ORIGINAL ARTICLE

Compositional profile of mucosal bacteriome of smokers and smokeless tobacco users

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Abstract

Introduction Smoked, and especially smokeless, tobacco are major causes of oral cancer globally. Here, we examine the oral bacteriome of smokers and of smokeless tobacco users, in comparison to healthy controls, using 16S rRNA gene sequencing.
Methods Oral swab samples were collected from smokers, smokeless tobacco users, and healthy controls ($n = 44$). Microbial DNA was extracted and the 16S rRNA gene profiled using the Illumina MiSeq platform. Sequencing reads were processed using DADA2, and taxonomical classification was performed using the phylogenetic placement method. Differentially abundant taxa were identified using DESeq2, while functional metagenomes based on KEGG orthology abundance were inferred using LIMMA.
Results A significantly higher microbial diversity was observed in smokeless tobacco users and smokers relative to controls ($P < 0.05$). Compositional differences in microbial communities were observed in all comparisons with healthy controls (PERMANOVA $P < 0.05$) but not between smokers and smokeless tobacco users. Levels of *Fusobacterium* spp., *Sacchari-bacterium* spp., and members of *Shuttleworthia* were elevated in smokers when compared to controls (BH *adj P* < 0.01). In addition, the relative abundance of three bacterial taxa belonging to genera *Fusobacterium* spp., *Catonella*, and *Fretibacterium* spp. was significantly increased in smokeless tobacco users relative to controls (BH *adj P* < 0.01). Major functional pathways significantly increased in smokeless tobacco users relative to both controls, and smokers were similar and involved amino acid metabolism including glutamate and aspartate biosynthesis and degradation (log FC > 1.5; BH *adj P* < 0.01).
Conclusions A distinct taxonomic and functional profile of oral microbiome in smokers and smokeless tobacco users as compared to healthy controls implicates a significant role of microbes and their metabolites in diseases associated with tobacco use including oral cancer.
Clinical relevance Future efforts in preventive, diagnostic, curative, and prognostic strategies for diseases associated with tobacco use in smokers and smokeless tobacco users could incorporate the oral microbiome.

Keywords Tobacco · Smoking · Smokeless tobacco · Microbiome · Microbiota · Oral microbiome

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Introduction

Despite a global decline in tobacco consumption, in Asian and African countries, it is rising, which is a significant public health concern [1, 2]. Although smoking accounts for much of this increase, smokeless tobacco (ST) is common in Asia and the Middle East and is used in many forms throughout the world [2, 3]. In particular, India has commercial products consisting of roasted, finely chopped tobacco with several other ingredients and flavoring agents called "Gutka" which is popular among young adults in India despite it being banned [4]. Added to this, India is the second-largest consumer of tobacco globally, and approximately 30% of adults use ST and 75% of these adults reside in rural areas [5, 6].

Several studies have indicated that the oral microbiome plays a key role in human health, and perturbations can influence disease progression [7, 8]. Host and environmental factors are known to alter the oral microbiome. This is particularly evidenced by the shift in abundance of certain oral bacteria in smokers compared with non-smokers [9–11]; nevertheless, one study did not find any difference between controls and smokers in various subsites except buccal mucosa which exhibited a lower alpha diversity in smokers [12]. However, the sample size from the latter study was too low for a definitive conclusion.

Smokeless tobacco products have been reported to have at least 30 carcinogens, including polycyclic aromatic hydrocarbons, tobacco-specific N-nitrosamines, aldehydes, and chemicals of known carcinogenic potential [13]. Therefore, it can be speculated to perturb the bacterial ecology of the oral cavity by direct toxicity, nutrient deprivation, or other mechanisms such as acid–base imbalance and alteration of salivary flow. Moreover, ST products are usually retained in the mouth for long hours and therefore in constant direct contact with oral mucosa and the associated microflora [14]. Thus, it may be expected that ST would have an impact on the survival and metabolic characteristics of oral bacteria. However, the currently available evidence is limited to laboratory data, e.g., in vitro studies have reported that nicotine affects the growth and viability of *Streptococcus mutans* in a dose-dependent manner [15, 16]. There is little evidence on the impact of smokeless tobacco on the diversity and abundance of the oral microbiota in humans.

Smokeless tobacco users have been reported to have poor oral hygiene and periodontitis, both of which are linked to oral microbiome dysbiosis and oral cancer risk [3, 17, 18]. Given the potential for tobacco use to exert influence over both the oral mucosa and associated microbes, it is important to determine the compositional and functional attributes of oral bacterial communities associated with smokeless tobacco use. This study aims to investigate the compositional and functional attributes of the oral bacteriome of smokeless tobacco users and smokers relative to controls by 16S r RNA metagenomic sequencing in an Indian population. A detailed understanding of these attributes associated with smokeless tobacco use can help to further elucidate their role in a range of oral, and possibly systemic, diseases.

Methods

Sample collection

This study was approved by the University of Hong Kong Ethics Review Board (UW 18–384) and Saveetha Dental College and Hospitals, Chennai (SRB/FACULTY/03/18/PHD/02). Written informed consent was obtained from all participants, and all methods in this study were performed in accordance with the relevant guidelines of the Declaration of Helsinki on biomedical research involving human subjects.

