have altered immune responses in the host and contributed to chronic inflammation and an impaired immune response (Ohlrich et al., 2010). This has been supported by an earlier study that associated DM as being a risk factor for periodontitis and contributes to increased prevalence, severity, and progression of periodontal disease (Kocher et al., 2018). It is therefore suggested that future longitudinal studies are performed to investigate the relationship or underlying mechanisms between the oral microbiome and the progression of chronic inflammatory This could assist to identify oral biomarkers associated with disease and could contribute to therapy and prevention. Furthermore, while the effect of AGE-RAGE interactions on the subgingival pathophysiology has been investigated in-depth, the effect of diabetes on the oral microbiome demands additional investigation.

## 6.5 Reference


Alberti, K.G., Eckel, M.M., Grundy, R.H., *et al*. 2009. Harmonizing the metabolic syndrome: A joint interim statement of the international diabetes federation task force on epidemiology and prevention; National heart, lung, and blood institute; American heart association; World heart federation; International . *Circulation*, 120(16): 1640–1645.

Andersen, C.J., Murphy, K.E. & Fernandez, M.L. 2016. Impact of obesity and metabolic syndrome on immunity. *Adv Nutr*, 7(1): 66–75.

Arimatsu, K., Yamada, H., Miyazawa, H., *et al*. 2014. Oral pathobiont induces systemic inflammation and metabolic changes associated with alteration of gut microbiota. *Scientific Reports*, 4: 1–9.

Beutler, J., Jentsch, H.F.R., Rodloff, A.C. & Stingu, C.S. 2019. Bacteremia after professional mechanical plaque removal in patients with chronic periodontitis. *Oral Dis*, 25(4): 1185–1194.

Buduneli, N. 2020. Biomarkers in Periodontal Health and Disease. *Biomarkers in Periodontal Health and Disease*: 25–36.

Casarin, R.C.V., Barbagallo, A., Meulman, T., *et al*. 2013. Subgingival biodiversity in subjects with uncontrolled type-2 diabetes and chronic periodontitis. *J Periodontal Res*, 48(1): 30–36.

Chistiakov, D.A., Orekhov, A.N. & Bobryshev, Y. V. 2016. Links between atherosclerotic and periodontal disease. *Exp Mol Pathol*, 100(1): 220–235. http://dx.doi.org/10.1016/j.yexmp.2016.01.006.

Chukkapalli, S.S., Velsko, I.M., Rivera-Kweh., *et al*. 2015. Polymicrobial oral infection with four periodontal bacteria orchestrates a distinct inflammatory response and atherosclerosis in ApoEnull mice. *PLoS ONE*, 10(11): 1–17.

Dewhirst, F.E., Chen, T., Izard, J., *et al*. 2010. The human oral microbiome. *J Bacteriol* 192(19): 5002–5017.

Enberg, N., Alho, H., Loimaranta, V. & Lenander-Lumikari, M. 2001. Saliva flow rate, amylase activity, and protein and electrolyte concentrations in saliva after acute alcohol consumption. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*, 92(3): 292–298.

Feres, F Teles, R. Teles, L. C Figueiredo, M.F. 2017. The subgingival periodontal microbiota in the aging mouth Magda. *Physiology & behavior*, 176(1): 139–148.

Ferroni, P., Roselli, M., Riondino, S., Cavaliere, F. & Guadagni, F. 2016. Insulin resistance as a predictor of venous thromboembolism in breast cancer. *Endocr Relat Cancer*, 23(6): L25–L28.

Caton, G., Armitage, J., Berglundh, G., et al. 2018. A new classification scheme for periodontal and peri-implant diseases and conditions – Introduction and key changes from the 1999 classification. *J Clin Periodontol*, 45(March): S1–S8.

Gao, L., Xu, T., Huang, G., Jiang, S., Gu, Y. & Chen, F. 2018. Oral microbiomes : more and more importance in oral cavity and whole body. *Protein & Cell*, 9(5): 488–500. https://doi.org/10.1007/s13238-018-0548-1.

Global Adult Tobacco Survey Collaborative Group. 2011. Tobacco Questions for Surveys: A Subset of Key Questions from the Global Adult Tobacco Surveys. http://www.who.int/tobacco/surveillance/en_tfi_tqs.pdf.

Grice, E.A. & Segre, J.A. 2013. The skin microbiome. *Nat Rev Microbiol*, 9(4): 244–253.

