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Oral Microbiome Differences Between Smokers and Non-Smokers Highlight a Small Unique Cluster

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Abstract

Smoking perturbs the oral environment leading to microbial dysbiosis that may contribute to adverse health outcomes. While smoking-related shifts in the oral microbiome have been increasingly studied, data from South Asian urban centers remain limited. In this study, we characterized the oral microbiome of Kathmandu residents to investigate differences between smokers and non-smokers. Using 16S rRNA sequencing targeting the V3–V4 regions, we profiled microbial composition at both the phylum and genus levels. Across all samples, Proteobacteria (~52%) and Firmicutes (~26%) were the dominant phyla, while Streptococcus (~15%) and Pseudomonas (~11%) were the most abundant genera. Microbial diversity at both alpha and beta levels did not differ significantly between smokers and non-smokers. However, subgroup analysis among smokers identified 64 differentially abundant genera distinguishing two smoker groups. One subgroup (Smoker-I) was enriched with several Gram-negative genera, including Aeromonas, Klebsiella, Enterobacter, Yersinia, Salmonella, Serratia, Vibrio, and Campylobacter, as well as rare genera like Nocardia, Gordonia, and Rhodococcus. These distinct microbial patterns suggest that varying levels of tobacco exposure selectively reshape oral microbiome composition and suggest that not all smokers are microbiologically alike. The emergence of distinct microbial signatures in smoker subpopulations points to potential differences in behavior, exposure, or health status that warrant further investigation. Incorporating microbiome stratification may offer new insights into differential health risks among smokers.

Keywords: 16S rRNA sequencing, Smoking, Oral microbiome, Microbial diversity, Buccal cavity, Microbial dysbiosis

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Introduction

Human microbiome consists of a plethora of microorganisms that reside both in internal and external body surfaces, and play essential roles in host physiology, including digestion [1], metabolism [2,3], and immune regulation [4]. The oral microbiome is the second most diverse microbial ecosystem, with over 700 bacterial species [5,6]. This complex microbial community is involved in maintaining oral health and has been increasingly associated with systemic diseases such as periodontitis, colorectal cancer, mumps, herpesvirus infections, HIV/AIDS, and varicella (chickenpox) [5–12]. Therefore, the oral microbiome has become a key area of research seeking to understand host–microbe interactions and their implications for human health and disease.

The heterogeneity of oral cavity microflora is influenced by a multitude of conditions including environmental components, dietary habits, and host genetic background [13,14]. Among these, tobacco smoking has been reported to dramatically influence the oral microbiome landscape [15–18]. Tobacco smoke introduces toxic chemicals and particulate matter that perturbs the oral environment leading to microbial dysbiosis and the growth of pathogenic taxa [17,19–23]. Cigarette smoking remains a widespread behavior with numerous adverse health outcomes, including respiratory diseases, cardiovascular diseases, and cancers [9]. These findings highlight the importance for further investigation regarding how smoking impacts the oral microbiome and its potential implications for health and disease.

Tobacco use remains a major public health concern in Nepal. In 2022, an estimated 5.4 million individuals (4.3 million males and 1.1 million females) were reported to be tobacco users [24–26]. Although tobacco consumption is highly prevalent, there has been limited research investigating its impact on the oral microbiome diversity in this population. The Kathmandu Valley, capital of Nepal, is a densely populated urban area which represents a unique setting for such studies. In

addition to high rates of tobacco use, Kathmandu is also characterized by some of the highest levels of air pollution in South Asia [27]. While the influence of these exposures on the oral microbiota remains largely unexplored, characterizing the oral microbiome of individuals in this area provides an opportunity to uncover how tobacco use may define microbial diversity.

In this study, we aim to profile the oral microbiota of Kathmandu residents to study the influence of tobacco use on microbial diversity. By comparing the oral microbiomes of smokers and non-smokers, we categorize the differences in microbial diversity and taxonomic structure related with smoking in this understudied population.

Materials and methods

Sample Collection and Sample Size

All procedures were conducted in accordance with the ethical standards of the Nepal Health Research Council (NHRC). Ethical approval for the collection of human buccal mucosa samples was obtained from the Institutional Review Board of Yeti Health Science Academy (Proposal ID Number: 2080/81-231) on 09 June 2023, which is registered with the NHRC.