The cohort consisted of subjects categorized into three distinct exposure groups: smokers who were using either bidis (thin, hand-rolled cigarettes composed of tobacco wrapped in a "tendu" or "temburni" leaf) or cigarettes ($n = 17$), smokeless tobacco users with or without areca nut ($n = 14$), and age-matched controls ($n = 13$). Study subjects were recruited from oral health/tobacco awareness camp visits conducted among factory workers by Saveetha Dental College and Hospitals in Chennai. To avoid gender confounding, women were not included as the percentage of women smokers is very low compared to males in most parts of India, including Tamil Nadu. Detailed information on tobacco use obtained from the subjects is presented in Table 1.

Each subject was asked to have refrained from smoking, drinking, or eating for at least 30 min before sample collection. Carbon monoxide (CO) values of the expired air of the smokers were measured using a "piCOSmokerlyzer" monitor (Bedfont, UK) to assess whether concentrations would be associated with any changes in bacterial diversity. The smokers were asked to hold their breath for 15 s and then blow into the disposable mouthpiece attached to the device. The CO concentration (ppm) is then displayed on the digital monitor. Sampling of the buccal mucosa of the subjects was conducted using Isohelix Swab SK-2S (Isohelix, UK) that comes as a kit incorporating the swab and collection tube. The kit has a unique cap design that allows the clinician to snap after sampling and push the whole swab head off the shaft into the collection tube and seal for safe containment. The swab was rubbed over both buccal mucosae multiple times firmly for 1 min, and immediately, the swab was sealed in the collection tube, then transported on ice to be stored

at −80 °C. The samples were subsequently transported to Rajiv Gandhi Centre for Biotechnology, Trivandrum, India, on dry ice and stored at −80 °C.

Sample processing and sequencing

Samples were allowed to thaw, and 2 ml of phosphate-buffered saline (PBS) (Gibco, Life Technologies, USA) was added to the tube containing the swab and incubated at 4 °C for 6 h. The tube was then vortexed for 15 s and pelleted at 7500 rpm for 10 min. DNA was then extracted by QIAamp DNA Mini Kit (Qiagen, Manchester, UK) according to the manufacturer's instructions with an added step of lysozyme treatment from the appendix protocol "Isolation of Genomic DNA from Gram-Positive Bacteria" from Qiagen Handbook (Qiagen, Manchester, UK). Viz, the pellet was treated with 180 µl of an enzyme solution (20 mg/ml lysozyme [Sigma Aldrich, St. Louis, USA] in 20 mM Tris–HCl [pH 8.0], 2 mM EDTA, and 1.2% Triton-X 100) at 37 °C for 1 h.

Sample integrity was assessed by electrophoresis on 1% agarose gel. DNA concentrations were measured with a Qubit Fluorometer (Invitrogen). Amplification of bacterial DNA to create 16S libraries was performed using PCR primers targeting the 16S rRNA gene V3–V4 (319F-806R), and the products were purified with AmpureXPbeads (Agencourt). The primer sequence is 341F-ACTCCTACGGGA GGCAGCAG and 806R-GGACTACGTGGGTATCTAAT. After quantification by real-time quantitative PCR (RT-qPCR) (EvaGreenTM), qualified libraries were sequenced on the Illumina MiSeq System using the PE300 reagent kit.

Bioinformatics and statistical analysis

The quality control (QC) filter was performed using DADA2, and the amplicons were paired and clustered [19]. Phylotype and gene inference analyses were performed by first aligning the quality-controlled query reads to the reference alignment with Infernal, then placing them on the phylogenetic reference tree with pplacer [20]. Taxonomical classification and gene inferences were based on edge placement and consensus identity with either internal or terminal nodes using PAPRICA as described in Bowman and Ducklow [21]. Estimation of alpha diversity was performed using the vegan library in R and visualized by box plots. Permutational multivariate analysis of variance (PERMANOVA) was used to compare the overall bacterial composition between the groups. A P value < 0.05 was considered of nominal statistical significance. Differential abundances of various edges and functional pathways were analyzed by DESeq2 [22]. Functional pathway prediction was performed based on KEGG orthology abundance inferred using LIMMA [23].

Results

Samples were collected from 20 subjects in each group. However, 16 samples had to be removed before data analysis because of low-quality sequencing reads with multiple noises. The final data consisted of samples from 17 smokers, 14 smokeless tobacco users, and 13 age-matched controls. Duration of tobacco use among the subjects ranged from 1 to 12 years. The demographic details of the subjects are provided in Table 1. Smokeless tobacco users either used tobacco alone or in combination with "pan masala," which is a mixture of areca nut with slaked lime, catechu, and other flavoring agents.