Griffen, A.L., Beall, C.J., Campbell, J.H., *et al*. 2012. Distinct and complex bacterial profiles in human periodontitis and health revealed by 16S pyrosequencing. *ISME Journal*, 6(6): 1176–1185. http://dx.doi.org/10.1038/ismej.2011.191.

Haffajee, A.D., Socransky, S.S., Patel, M.R. & Song, X. 2008. Microbial complexes in supragingival plaque. *Oral Microbiol Immunol*, 23(3): 196–205.

Han Yiping. 2015. Fusobacterium nucleatum: a commensal turned pathogen. *Curr Opin Microbiol*, 1(23): 141–147.

Hanioka, T., Morita, M., Yamamoto, T., *et al*. 2019. Smoking and periodontal microorganisms. *Jpn Dent Sci Rev*, 55(1): 88–94. https://doi.org/10.1016/j.jdsr.2019.03.002.

Haririan, H., Andrukhov, O., Bertl, K., *et al.* 2014. Microbial Analysis of Subgingival Plaque Samples Compared to That of Whole Saliva in Patients With Periodontitis. *J Periodontol*, 85(6): 819–828.

Henne, K., Fuchs, F., Kruth, S., Horz, H.P. & Conrads, G. 2014. Shifts in Campylobacter species abundance may reflect general microbial community shifts in periodontitis progression. *J Oral Microbiol*, 6(1): 6–11.

Homann, N., Tillonen, J., Meurman, J.H., *et al*. 2000. Increased salivary acetaldehyde levels

in heavy drinkers and smokers: A microbiological approach to oral cavity cancer. *Carcinogenesis*, 21(4): 663–668.

Huang, C. & Shi, G. 2019a. Smoking and microbiome in oral, airway, gut and some systemic diseases. *J Transl Med*, 17(1): 1–15. https://doi.org/10.1186/s12967-019-1971-7.

Janket, S.J., Baird, A.E., Chuang, S.K. & Jones, J.A. 2003. Meta-analysis of periodontal disease and risk of coronary heart disease and stroke. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*, 95(5): 559–569.

Jansson, L. 2008. Association between alcohol consumption and dental health. *J Clin Periodontol*, 35(5): 379–384.

Ji, S., Kim, Y., Min, B.M., Han, S.H. & Choi, Y. 2007. Innate immune responses of gingival epithelial cells to nonperiodontopathic and periodontopathic bacteria. *J Periodontal Res*, 42(6): 503–510.

Jiang, Y., Zhou, X., Cheng, L. & Li, M. 2020. The Impact of Smoking on Subgingival Microflora: From Periodontal Health to Disease. *Front Microbiol,* 11(January): 1–13.

Kantorski, K.Z., de Souza, D.M., Yujra, V.Q. ,*et al.* 2007. Effect of an alcoholic diet on dental caries and on Streptococcus of the mutans group: Study in rats. *Braz Oral Res*, 21(2): 101–105.

Karabudak, S., Ari, O., Durmaz, B., *et al*. 2019. Analysis of the effect of smoking on the buccal microbiome using next-generation sequencing technology. *J Med Microbiol*, 68(8): 1148–1158.

Kato, I., Vasquez, A.A., Moyerbrailean, G., *et al*. 2016. Oral microbiome and history of smoking and colorectal cancer. *J Epidemiol Res*, 2(2): 92–101.

Keijser, B.J.F., Zaura, E., Huse, S.M., *et al.* 2008. Pyrosequencing analysis of the oral microflora of healthy adults. *J Dent Res*, 87(11): 1016–1020.

Kengne, A.P., Erasmus, R.T., Levitt, N.S. & Matsha, T.E. 2017. Alternative indices of glucose homeostasis as biochemical diagnostic tests for abnormal glucose tolerance in an African setting. *Prim Care Diabetes*, 11(2): 119–131. http://dx.doi.org/10.1016/j.pcd.2017.01.004.

Khader, Y.S., Albashaireh, Z.S.M. & Alomari, M.A. 2004. Periodontal Diseases and the Risk of Coronary Heart and Cerebrovascular Diseases: A Meta-Analysis. *J Periodontol*, 75(8): 1046–1053.

Kocher, T., König, J., Borgnakke, W.S., Pink, C. & Meisel, P. 2018. Periodontal complications of hyperglycemia/diabetes mellitus: Epidemiologic complexity and clinical challenge. *Periodontol 2000*, 78(1): 59–97.

Krisanaprakornkit, S., Kimball, J.R., Weinberg, A., *et al.* 2000. Inducible expression of human β-defensin 2 by Fusobacterium nucleatum in oral epithelial cells: Multiple signaling pathways and role of commensal bacteria in innate immunity and the epithelial barrier. *Infect Immun*, 68(5): 2907–2915.

