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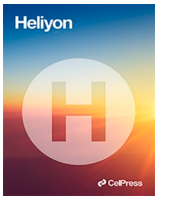

Research article

The effect of cigarette smoking on the oral microbiota in a South African population using subgingival plaque samples

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ABSTRACT

Disturbances in the oral microbiota 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 in a South African population using subgingival plaque samples. From the 128 recruited participants, 57 were identified as smokers (serum cotinine: >15 ng/ml). Analysis of 16S rRNA gene sequencing demonstrated significant differences between the two groups with a reduced abundance of Actinobacteria in smokers. Fusobacterium and Campylobacter were found in higher abundance, while a lower abundance of Leptotrichia, Actinomyces, Corynebacterium, and Lautropia were observed. This study highlighted significant differences in the oral microbiota of smokers, indicating an abundance of anaerobic gram-negative bacteria. These findings suggest that smoking allows certain oral microorganisms to gain dominance, thereby predisposing individuals to periodontal disease development and progression.

1. Introduction

Cigarette smoking has become a major global health and economic concern, contributing to a rapid increase in non-communicable diseases, especially in low- and middle-income countries. It is estimated that 21 % of mortalities in these countries are due to cardiovascular disease (CVD) [1,2]. Mortality due to tobacco intake is steadily increasing. Data from an epidemiological study demonstrated the relationship between smoking and CVDs and showed that smoking is linked to an estimated 20 % of deaths from CVD [3]. Cigarettes contain chemical toxins that directly access the oral microbiota and cause oral dysbiosis, which results in the depletion of vital oral microbes and promotes colonization by disease-causing pathogens, which ultimately initiate the onset of periodontal disease [4]. Cigarette smoking plays an important role in the host-pathogen interaction in the oral cavity, which includes responses to cell-mediated and humoral immune responses, encouraging dysbiosis in the oral microbiota. This dysbiosis depletes important oral

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microbiota, which may lead to bacteria becoming more harmful or less susceptible to immune defenses, potentially leading to more severe infections or increased resistance to treatment [5–7]. Similarly, smoking is associated with systemic inflammation, heightened platelet activation, and disrupted vascular smooth muscle function [8]. Chronic inflammation coupled with disrupted metabolic pathways, such as those related to low-density lipoprotein (LDL) cholesterol metabolism, cause atherosclerotic plaque accumulation in blood vessels, thus compromising endothelial and vascular functions [9,10]. Vascular dysregulation and abnormal platelet function contribute to the development and progression of CVDs [3]. Periodontitis has been linked to CVD risk, and research has shown that individuals who smoke cigarettes are more likely to develop both CVDs and periodontitis [11–13]. Dysbiosis and the associated inflammatory responses are associated with periodontitis [14]. Interestingly, the relationship between smoking, chronic inflammation, and bacterial disruptions may contribute to the development of CVDs and metabolic diseases [15,16].

Although tobacco use has declined since 2000, the World Health Organization (WHO) estimates that over 1.2 billion people will still smoke by 2025, and that many of these individuals reside in low- and middle-income countries [17]. In South Africa, government policies attempting to reduce smoking in the population have been successful; however, recent reports suggest that 17.6 % of adult South Africans still smoke tobacco [18]. Despite the dissemination of educational material regarding the harmful effects of smoking and secondary cigarette smoke inhalation, the habit persists at an increasing rate. Smoke inhalation, whether passive or active, rapidly disperses toxins within the epithelial lining fluid, leading to systemic absorption. Moreover, reactive oxygen species (ROS) are produced during active cigarette smoking due to the combustion process [19]. Research has shown that oxidative stress plays a pivotal role in the pathogenesis of pulmonary diseases and metabolic dysfunction [20,21], and correlates with the composition of the oral microbiome [22].

The arrangement of the oral microbiome can be affected by numerous factors, including the environment, antibiotic use, diet, alcohol, and tobacco use [23]. Studies regarding tobacco products in the European Union have revealed that cigarettes contain toxins and several microorganisms, including both soil and human bacteria [24,25], while others have observed that tobacco can suppress the innate immune system, including the activation of natural killer cells and neutrophils [26]. These studies have consequently led to the hypothesis that tobacco use can directly or indirectly affect microorganisms inhabiting the oral cavity [27]. Therefore, this study aimed to investigate the oral microbiota in smokers and non-smokers to identify whether smoking allows for the distribution of harmful microbiota, which may increase the risk of developing oral diseases and lead to systemic diseases, such as CVD.