The oral bacteriome profile is different in smokeless tobacco users and smokers compared to controls

To evaluate alterations in microbiota structure between the groups, we measured microbial alpha diversity using the Shannon index, Simpson index, and Pielou's evenness. Significantly higher diversity was observed in smokeless tobacco users and smokers relative to controls ($P < 0.05$; Fig. 1). The beta diversity was visualized using principal component analysis (PCoA) and canonical analysis of principal coordinates (CAP) as well as compared using

Table 1 Demographic characteristics of the three distinct groups

Parameter	Smokers	Chewers	Controls
Total no. of subjects	17	14	13
Age, mean (years)	33.05	32.92	33.69
Standard deviation (SD)	±3.91	±3.54	±4.66
Duration of use (years)			
<2	2	0	NA
2–5	7	8	
6–10	4	4	
>10	4	2	
Alcohol habit			
Yes	5	5	5
No	12	9	8
Types of smoking		NA	NA
Cigarettes	3		
Bidis	5		
Both	9		
Types of smokeless tobacco	NA		NA
Tobacco alone		6	
Tobacco + Pan masala		8	
Betel leaf and tobacco		0	
CO values (ppm)		NA	NA
<7*	5		
>7*	12		

Fig. 1 Box plots illustrating alpha diversity indices (Simpson index, Shannon diversity, and Pielou's evenness) in the microbiome of controls, smokeless tobacco users, and smokers samples. Median values and interquartile ranges have been indicated in the plots. ** indicates a significant difference between controls and smokers (P value < 0.05). *** indicates significant difference between controls and smokeless tobacco users (P value < 0.001)
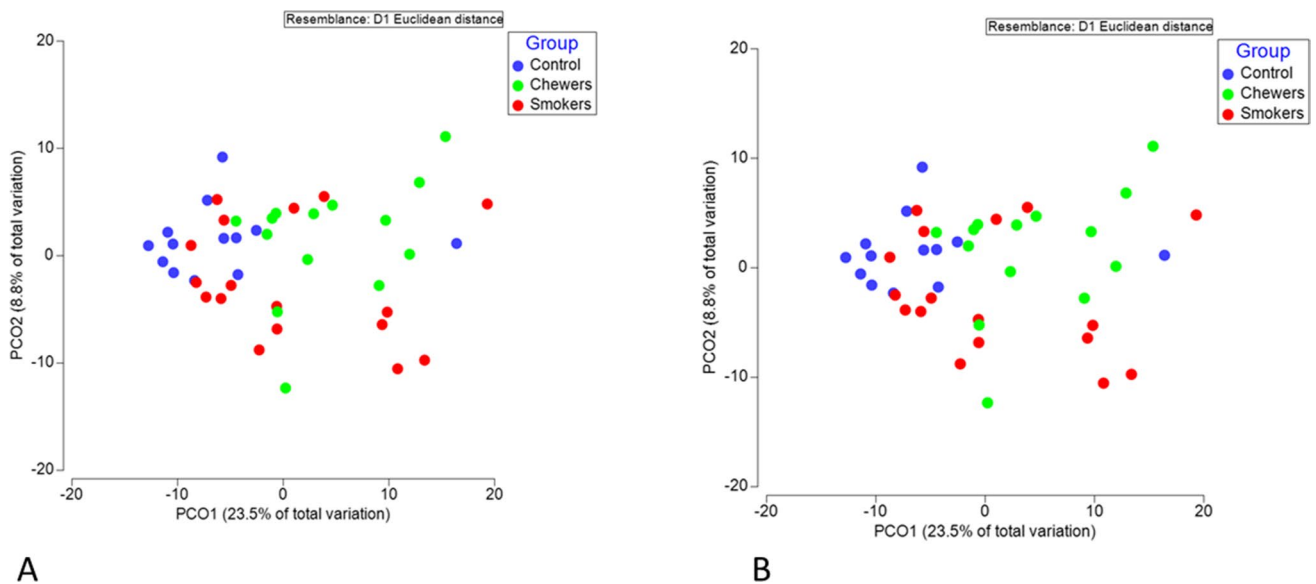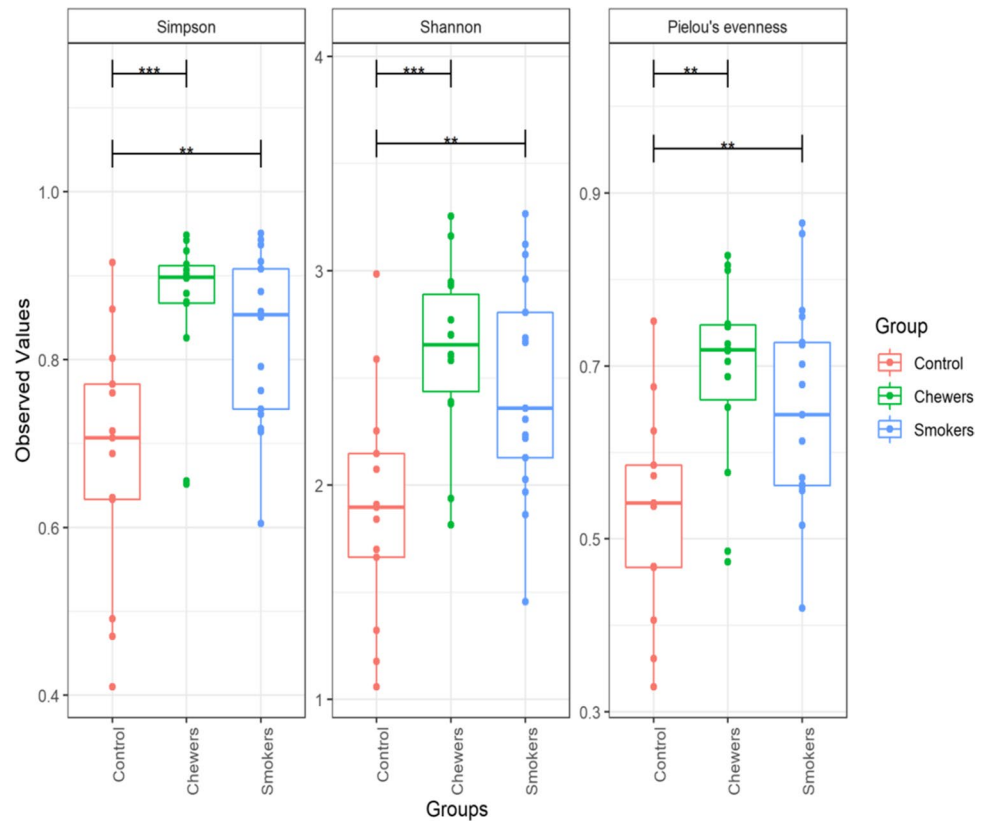