Samples were collected from adult males aged 18 to 64 years residing in Kathmandu. Inclusion criteria required participants to have abstained from antibiotic use for at least two months prior to sampling and not to be undergoing any immunosuppressive therapy at the time of collection. Additionally, participants refrained from alcohol consumption for at least 12 hours before sample collection. A total of 25 samples were collected, comprising 15 smokers and 10 non-smokers. Smokers were defined as individuals who had consumed at least one tobacco product within the past three days and had a regular smoking history of at least one year. Non-smokers were defined as individuals who had been tobacco-free for one year or more.

To standardize the sample collection process, participants were instructed to rinse their mouths thoroughly with clean water prior to sample collection. The buccal cavity was then gently swabbed for one minute using a sterile sample collection swab. To ensure the reliability of results, duplicate samples were collected from each participant. Swabs were immediately placed into cryogenic storage vials containing 3 mL of cold phosphate-buffered saline (PBS, pH 7.0). Unless immediate analysis was required, samples were stored overnight at 4°C prior to further processing. Short term storage of samples at 4°C helps avoid freeze-thaw cycles and have been shown to have minimal impact in bacterial biodiversity [28].

DNA Isolation and Validation

Collected samples were vortexed for 2 minutes to ensure thorough homogenization. Genomic DNA was extracted using Quick-DNA/RNA™ Pathogen Miniprep kit (Zymo Research, Cat. No.: R1042) following the manufacturer's protocol. The extracted DNA was analyzed on a 1.5% agarose gel to evaluate its integrity, size, quantity, and overall extraction quality.

PCR Amplification

16S metagenomic sequencing library preparation was performed based on the Illumina's standard protocol with necessary modifications. Two universal primers with the sequence 5'-CCTACGGGNGGCWGCAG-3' for forward and 5'-GACTACHVGGGTATCTAATCC-3' for reverse were used to amplify the V3-V4 region of the 16s rRNA gene [29]. The targeted amplicon size was approximately 550bp. The 25µL PCR mixture contained 12.5µL Kappa (2X; KAPA HiFi HotStart Library Amp Kit Ref: 07958960001), 5 µL 16s_F_illumina i.e., forward primer (1 pmol/µL), 5 µL 16s_R_illumina i.e., reverse primer (1 pmol/µL), and 2.5 µL template (1 pmol/µL).

PCR amplification was carried out using the following thermal cycling conditions: an initial denaturation at 95°C for 3 minutes; followed by 35 cycles of denaturation at 98°C for 30 seconds, annealing at 65°C for 25 seconds, and extension at 72°C for 20 seconds; with a final extension at 72°C for 5 minutes. The amplified products were again visualized on a 1.5% agarose gel for quality control confirming size of amplicon to be ~550bp.

To ensure that only the targeted amplicons were taken to the further library preparation step, the amplicons were purified using AMPure XP beads following the manufacturer's protocol. This ensures the removal of any free primers, nucleotides, or any inhibitors from the PCR reaction that could interfere with the library preparation step. The concentration of the purified amplicons were measured using a Qubit 3.0 Fluorometer with Qubit™ dsDNA BR Assay Kit (Cat. No.: Q32850) following the manufacturer's instructions. The quantified amplicons were then used to construct the library using NEBNext Companion Module for Oxford Nanopore and Oxford Nanopore Native Barcoding Kit (SQK-NBD114.24), adhering to standard protocols. Sequencing was performed on the MinION platform (Oxford Nanopore Technologies) using a Flongle flow cell at the Center for Molecular Dynamics Nepal (Thapathali, Kathmandu, Nepal).

Data Analysis

The obtained FASTQ format files of sequenced data were processed using the wf-metagenomics pipeline from epi2me-labs (https://github.com/epi2me-labs/wf-metagenomics), executed via the Nextflow workflow manager in a Linux environment. Specifically designed for Oxford Nanopore sequencing data, this workflow first removed host-derived sequences by mapping reads against a reference host genome. The remaining reads were then categorized using Kraken2(30,31], a k-mer-based taxonomic assignment program that aligned the reads to a reference library of bacterial, fungal, and viral genomes, enabling microbial species identification in the samples. The pipeline was executed in real-time mode, processing readings in batches of 1000 with a read limit of 4000, streamlining the analysis. The final output included an HTML report containing a taxonomic abundance table that summarized the relative abundance of detected microbial species.