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 (CPUT), Bellville South, Cape Town, South Africa. Ethical approval for the study was obtained from the Research Ethics Committees of the 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 microbiota study (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 VMH study, in which detailed procedures are described [28]. In this case-control study, 128 participants were selected from the 1989 individuals who had participated in the 2014/2016 VMH study. Anthropometric measurements, blood pressure measurements, and oral glucose tolerance tests (OGTTs) were performed on all participants. Plasma glucose, glycated hemoglobin (HBA1c), 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 wooden toothpick as previously described [29].

2.3. Smoking assessment

The STEPwise questionnaire following guidelines established in 2011, was used to assess the smoking status of all participants [30]. First, participants indicated their smoking status in the questionnaire. Second, blood tests were conducted to determine cotinine levels of the participants. Third, serum cotinine levels >15 ng/ml were used to validate smokers [31,32].

2.4. Plaque sample collection and periodontal assessment

Bleeding on probing (BP) was recorded for each tooth circumference and was recorded as absent or present. Regarding pocket depth (PD), each tooth was probed in its entirety and its highest measurement was then recorded. These assessments followed the guidelines outlined by the World Health Organization (2016) and the Community Periodontal Index. The plaque samples were collected using a wooden toothpick as previously described [29]. All toothpicks were sterilized by autoclaving following correct standards and a registered qualified oral hygienist collected the sample. Briefly, plaque samples were collected by inserting the wood toothpick into the subgingival crevice between the maxillary second premolar and the first upper molar. Four toothpick samples were collected from both sides of the mouth and stored separately at −80 °C until use.

2.5. DNA extraction and 16S rDNA gene amplicon sequencing

DNA from two pooled plaque toothpicks was extracted using a DNA extraction kit from Zymo Quick-DNA Fungal Bacterial Miniprep KIT (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. Ion 16S™ Metagenomic DNA (mgDNA) (Thermo Fisher Scientific, Waltham, MA, USA) was quantified using the Qubit™ 1 × dsDNA HS (high sensitivity) assay kit (Thermo Fisher Kit; MAN0017455 Rev. A.0). The NanoDrop ND-1000 was used to measure the purity of the mgDNA samples. LabChip GXII Touch and a DNA Reagent Kit (PerkinElmer, Waltham, MA, USA) were used for DNA sizing, quantitation, and purification according to the protocol provided (CLS140166 Rev. C; Supplementary Report: Genomic DNA [gDNA] Quality Control) using the Qubit 4.0 Fluorometer.

The Ion 16S Metagenomics Kit was used for amplification of the hypervariable region from the polybacterial subgingival DNA samples, following the MAN0010799 REV C.0 protocol. Target amplification was performed using 2 μL of mgDNA across two cycles with two primer pools using the SimpliAmp Thermal Cycler (Thermo Fisher Scientific). After verifying the amplification (using polymerase chain reaction; PCR), primer 1 (V2-4-8) and primer 2 (V3-6-7-9) pools were combined for each sample. Combining the two primer pools allows identification of bacteria from complex mixed populations. The amplified fragments were then sequenced on the Ion GeneStudio S5 system and analyzed using the metagenomics workflow in the Ion Reporter™ Software. Agencourt AMPure XP reagent was used for purification of eluted DNA. The Qubit 1 × dsDNA HS assay kit was used to quantify the purified amplicons using the Qubit 4.0 Fluorometer following the MAN001 7455 Rev. A.0 protocol.

2.5.1. Library preparation

The NEXTflex DNA Sequencing Kit was used to prepare the library. Thereafter, 100 ng of the pooled amplification product was used for each sample following the Bio Scientific Corporation protocol (v 15.12). 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 manufacturer's protocol (CLS145098 Rev. E).

2.5.2. Template preparation, enrichment, sequencing, and analysis

Library dilution was conducted by targeting a 10 pM concentration. Thereafter, the diluted 16S barcoded libraries were combined in equimolar amounts for template preparation using the Ion 510, Ion 520, and Ion 530 Chef Kit (Ion Chef Kit, Thermo Fisher Scientific). Briefly, 25 μL of the pooled library was loaded into the Ion Chef liquid handler with reagents, solutions, and supplies according to the manufacturer's 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 Kit was used to run massive parallel sequencing according to the manufacturer's protocol. 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 assignments were performed with specific software that groups sequences of very high similarity (97 %) using the database Quantitative Insights into Molecular Ecology (QIIME2), which selects operational taxonomic units (OTUs) and assigns taxonomic identities based on comparisons to sequences from the Greengenes reference database.