A

B

Fig. 2 **A** Principal component analysis (PCoA) and **B** canonical analysis of principal components (CAP) of β diversity in three groups. Log-transformed data were used for analysis. In PCoA, the first two principal components capture 8% (PCA1) and 23% (PCA2) of variance, respectively. CAP plot is a supervised PCoA, where the group was used as a dependent variable. The separation between the three groups remained significant ($P=0.002$, PERMANOVA). Each point corresponds to the composition of the microbiome in a sample in a group corresponding to the color coding

PERMANOVA global and pair-wise test (Fig. 2). PER-MANOVA remained significant overall, and pairwise comparisons demonstrated that the composition of controls was significantly different from that of smokeless tobacco users ($P = 0.003$) and smokers ($P = 0.024$). We also assessed whether any pattern of association could be detected between carbon monoxide values and oral microbiome in smokers; however, there was no specific clustering associated with CO levels (Supplementary Fig. 1).

The number of OTU in each group is as follows: chewer, 103; controls, 95; and smokers, 102. Overall, phyla *Fusobacteria*, *Firmicutes*, *Proteobacteria*, *Bacteriodetes*, *Actinobacteria*, and *Candidatus Saccharibacteria* accounted for the bulk of the bacteriome. Genera *Fusobacterium*, *Leptotrichia*, *Streptococcus*, *Saccharibacterium*, *Rothia*, *Capnocytophaga*, *Campylobacter*, *Atropobium*, *Fretibacterium*, *Streptophyta*, and *Parvimonas* were the most abundant in our samples. The abundances of the various taxa at phyla are illustrated in Fig. 3, and genus levels are illustrated in Fig. 4.

Smokers and smokeless tobacco users were associated with changes in specific oral taxa and inferred functional pathways relative to controls

Pairwise comparison by DeSeq2 analysis between smokeless tobacco users and controls illustrated three taxa belonging to genus *Fusobacterium*, *Catonella*, and *Fretibacterium* to be significantly increased in smokeless tobacco users relative to controls (Fig. 5). An increase in four taxa belonging to genus *Fusobacterium* and *Campylobacter* was identified in smokeless tobacco users in comparison with smokers. Three taxa that belong to the genus *Fusobacterium*, *Shuttleworthia*, and *Saccharibacterium* were elevated in smokers in comparison to controls. Thus, the use of tobacco, regardless of smokeless or smoking, altered the bacterial composition of the oral cavity and with a notable increased abundance of members belonging to genus *Fusobacteria*.