Statistical Analysis

Alpha (microbial diversity within a sample) and beta (microbial diversity between the samples) diversity were computed from the taxonomic abundance table and visualized using the R programming language [32] with the vegan (31] and betapart [33] packages. The Mann-Whitney U Test was used to compare alpha diversity metrics between groups. The U statistic was used to assess the differences in the Shannon index, Simpson index, and Inverse Simpson index of microbial alpha diversity within samples between groups. Likewise, beta diversity was assessed using the Bray-Curtis dissimilarity index, followed by analysis of variance (ANOVA) to assess statistical differences between groups, with p-values used to evaluate the significance of these differences. A p-value less than 0.05 was considered to be significant.

Results

Identification of oral microbiome of Kathmandu residents

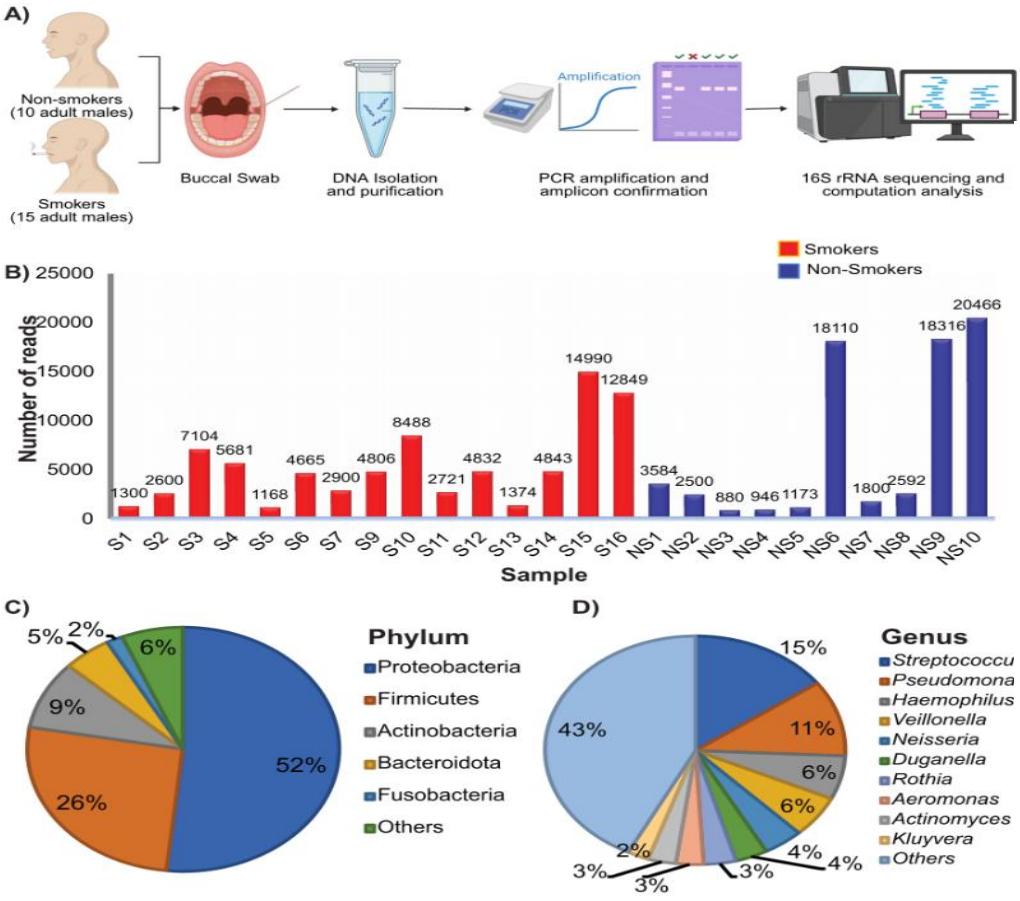

Figure 1. Identification of oral microbiome of Kathmandu residents. A) Methodology for identification of microbial diversity in the buccal cavity of Kathmandu residents using 16s rRNA sequencing. B) Bar graph showing the number of reads for each barcoded sample. Smokers are shown in red and non-smokers in blue. C-D) Pie charts illustrating the taxonomic composition at the phylum (C) and genus (D) levels of bacterial communities from samples collected from Kathmandu residents.