2.6. Statistical analysis

We used SPSS v.26 (IBM Corp, 2019) for data analysis. The data was tested for normality using Normal Q-Q Plots. The results were reported as mean ± standard deviation, median (25th and 75th percentiles), and counts (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. Microbiota data were presented in terms of relative abundance percent for phyla, genus, and species. Those comprising ≤1

Table 1
General characteristics of participants according to smoking status.

	Non-smoker n = 66	Smoker n = 57	*p*-value
Male, n (%)	21 [32]	13 [23]	0.265
Female, n (%)	45 (68.2)	44 (77.2)	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
Fasting plasma glucose, (mmol/L)[a]	5.70 (4.90; 7.63)	5.60 (4.70; 7.30)	0.544
Post 2-h glucose, (mmol/L)[a]	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
us-C-reactive Protein, (mg/L)[a]	3.75 (1.50; 8.59)	5.90 (2.07; 11.38)	0.088
ALT, (IU/L)[a]	20.0 (14.00; 27.00)	22.00 (13.00; 31.00)	0.944
AST, (IU/L)[a]	21.0 (17.25; 27.75)	25.00 (20.00; 33.00)	0.011
Cotinine, (ng/mL)[a]	10 (10; 10)	259 (183; 348)	<0.001
γ-Glutamyltransferase, IU/L[a]	31 (22; 57)	38 (26; 72)	0.164

[a] Median (25th and 75th percentiles).

% 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 phylum, genus, or species. Chao1, Shannon, and Simpson indices were used to determine alpha diversity, and EMPeror (v0.9.60) was used for principal coordinate analysis (PCA) to visualize beta diversity. Raw sequence data and taxonomy assignments were performed using QIIME 2. A multivariate logistic regression analysis was conducted to assess the presence of oral microbiome species in smokers compared to non-smokers. Various models were used, namely Model 1: Crude; Model 2: included age and sex; Model 3: included age, sex, and body mass index (BMI), Model 4: included age, sex, BMI, and bleeding. Values of $p < 0.05$ were used to characterize statistically significant results.

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.

The general characteristics of participants are indicated in Table 1. The average age of participants was similar between the two groups, i.e., smokers (46.7 years) and non-smokers (47.7 years). Furthermore, there were more female smokers (77.2 %) in comparison to non-smokers (68.2 %). 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. No significant difference was observed in the demographics, anthropometric measurements, or biochemical parameters between the two groups. Ultrasensitive C-reactive protein (CRP), an indicator of inflammation, was slightly elevated in smokers ($p = 0.088$). Furthermore, when assessing both the gingival bleeding and periodontal status (pocket depth) of smokers and non-smokers we found no significance among these groups ($p \geq 0.350$).

Based on the Chao Index, the alpha diversity appeared lower in smokers compared to non-smokers (Table 2). However, non-significance in species diversity was observed in smokers ($p = 0.05$) while using the Shannon Diversity Index.

The five most abundant phyla observed across all participants were *Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria,* and *Fusobacteria*, which constituted more than 98 % of the total number of phyla (see Fig. 1). *Actinobacteria* was the only phylum that appeared significantly lower in smokers ($p < 0.001$; Fig. 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, genera *Fusobacterium* ($p = 0.002$) and *Campylobacter* ($p = 0.010$) were seen in abundance, while a reduced significance was observed in *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 *Fusobacterium nucleatum* ($p < 0.001$), *Campylobacter gracilis* ($p = 0.010$), *Veilonella rogosae* ($p = 0.001$), *Fusobacterium canifelinum* ($p = 0.009$), and *Actinomyces odontolyticus* ($p = 0.003$), were significantly enriched among smokers, while *Campylobacter matruchotii* ($p = 0.002$), *Actinomyces dentalis* ($p = 0.033$), *Actinomyces naeslundii* ($p = 0.010$), *Campylobacter sputigena* ($p = 0.042$), and *Streptococcus sanguinis* ($p = 0.016$) were less abundant in smokers (Table 3).

In multivariable logistic regressions, the species *Fusobacterium canifelinum, Campylobacter gracilis, F. nucleatum, A. odontolyticus*, and *V. rogasae* were associated with higher odds of being present in smokers (odds ratio (OR) ≥1.07, 95 % confidence interval (CI): >1.03–1.12, $p \leq 0.012$), whilst *Actinomyces 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, $p \leq 0.040$) in crude models (Table 4).

4. Discussion

This case-control study compared the oral microbiota of 57 smokers and 66 non-smokers. We observed significant differences in the distribution of bacteria within the oral microbiota 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 favors gram-negative bacteria (gnb).