To infer the functional potential based on the bacterial community profiles deduced from the 16S rRNA gene
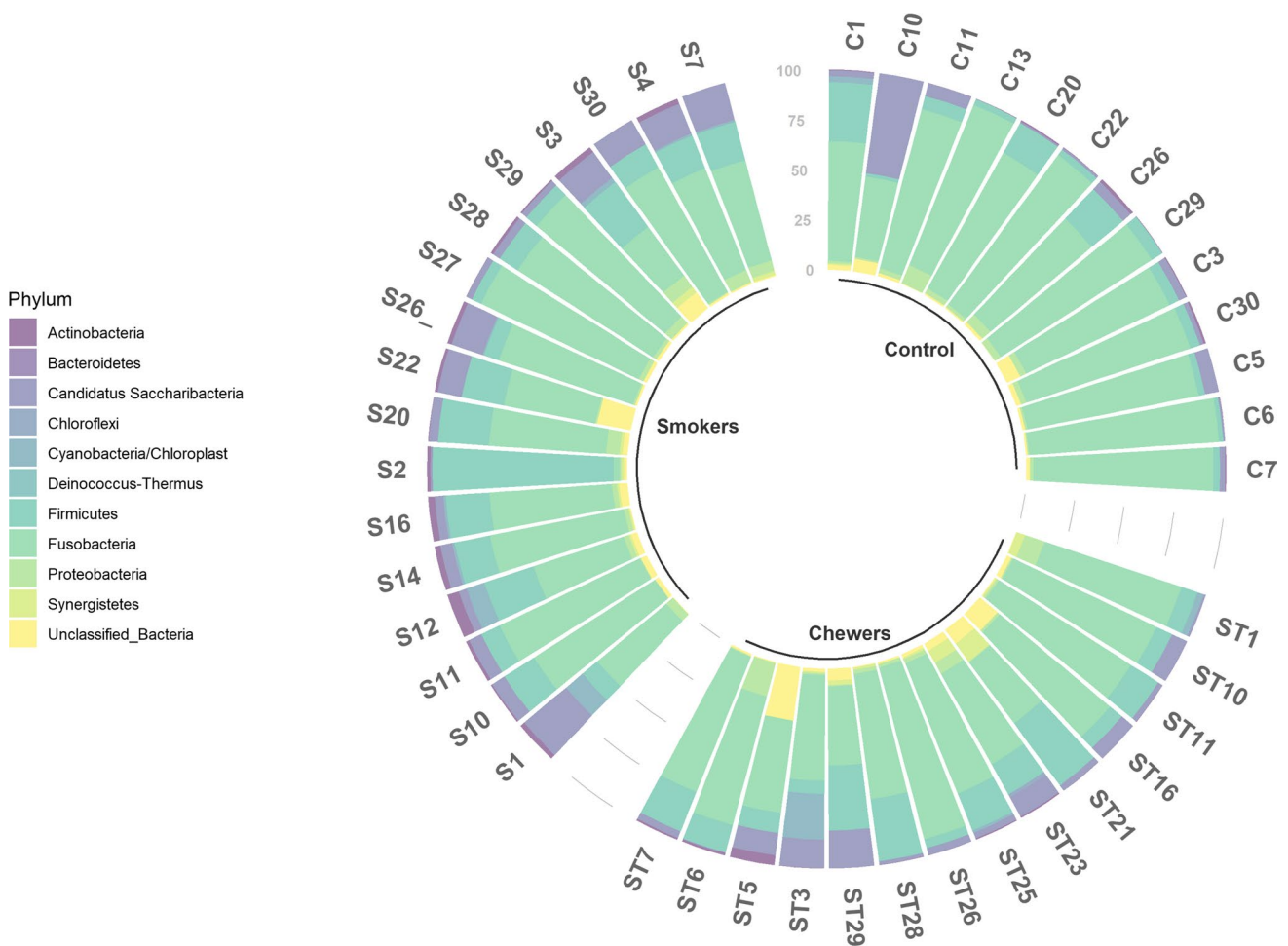

Fig. 3 Circular plot illustrating the distribution of various phyla in the three groups of subjects
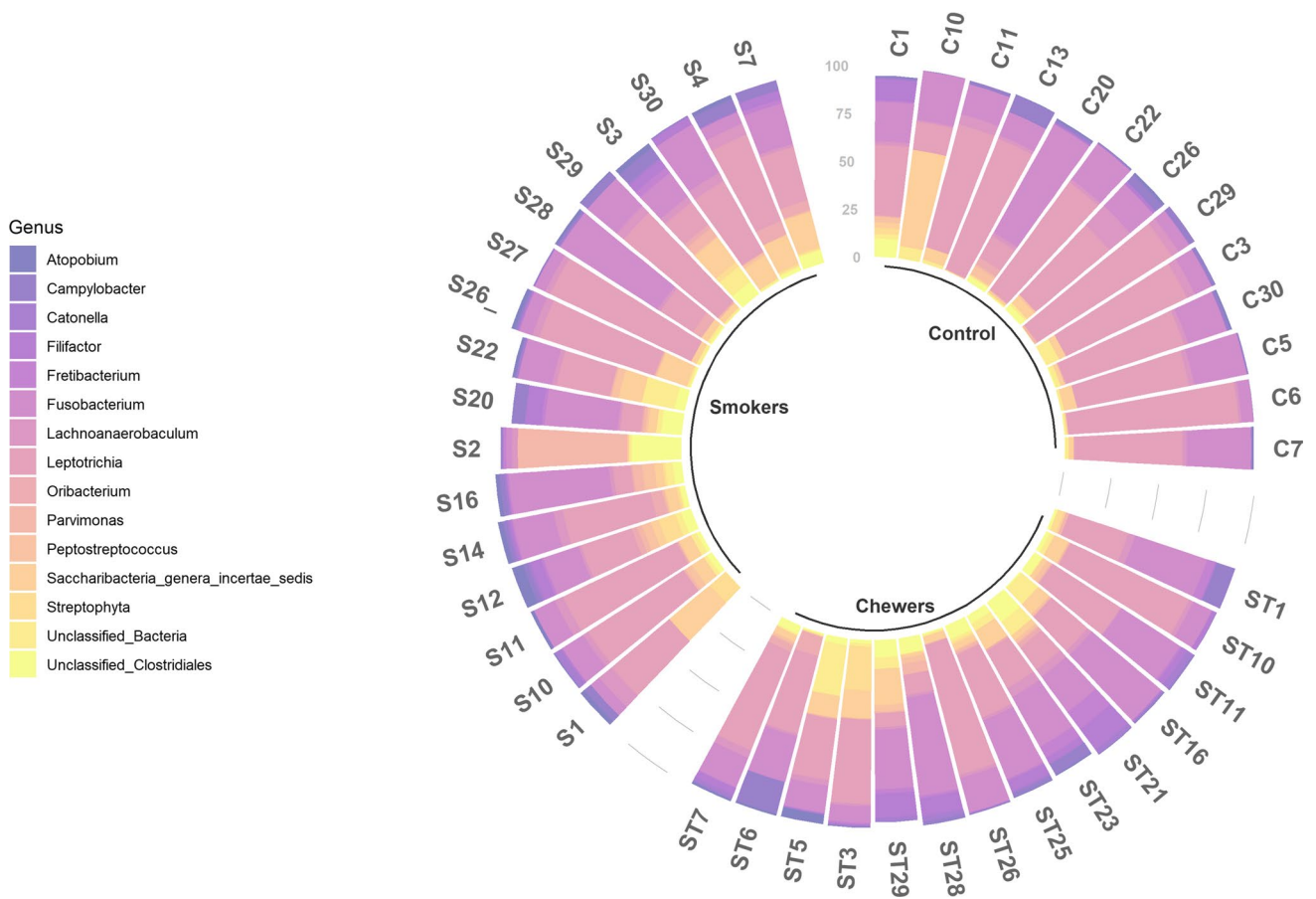
Fig. 4 Circular plot illustrating the distribution of various genera in three groups