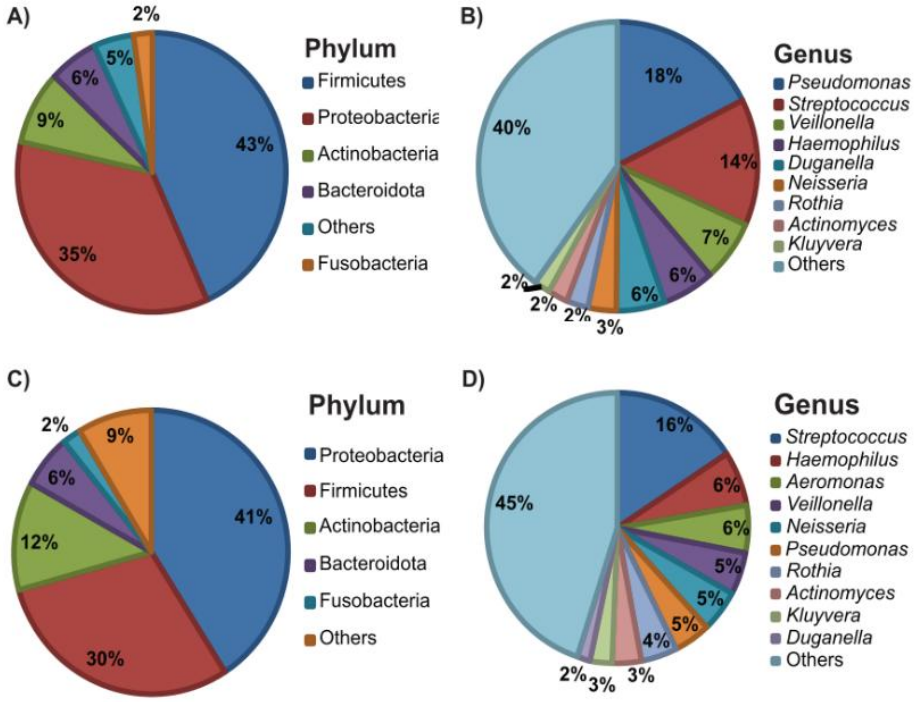

Figure 2. Comparison of microbial composition in the buccal cavity between non-smokers and smokers**. A-B)** Pie charts illustrating taxonomic composition at the phylum **(A)** and genus **(B)** levels of bacterial communities in non -smokers. **C-D)** Pie charts illustrating taxonomic composition at the phylum **(C)** and genus **(D)** level compositions of bacterial communities in smokers.

To investigate microbial diversity in the buccal cavity of Kathmandu residents, buccal swabs were collected from male participants and subjected to 16S rRNA gene sequencing. This study aimed to determine whether tobacco use influences microbial diversity and composition in the oral cavity. Participants were selected based on specific inclusion criteria (see Methods) and categorized as smokers (n = 15) or non-smokers (n = 10) according to their tobacco usage. Genomic DNA was extracted from each swab to profile the microbial community, targeting the V3–V4 hypervariable regions of the 16S rRNA gene (**Figure 1A**). Sequencing data were processed using the wf-metagenomics pipeline from Epi2me Labs, which removed host-derived reads and assigned microbial taxa using Kraken2. The number of reads obtained per sample varied substantially among individuals, regardless of smoking status (**Figure 1B**).

Microbial composition was characterized at both the phylum and genus levels, revealing the dominant taxa present across samples. The most abundant phyla were Proteobacteria (~52%) and Firmicutes (~26%), consistent with previous reports of oral microbiota (**Figure 1C**) (34]. At the genus level, *Streptococcus* (~15%) and *Pseudomonas* (~11%) were the most prevalent in the buccal cavity of Kathmandu residents (**Figure 1D**). We further characterized the phylum (**Figure 2A, 2C**) and genus (**Figure 2B, 2D**) for non-smokers and smokers separately. While the overall phylum-level composition was similar between the two groups, notable differences emerged at the genus level. Pseudomonas accounted for 18% of the genera in non-smokers but only 5% in smokers, whereas Aeromonas represented approximately 6% of the genera in smokers and was not a large group in non-smokers. These taxonomic profiles offer a foundational overview of the oral microbiome structure and suggest that varying levels of tobacco exposure may selectively enrich or deplete specific microbial genera, contributing to distinct microbial signatures in smokers and non-smokers.