In smokers, we observed a significant reduction in *Actinobacteria*, which are gram-positive (gpb) anaerobic/aerobic bacteria, predominantly inhabiting the oral cavity and forming part of the commensals of the skin [33,34]. However, this finding contradicts the results obtained by other investigators [23,25,35], who have all reported an abundance of *Actinobacteria* phylum. Mason et al. reported an increase of anaerobes and a decrease of aerobes in smokers [36]. In support of this, we also observed depletion in aerobes, such as *Corynebacterium* (aerobe/anaerobic) and *Actinomyces* (gram-positive rod-shaped), which are associated with biofilm and plaque formation. Both these genera fall under the phylum *Actinobacteria.*

Although smoking creates an anaerobic environment through oxygen deprivation [35], our study noted that certain anaerobic bacteria were reduced, namely *Leptotrichia* and *Lautropia*. Both genera are gram-negative rod-shaped (gnb) facultative anaerobic

Table 2
Alpha diversity in species indices according to smoking status.

	Smoking status		*p*-value
	No	Yes	
Number of taxa	273	250	
Shannon	4.232	4.228	0.051
Chao1	273	250	<0.001
Simpson	0.0297	0.0283	0.511

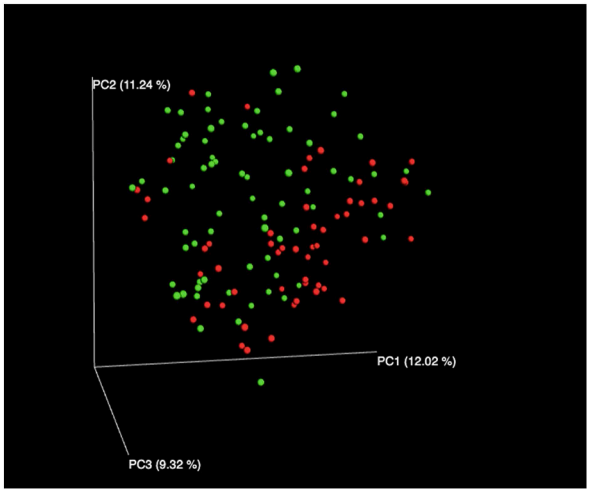

Fig. 1. Beta diversity comparisons of microbial communities in smokers and non-smokers. Smokers (red) and non-smokers (green) are shown to determine Bray–Curtis distances. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

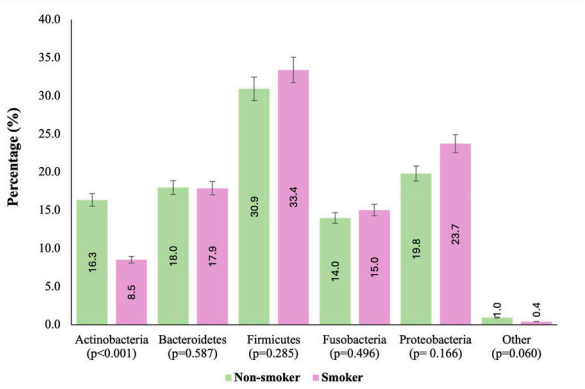

Fig. 2. Relative percentage abundance of phyla in smokers and non-smokers.

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 [25], which reported that *Corynebacterium* and *Leptotrichia* levels were significantly decreased in smokers, while other studies have reported enrichment of these genera [37,38].

The genera *Fusobacterium* and *Campylobacter* were both enriched in smokers. Both are gnb anaerobic microorganisms that have been linked with periodontal disease progression. *Fusobacterium* plays a vital role in dental biofilm formation and may explain why smoking has been shown to promote the formation of biofilm [39,40]. This genus, especially the species *F. nucleatum,* has also been associated with systemic diseases, including CVDs [41] and uncontrolled type-2 diabetes [42]. Similar to our findings, others have also reported an abundance of both *Fusobacterium* and *Campylobacter* in smokers and an association with the development of periodontal disease [37,43–45].

The species *F. nucleatum, C. gracilis, V. rogosae, F. canifelinum*, and *A. odontolyticus,* were enriched amongst smoking subjects in our study. Apart from *A. odintolyticus,* these species are gram-negative anaerobic microbiota that reside as commensals in the oral cavity. *F. nucleatum* is reportedly an important pathogen in severe periodontal disease, including gingivitis, whereas *F. canifelinum* plays a vital role in the subgingival biofilm function [46,47]. *C. gracilis* resides in deep sites of the oral cavity and is associated with different stages of periodontitis, including progression [48,49]. However, *V. rogosae* are commensals of saliva and reside on the dorsal and lateral surfaces of the tongue, but their association with smoking and periodontitis remains controversial [50,51]. Further, *A. odontolyticus* is a purple-complex bacteria known to be associated with the adherence and congregation of oral microorganisms, allowing bridging to both orange- and red-complex bacteria [52]. Further studies have associated this species of bacteria with chronic periodontitis and a role in plaque composition alteration leading to disease [53].