Kumar, P.S., Matthews, C.R., Joshi, V., de Jager, M. & Aspiras, M. 2011a. Tobacco smoking affects bacterial acquisition and colonization in oral biofilms. *Infect Immun*, 79(11): 4730–4738.

Lamster, I.B. & Pagan, M. 2017. Periodontal disease and the metabolic syndrome. *Int Dent J*, 67(2): 67–77.

Linden, G.J., Lyons, A. & Scannapieco, F.A. 2013. Periodontal systemic associations : review of the evidence. *J Periodontal,* 84: 8–19.

Lõivukene, K., Pähkla, E.R., Koppel, T., Saag, M. & Naaber, P. 2005. The microbiological status of patients with periodontitis in southern Estonia after non-surgical periodontal therapy., *Stomatologija*, 7(2): 45–47.

Long, J., Cai, Q., Steinwandel, M., *et al* . 2017. Association of oral microbiome with type 2 diabetes risk. *J Periodontal Res*, 52(3): 636–643.

Lu, M., Xuan, S. & Wang, Z. 2019. Oral microbiota: A new view of body health. *Food Science and Human Wellness*, 8(1): 8–15. https://doi.org/10.1016/j.fshw.2018.12.001.

M. Kilian, Chapple, I.L.., Hannig, M., *et al*. 2016. The oral microbiome - An update for oral healthcare professionals. *Bri Dent J*, 221(10): 657–666.

Macuch, P.J. & Tanner, A.C.R. 2000. Campylobacter species in health, gingivitis, and periodontitis. *J Dent Res*, 79(2): 785–792.

Mager, D.L., Ximenez-Fyvie, L.A., Haffajee, A.D. & Socransky, S.S. 2003. Distribution of selected bacterial species on intraoral surfaces. *J Clin Periodontol*, 30(7): 644–654.

Mason, M.R., Preshaw, P.M., Nagaraja, H.N., *et al*. 2015a. The subgingival microbiome of clinically healthy current and never smokers. *ISME Journal*, 9(1): 268–272.

Matsha, T.E., Hassan, M.S., Hon, G.M., *et al.* 2013. Derivation and validation of a waist circumference optimal cutoff for diagnosing metabolic syndrome in a South African mixed ancestry population. *Int J Cardiol*, 168(3): 2954–2955. http://dx.doi.org/10.1016/j.ijcard.2013.03.150.

Matsha, T.E., Prince, Y., Davids, S., *et al.* 2020. Oral Microbiome Signatures in Diabetes Mellitus and Periodontal Disease. *J Dent Res* 99(6): 658–665. https://doi.org/10.1177/0022034520913818.

McCracken, E., Monaghan, M. & Sreenivasan, S. 2018. Pathophysiology of the metabolic syndrome. *Clin Dermatol*, 36(1): 14–20. http://dx.doi.org/10.1016/j.clindermatol.2017.09.004.

McLean, J.S. 2014. Advancements toward a systems level understanding of the human oral microbiome. *Front Cellul Infect Microbiol*, 4(JUL): 1–13.

Medikeri, R.S., Lele, S.V., Jain, P.M., Mali, P. & Medikeri, M.R. 2015. Quantification of selenomonas sputigena in chronic periodontitis in smokers using 16S rDNA based PCR analysis. *J Clin Diag Res* 9(4): ZC13–ZC17.

Moon, J.H., Lee, J.H. & Lee, J.Y. 2015a. Subgingival microbiome in smokers and non-smokers in Korean chronic periodontitis patients. *Mol Oral Microbiol*, 30(3): 227–241.

Ogawa, T., Honda-Ogawa, M., Ikebe, K. ,*et al*. 2017. Erratum to 'Characterizations of oral microbiota in elderly nursing home residents with diabetes'. *J Oral Sci*, 60(1): 163.

Ohlrich, E.J., Cullinan, M.P. & Leichter, J.W. 2010. Diabetes, periodontitis, and the subgingival microbiota. *J Oral Microbiol*, 2(2010): 1–8.

Pirkle, J.L., Flegal, K.M., Bernert, J.T., Brody, D.J., Etzel, R.A. & Maurer, K.R. 1996. Exposure of the US population to environmental tobacco smoke: The Third National Health and Nutrition Examination Survey, 1988 to 1991. *JAMA,* 275(16): 1233–1240.