Fig. 5 Differentially abundant taxa identified in smokeless tobacco users, smokers, and controls using DESeq2. Each side of the "triangle" represents pair-wise comparison. The colors of the boxes indicate the group, which shows the increased presence of those taxa listed relative to the other group in comparison
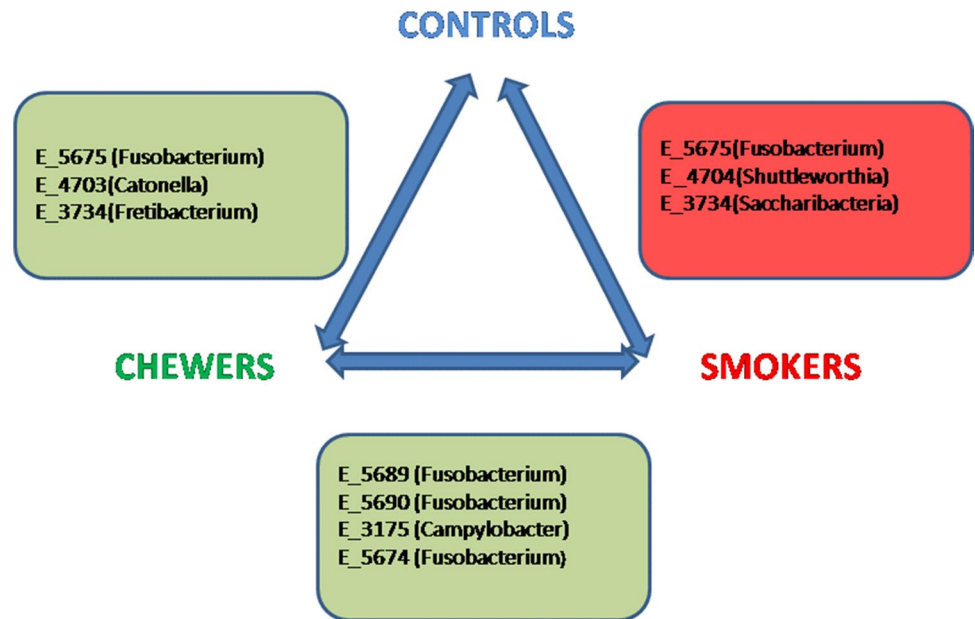
sequences, we used the PAPRICA-based prediction method. Pair-wise comparisons of the groups were performed using LIMMA to determine which pathways are differentially expressed. Functional pathways that are abundant in each of the study groups are shown in Fig. 6 and are significantly different. Seven pathways were more abundant in smokers compared to controls. Importantly, ten pathways were significantly increased in smokeless tobacco users relative to both controls and smokers (Fig. 6). These were similar and related primarily to amino acid metabolism, including glutamate and aspartate biosynthesis and degradation. Smokers showed an increase in pathways related to nucleotide biosynthesis and glycine metabolism relative to controls.

Discussion

To the best of our knowledge, this is the first study comparing the oral microbiome of smokeless tobacco (ST) users and smokers relative to normal controls using 16S r RNA metagenomic sequencing. Our data illustrate that the use of ST increases bacterial diversity and alters bacterial functional pathways. These are important observations as ST is a significant risk factor for the development of oral cancer and oral, potentially malignant, disorders.