The differences in the oral microbiota 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. Therefore, it is suggested that smoking

Table 3
Relative percentage 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	1.39	0.99	0.082
				P. veroralis	1.13	0.85	0.087
				S. 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

Bold font indicates bacteria that were differentially distributed in smokers.

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: age and sex; Model 3: age, sex, and BMI; Model 4: age, sex, BMI, and bleeding; *calculated for 0.1-unit increase.

produces a variation in bacterial communities through various mechanisms. Research has shown that oral microbial species diversity is conserved in healthy individuals, while abundance is expected to differ in pathological conditions [25,54]. This concept has been supported by researchers who have shown alterations in the abundance of selected oral microbiota in smokers compared with non-smokers. The results from Mason et al. demonstrated variation across all taxonomic levels [36]. In our principal coordinate analysis, we discovered distinct clustering of the microbial communities based on smoking status.

The variability in the oral microbiota of smokers is controversial, with many researchers recording different findings [45,55]. These differences may be due to various factors, such as diet, pH changes, interactions among microorganisms, gene mutations, gene transfers, and different locations and methods of sampling [56,57]. In our study, subgingival plaque was collected using the toothpick method, while other studies have collected samples using oral washes and buccal swabs [23]. Furthermore, the techniques utilized 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 utilized the 454 Roche FLX Titanium pyrosequencing system [23]. Variations in methodologies may have further contributed to discordant results. Studies have shown that ethnicity may be a contributing factor to differences in the oral microbiota [58].

This study is limited by the fact that it was a case-control study with a small sample size. Further longitudinal studies on larger cohorts are recommended to thoroughly investigate the effects of smoking on oral microbiota and health. Future research should include novel approaches, such as log ratios and differential ranking methodologies to rank differential abundance using multinomial regression analysis, [59]. Furthermore, we acknowledge that the marker gene is limited by its low taxonomical resolution of 16S NGS sequence reads and we recommend that other in-house genes, such as RpoB, should be included in future studies [60,61]. Despite these limitations, we can conclude that the subgingival microbiota 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.

Socransky et al. divided periodontopathogenic bacteria into complexes depending on their properties and pathogenicity. They stated that green- and orange-associated complexes are known as “early colonizers”, thus forming the basis for colonizing the gingival sulcus (the space between the tooth and the surrounding gum tissue) with other periodontitis-associated bacteria [62]. Moreover, the orange species complex forms the bridge or link between the early colonizers and the highly pathogenic bacteria of the red complex. Our results show that smoking increases populations of red- and orange-complex bacteria, *F. nucleatum* and *C. gracilis*, and lowers populations of purple complex bacteria, such as *Actinomyces* species [63]. The pathogenic potential of these marker bacteria is significantly increased due to the production of various toxins and enzymes [64]. Our findings suggest that changes in the diversity and communities of the oral microbiota in South African smokers may create an environment that promotes periodontal disease development [35,40]. Our results serve as a foundation for future research to determine the link between smoking, oral health, and cardiovascular diseases.

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Compliance with ethical standards

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 (CPUT/HW-REC 2017/H31). A written consent form was signed by all participants after all procedures had been fully explained in a language of their choice.

Data availability statement

The datasets generated and/or analyzed during the current study are not publicly available due to the terms of consent to which participants agreed, but are available from the principal investigator of the main study upon reasonable request. The sequence data used to support the findings of this study have been deposited in the SRA BioProject database (accession number: PRJNA723337).

CRediT authorship contribution statement

Yvonne Prince: Writing – original draft, Methodology, Investigation, Formal analysis. **Glenda M. Davison:** Writing – review & editing, Supervision, Investigation, Formal analysis. **Saarah F.G. Davids:** Visualization, Methodology, Investigation, Formal analysis, Data curation. **Rajiv T. Erasmus:** Writing – review & editing, Supervision, Investigation, Conceptualization. **Andre P. Kengne:** Writing – review & editing, Methodology, Investigation. **Shanel Raghubeer:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Tandi E. Matsha:** Writing – review & editing, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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