Preza, D., Olsen, I., Willumsen, T., *et al.* 2009. Microarray analysis of the microflora of root caries in elderly. *Eur J Clin Microbiol Infect Dis*, 28(5): 509–517.

Reddy, P., Zuma, K., Shisana, O., *et al*. 2015. Prevalence of tobacco use among adults in South Africa : Results from the first South African National Health and Nutrition Examination Survey. , 105(8).

Saeb, A.T.M., Al-Rubeaan, K.A., Aldosary, K., *et al.* 2019. Relative reduction of biological

and phylogenetic diversity of the oral microbiota of diabetes and pre-diabetes patients. *Microb Pathog*, 128(January): 215–229. https://doi.org/10.1016/j.micpath.2019.01.009.

Sapkota, A.R., Berger, S., Vogel, T.M., Reynolds, C.R.J. & Co, T. 2010. Human Pathogens Abundant in the Bacterial Metagenome of Cigarettes., *Environ Health Perspect,* 118(3): 351–356.

Sato, T., Watanabe, K., Kumada, H., Toyama, T., Tani-Ishii, N. & Hamada, N. 2012. Peptidoglycan of Actinomyces naeslundii induces inflammatory cytokine production and stimulates osteoclastogenesis in alveolar bone resorption. *Arch Oral Biol*, 57(11): 1522–1528. http://dx.doi.org/10.1016/j.archoralbio.2012.07.012.

Sharma, N., Bhatia, S., Singh Sodhi, A. & Batra, N. 2018. Oral microbiome and health. *AIMS Microbiology*, 4(1): 42–66. http://www.aimspress.com/article/10.3934/microbiol.2018.1.42.

Shchipkova, A.Y., Nagaraja, H.N. & Kumar, P.S. 2010. Subgingival microbial profiles of smokers with periodontitis. *J Dent Res*, 89(11): 1247–1253.

Shepherd, S. 2011. Alcohol consumption a risk factor for periodontal disease. *Evid Based Dent*, 12(3): 76. http://dx.doi.org/10.1038/sj.ebd.6400808.

Si, J., Lee, C. & Ko, G. 2017. Oral Microbiota: Microbial Biomarkers of Metabolic Syndrome Independent of Host Genetic Factors. *Frontiers in Cellular and Infection Microbiology*, 7(December): 1–11. http://journal.frontiersin.org/article/10.3389/fcimb.2017.00516/full.

Si, J., Lee, C. & Ko, G.P. 2017. Oral microbiota: Microbial biomarkers of metabolic syndrome independent of host genetic factors. *Front Cell Infect Microbiol*, 7(DEC): 1–11.

Signat, B., Roques, C., Poulet, P. & Duffaut, D. 2011. Role of Fusobacterium nucleatum in periodontal health and disease. *Curr Issues Mol Biol*, 13(2): 25–36.

Signoretto, C., Bianchi, F., Burlacchini, G., *et al.* 2010. Drinking habits are associated with changes in the dental plaque microbial community. *J Clin Microbiol*, 48(2): 347–356.

Slattery, M.L., Hunt, S.C., French, T.K., Ford, M.H. & Williams, R.R. 1989. Validity of cigarette smoking habits in three epidemiologic studies in Utah. *Prev Med*, 18(1): 11–19.

Tefiku, U., Popovska, M., Cana, A., *et al.* 2020. Determination of the Role of Fusobacterium Nucleatum in the Pathogenesis in and Out the Mouth. *Prilozi*, 41(1): 87–99.

Ursell, L.K., Metcalf, J.L., Parfrey, L.W. & Knight, R. 2012. Defining the human microbiome.

*Nutr Rev*, 70(SUPPL. 1).

Valour, F., Sénéchal, A., Dupieux, C., *et al*. 2014. Actinomycosis: Etiology, clinical features, diagnosis, treatment, and management. *Infect Drug Resist*, 7: 183–197.

Vielkind, P., Jentsch, H., Eschrich, K., Rodloff, A.C. & Stingu, C.S. 2015. Prevalence of Actinomyces spp. in patients with chronic periodontitis. *Int J Med Microbiol*, 305(7): 682–688. http://dx.doi.org/10.1016/j.ijmm.2015.08.018.

Wade, W.G. 2013. The oral microbiome in health and disease. *Pharmacol Res*, 69(1): 137–143. http://dx.doi.org/10.1016/j.phrs.2012.11.006.

Willis, J.R. & Gabaldón, T. 2020. The human oral microbiome in health and disease: From sequences to ecosystems. *Microorganisms*, 8(2): 1–28.