Quantifying diversity is of central importance for the study of structure, function, and evolution of microbial communities, and Shannon and Simpson diversity indices are recommended to compare microbial diversity [24]. We found that the use of tobacco (smoking and smokeless) was significantly associated ($P < 0.05$) with greater diversity in the oral microbiome. Similar observations have been reported by others regarding smoking [25, 26]. Several hypotheses could be suggested based on the known impacts of tobacco on oral bacteria. These include immunomodulatory effects such as significant loss of antibody responses and defects in antigen mediated signaling [27], changes to oxygen tension [28], microbial attachments to mucosal surfaces [29], impaired functions of oral polymorphonuclear leukocytes [30], and even direct toxic effects [31]. It is postulated that a healthy niche, as part of homeostasis, has more members from the same species instead of several species, but when a niche is altered, e.g., due to smoking, the niche is susceptible to colonization by potential pathogens [32]. This is likely to be applicable to the use of smokeless tobacco as well. Overall, a stable microbiome is associated with oral health, and an increase in diversity induced by tobacco could contribute to dysbiosis [32].

There are few laboratory studies on the effects of smokeless tobacco on oral bacteria, and we are not aware of any
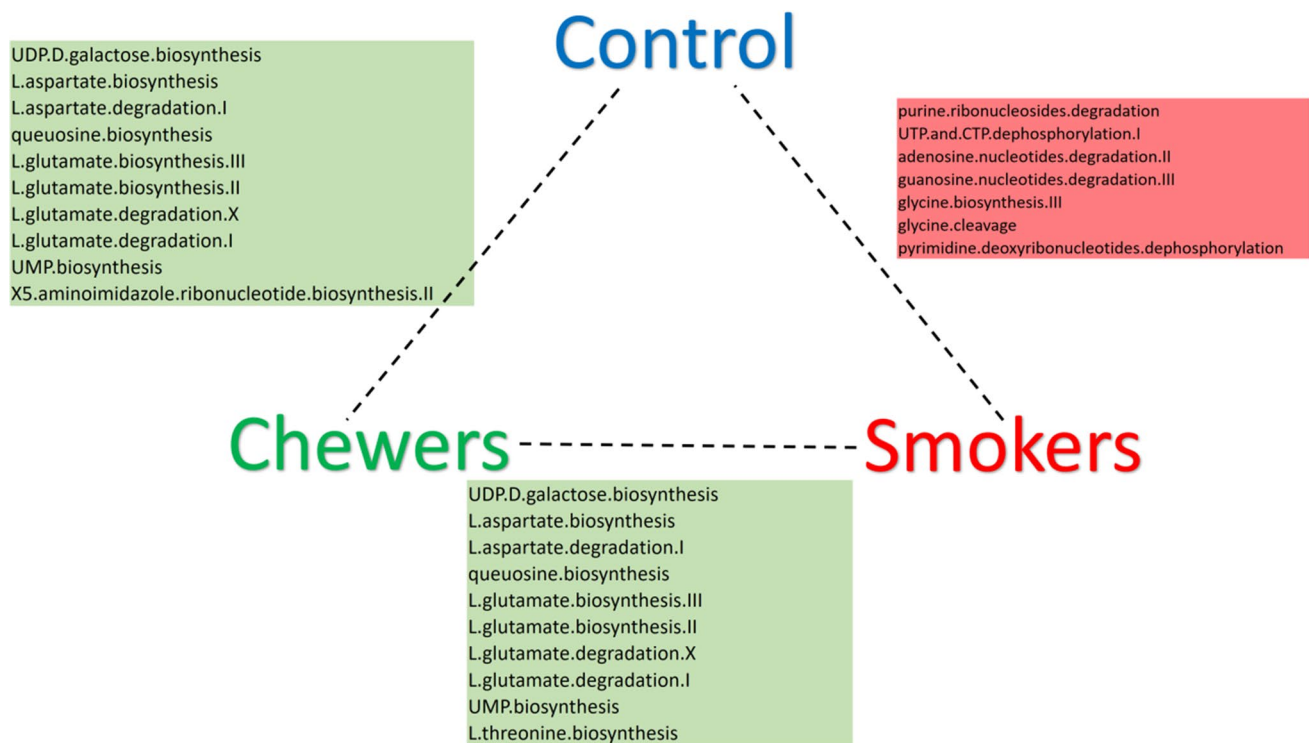

Fig. 6 Differentially abundant functional pathways identified in smokeless tobacco users, smokers, and controls using DESeq2. Each side of the "triangle" represents pair-wise comparison. The color of the boxes indicates the group which shows the increased presence of those metabolic pathways listed relative to the group in comparison