World Health Organization. 2019. *Report on Global Tobacco Epidemic*. https://apps.who.int/iris/bitstream/handle/10665/326043/9789241516204-eng.pdf?ua=1&ua=1.

Wu, J., Li, M. & Huang, R. 2019. The effect of smoking on caries-related microorganisms. *Tob Induc Dis*, 17(April).

Wu, J., Peters, B.A., Dominianni, C., *et al.* 2016. Cigarette smoking and the oral microbiome in a large study of American adults. *The ISME Journal*, 10: 2435–2446. www.nature.com/ismej 27 February 2020.

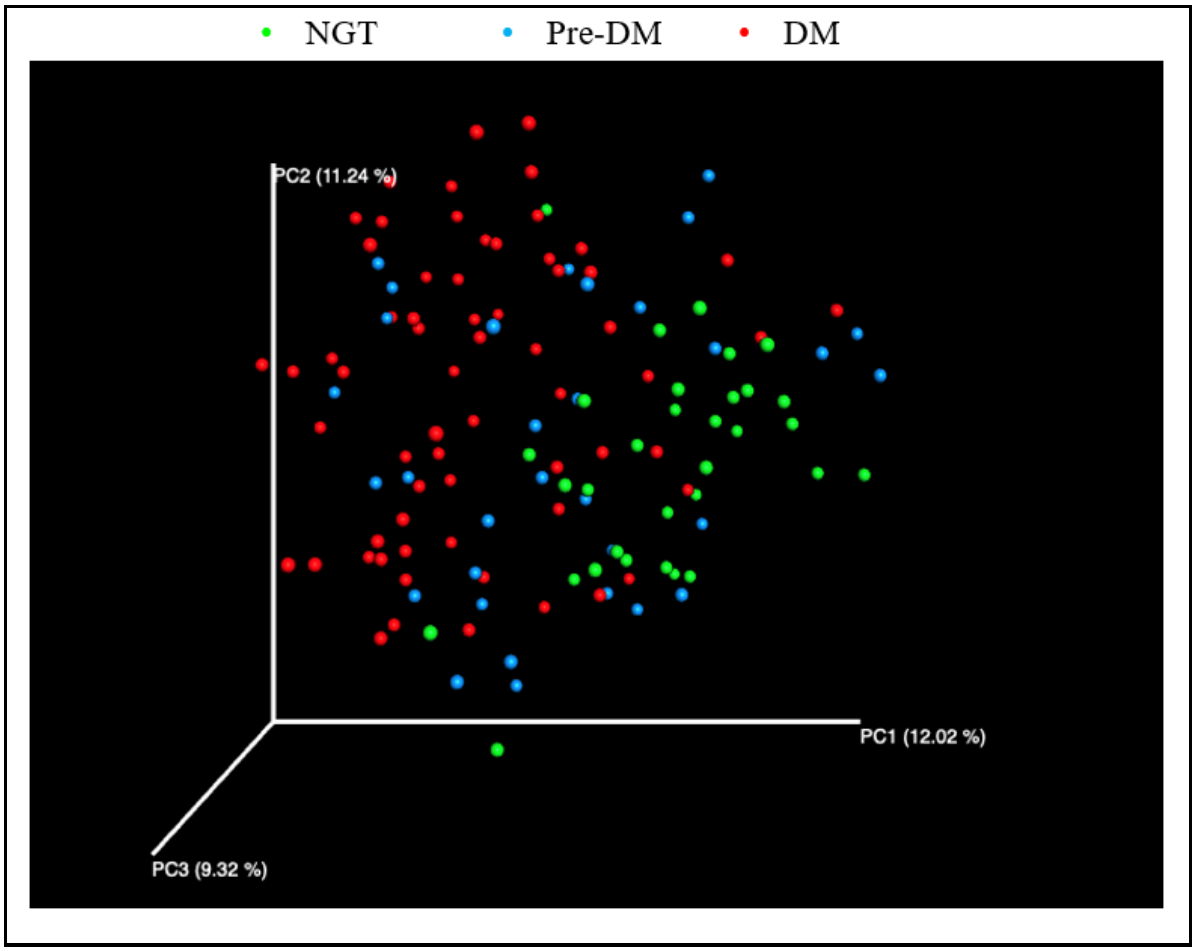
Yost, S., Duran-Pinedo, A.E., Teles, R., Krishnan, K. & Frias-Lopez, J. 2015. Functional signatures of oral dysbiosis during periodontitis progression revealed by microbial metatranscriptome analysis. *Genome Med*, 7(1): 1–19.

Yu, Q., Huang, S., Xu, T.T., Wang, Y.C. & Ju, S. 2020. Measuring Brown Fat Using MRI and Implications in the Metabolic Syndrome. *J Mag Reson Imaging*, Oct 12.

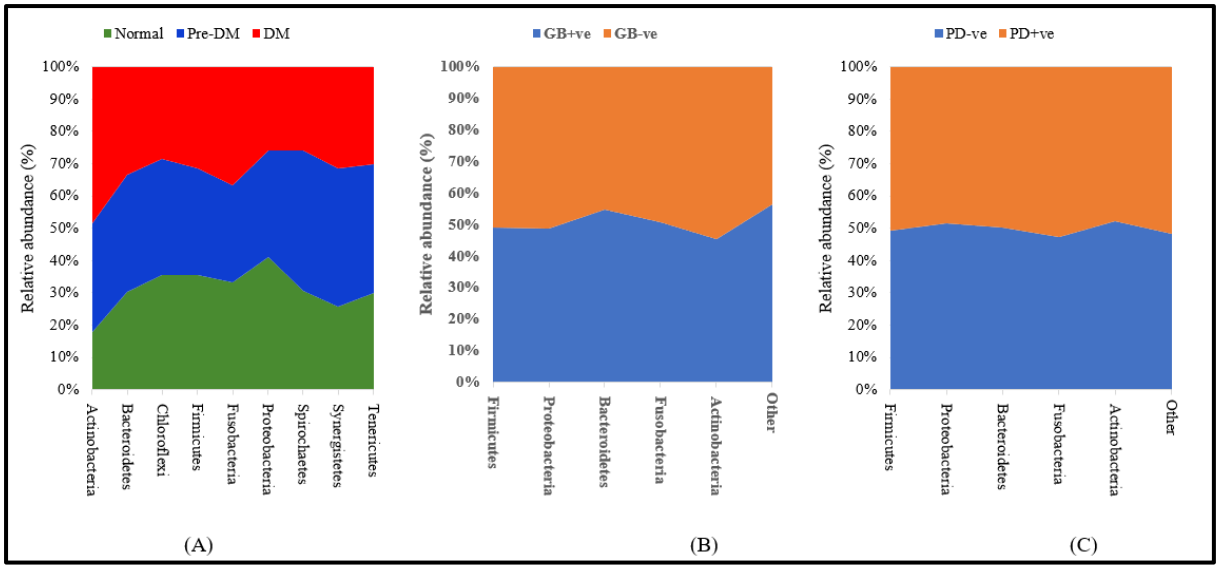
Zhong, E.F., Chang, A., Stucky, A., *et al.* 2020. Genomic analysis of oral lichen planus and related oral microbiome pathogens. *Pathogens*, 9(11): 1–13.

**Supplementary Appendix Table 2.** Alpha diversity in species indices according to glycaemic status

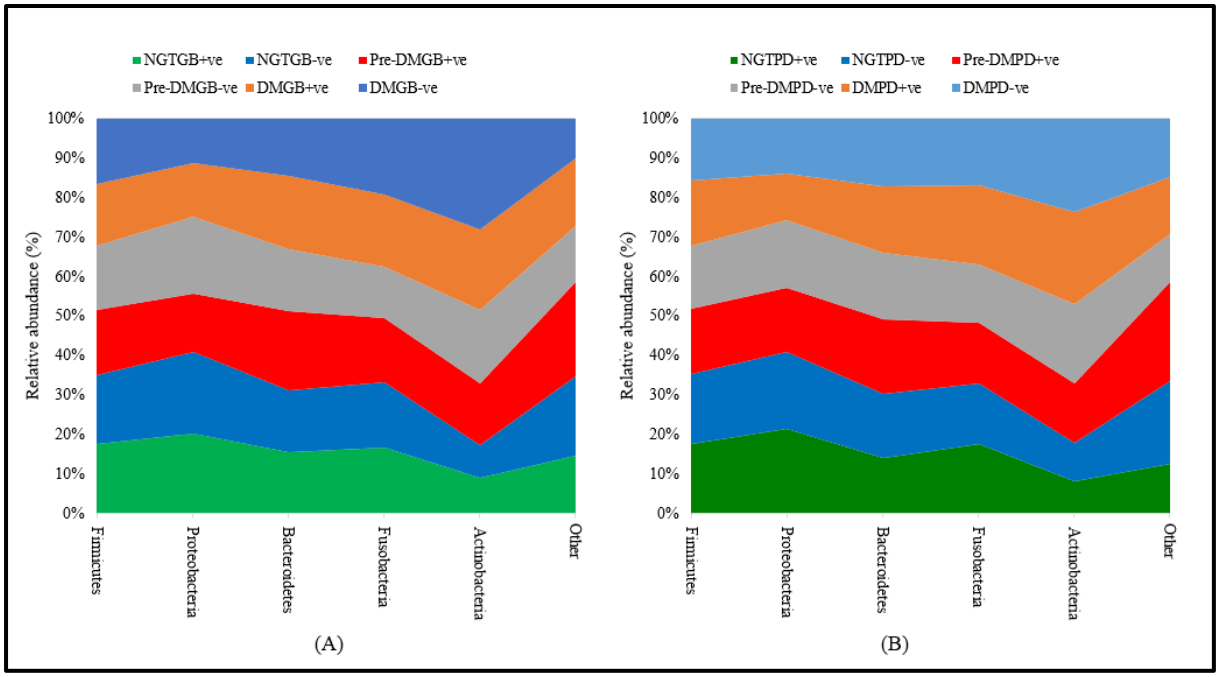
| | NGT | Pre-DM | DM |
|---|---|---|---|
| Number of taxa | 555 | 531 | 503 |
| Shannon | 3.4559 | 3.4506 | 4.1460 |
| Chao1 | 555 | 531 | 503 |
| Simpson | 0.9682 | 0.9679 | 0.9840 |