study to date that has investigated the effects of smokeless tobacco use on the human oral mucosal microbiome. In a previous in vitro study, fermentation of smokeless tobacco by microbes was observed to increase the production of nitrosamines, signifying clear carcinogenic potential [33]. Moreover, smokeless tobacco has been shown to have a dose-dependent impact on the composition of the oral microbiome in the golden hamster, and different taxa showed different responses over a 4-week period [34]. In this study, the authors reported an increase in the relative abundance of eighteen genera including *Streptococcus*, *Actinomyces*, *Granulicatella*, *Eubacterium*, and *Oribacterium* as well as a decrease in five genera including *Bacteroides*, *Dialister*, and *Leptotrichia*. Our results indicate an increased abundance of *Fusobacterium* spp. in tobacco smokeless tobacco users relative to controls and smokers. *Fusobacterium* spp. are facultative anaerobes, and their cell viability is enhanced in the presence of smokeless tobacco in vitro which may explain their presence in smokeless tobacco users relative to the other two groups. *Fusobacterium nucleatum*, a significant species belonging to this genus, has been regarded as an opportunistic pathogen, is known for its coaggregation properties, and has been shown to act as a bridge between otherwise non-aggregating bacteria [35]. Beyond the facilitation of simple adherence, it has also been shown to benefit the community by facilitating the survival of obligate anaerobes in an aerated environment [35–37]. Additionally, they are capable of growth in acidic conditions and neutralize the acidic microenvironment through amino acid catabolism [36, 37]. Fusobacterium proteins induce lymphocyte death which may contribute to oncogenesis and tumor progression [37]. Several in vitro and in vivo studies have highlighted the role of *Fusobacterium nucleatum* in colorectal carcinogenesis and hence are implicated; they are currently being regarded as "oncobacteria," contributing in multifaceted ways [37]. Interactions with other members of the microbial community are also important [38] and need to be studied in the pathogenesis of various tobacco-associated disorders of the oral cavity. Apart from Fusobacterium spp., we have observed increased abundances of bacteria belonging to genus *Fretibacterium* (phylum *Synergistetes*) and *Catonella* (phylum *Firmicutes)* in smokeless tobacco users relative to controls. Even though these have been previously reported as associated with periodontitis in smokers, literature regarding their pathogenic characteristics is limited as these are relatively recently characterized organisms [39, 40].

Our data also suggests that there is an increase in the abundance of anaerobes including *Fusobacterium* spp. in smokers. Our results are also in agreement with previous studies on salivary fluids [9–11, 41] that suggested alterations in the abundance of bacteria favoring an anaerobic microenvironment in smokers relative to controls. This is explicable as buccal mucosal surfaces are constantly bathed in saliva. However, it has been shown that smokers demonstrated diverse colonization of plaque biofilms with genera *Fusobacterium*, *Cardiobacterium*, *Synergistes*, and *Selenomonas* colonizing the early biofilms of smokers, depicting that smoking favors the acquisition and colonization of anaerobic bacteria in plaque biofilms [42]. Though buccal mucosa is a distinct microbial niche in comparison to plaque biofilm on the tooth surface, it seems rational to assume that the impact of the smoking caused by the various compounds in cigarette smoke is essentially the same in all microenvironments.

In addition, we report for the first time the significantly increased abundance of genus *Saccharibacterium* and *Shuttleworthia* in smokers compared to controls. *Saccharibacterium* is a relatively complex genus of phylogenetically diverse microorganisms which prefer anaerobic conditions with the capability of degradation of several organic compounds, including sugars, independent of the presence of oxygen [43], whereas *Shuttleworthia* is a recently identified obligate anaerobe [44]. This is consistent with in vitro studies which show that tobacco smoke generates anaerobic conditions [45] and reduces aerobic respiration [46].

Our analysis of inferred functions of oral bacteriome indicates further differences in tobacco users (smokers and smokeless tobacco users) relative to controls, who exhibited a greater abundance of glycolytic metabolism pathways. Most of the elevated pathways in smokeless tobacco users and smokers relate to amino acid metabolism. Aromatic amino acid catabolism in bacteria leads to the generation of excessive toxic metabolites including putrescine, spermidine, cresols, indoles, and phenols [47–50]. Amino acid deamination also leads to the consumption of more oxygen [47]. Moreover, amino acid metabolites, including polyamines and ammonia, have been shown to modulate cell signaling pathways, neutrophil gene transcription, apoptosis, and cytokine release [47–50]. In addition to amino acid metabolism, purine and pyrimidine metabolism were also increased in smokers relative to controls. A recent study reported that microbiome traits differ in smokers without and with head and neck cancer, with the degradation of amines and other xenobiotics prevailing in the latter group [51].

While other studies have used self-reported questionnaires about smoking habits, we validated the smoking status of our subjects by CO measurement. However, we found no associations between CO values and the bacteriome data, perhaps because of the modest sample size (Supplementary Fig. 1). The study is underpowered to detect subtle changes with subtypes and frequency of tobacco use: our smokers were using either cigarette and/or bidi; smokeless tobacco users were commercial gutka users.

Conclusion

Our findings suggest that tobacco, irrespective of the mode of use, create a microenvironment favoring anaerobes. While taxonomic similarities were noted between the oral microbiome of smokers and ST users, overall, the inferred functional pathways of the bacteriome of ST users favor amino acid metabolism over saccharolytic metabolism. Genera involved in these shifts are increasingly associated with oncogenesis in the mouth and elsewhere in the gastrointestinal tract, implying that studies of the oral microbiome might have predictive value, and that good oral hygiene or antimicrobial treatment might reduce the risk of cancer.

Declarations

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

Conflict of interest The authors declare no competing interests.

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