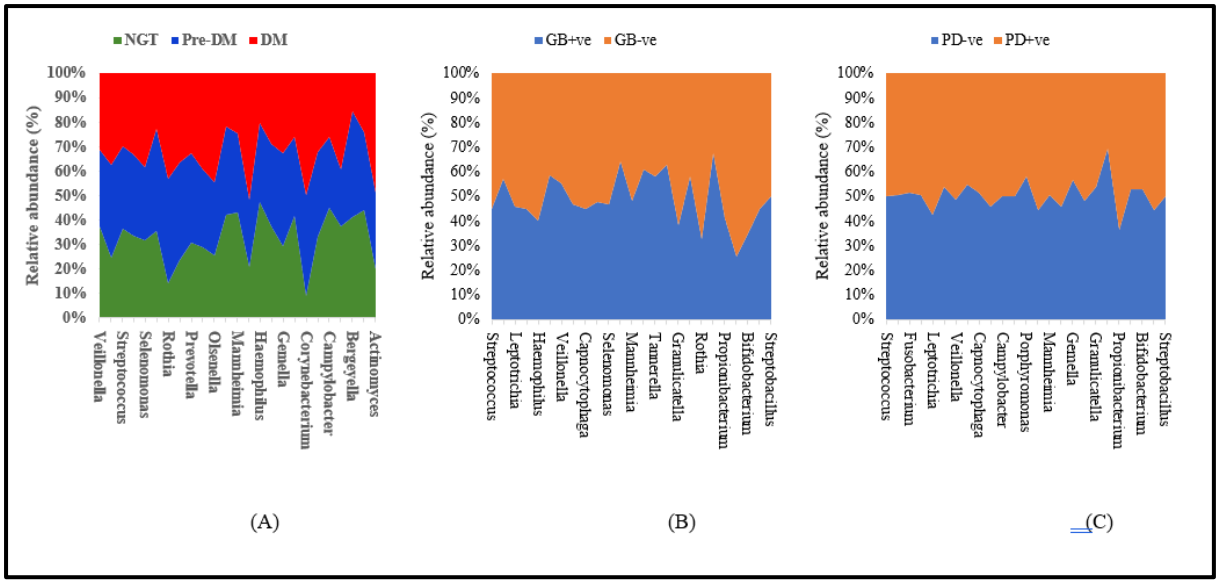

**Supplementary Appendix Figure 1.** Beta diversity comparisons of microbial communities in the diabetes mellitus (DM), prediabetes (Pre-DM) and normoglycaemia (NGT). Principal coordinate analysis (PCoA) plots for DM (red), Pre-DM (blue) and NGT (green) are shown to determine Bray–Curtis distances.



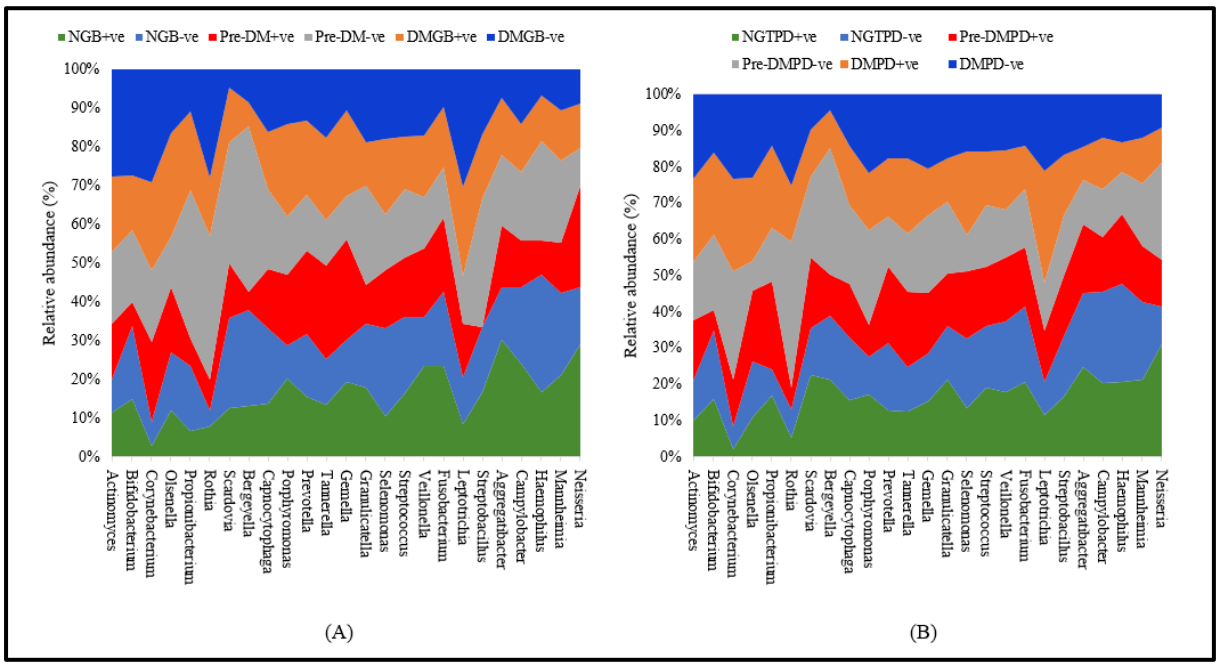

**Supplementary Appendix Figure 2.** Composition of the oral microbial community at phylum as the percentage of relative abundance in individuals with (A), normotolerant (NGT), prediabetes (Pre-DM) and diabetes (DM) individuals; (B), gingival bleeding on probing (GB+ve) and no gingival bleeding on probing (GB-ve); (C), pocket depth ≥ 4mm (PD+ve) and pocket depth <4mm (PD-ve).




**Supplementary Appendix Figure 3.** Composition of the oral microbial community at phylum as the percentage of relative abundance in individuals with (A), normal glucose tolerance with or without gingival bleeding on probing (NGT GB+ve, NGT GB-ve); prediabetes with or without gingival bleeding on probing (Pre-DM GB+ve, Pre-DM GB-ve) versus; diabetes mellitus with or without gingival bleeding on probing (DM GB+ve, DM GB-ve). (B), normal glucose tolerance with or without pocket depth ≥ 4mm (NGT PD+ve, NGT PD-ve); Pre-DM PD+ve, Pre-DM -ve; DM PD+ve, DM PD-ve.




**Supplementary Appendix Figure 4.** Composition of the oral microbial community at genus as the percentage of relative abundance in individuals with (A), normotolerant (NGT), prediabetes (Pre-DM) and diabetes (DM) individuals; (B), gingival bleeding on probing (GB+ve) and no gingival bleeding on probing (GB-ve); (C), pocket depth ≥ 4mm (PD+ve) and pocket depth <4mm (PD-ve).




**Supplementary Appendix Figure 5.** Composition of the oral microbial community at genus as the percentage of relative abundance in individuals with (A), normal glucose tolerance with or without gingival bleeding on probing (NGT GB+ve, NGT GB-ve); prediabetes with or without gingival bleeding on probing (Pre-DM GB+ve, Pre-DM GB-ve) versus; diabetes mellitus with or without gingival bleeding on probing (DM GB+ve, DM GB-ve). (B), normal glucose tolerance with or without pocket depth ≥ 4mm (NGT PD+ve, NGT PD-ve); Pre-DM PD+ve, Pre-DM -ve; DM PD+ve, DM PD-ve.