Alpha and Beta Diversity Analyses Reveal Comparable Microbial Richness and Evenness Between Smokers and Non-Smokers

Next, we assessed the microbial diversity within-sample, or alpha diversity using four different indices - Shannon index (diversity index that considers both number of species and evenness of their distribution), Simpson index (probability that two random sample are from the same community), Inverse Simpson index and Species richness (number of species per sample) [35]. These metrics capture both the number of unique taxa present and how evenly they are distributed within each sample. Comparing the alpha diversity between smokers and non-smokers, we observed no significant differences in alpha diversity values (**Figure 3A-D**). This indicates that the overall richness and evenness of the microbial communities are largely maintained regardless of smoking status in our cohort.

To further evaluate differences in overall microbial community structure between smokers and non-smokers, we performed beta diversity analysis using the

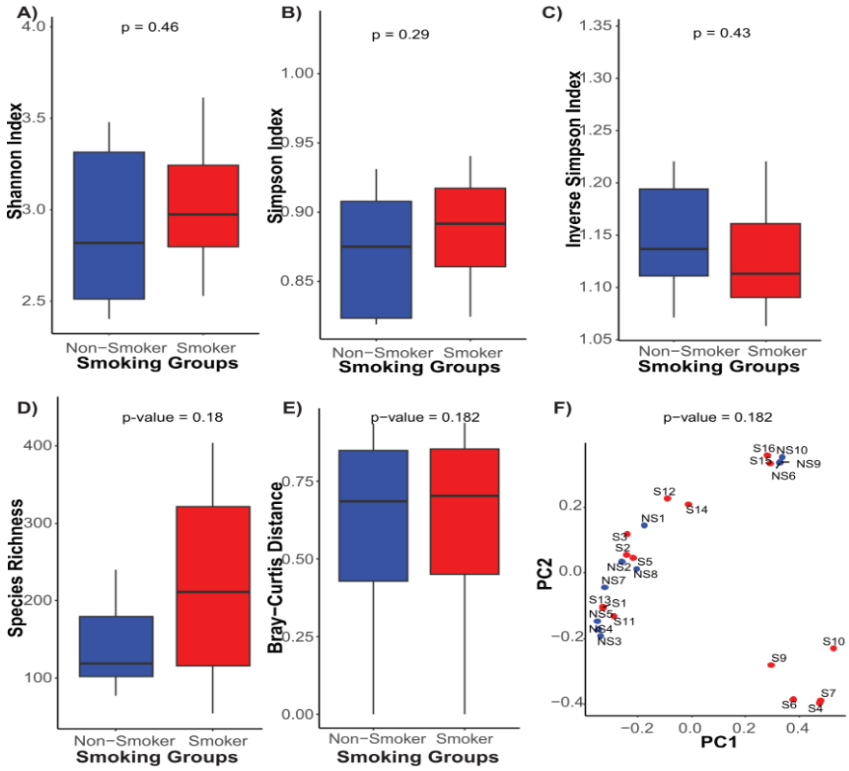

Figure 3. Comparison of alpha and beta diversity of buccal microbial communities in non-smokers and smokers. **A-C)** Box-and-whisker plots comparing alpha diversity between non-smokers (blue) and smokers (red): **A)** Shannon-index B) Simpson-index **C)** Inverse Simpson Index **D)** Species Richness **E-F)** Comparison of beta diversity between non-smokers (blue) and smokers (red) using the Bray-Curtis dissimilarity index: **E)** Box-and-whisker plot showing pairwise dissimilarity between samples **F)** Principal Coordinates Analysis (PCoA) plot visualizing clustering patterns of microbial communities

Bray–Curtis dissimilarity metric. We first visualized the distribution of Bray–Curtis dissimilarities within and between smoker and non-smoker groups using box plots (**Figure 3E**). Permutational multivariate analysis of variance (PERMANOVA) confirmed no statistically significant difference in beta diversity between smokers and non-smokers (R^2 = 0.062, F = 1.53, p = 0.184), indicating that tobacco use did not substantially alter the overall microbial community composition in the buccal cavity. Next, Principal Coordinates Analysis (PCoA) based on Bray–Curtis distances was performed to explore compositional patterns in multivariate space (**Figure 3F**). These plots showed considerable overlap between smokers and non-smokers distribution, with one small distinct cluster of smokers (S4, S6, S7, S9, S10). To assess whether technical variation could account for this heterogeneity between the smoking group, we examined the sequencing depth across samples and found no apparent differences between Smoker-I and other groups, suggesting that the clustering is unlikely to be driven by sequencing depth (**Figure 1B, 3F**). Hence, our study reveals that while the oral microbial communities of smokers and non-smokers are broadly similar in their diversity and composition, there is some heterogeneity within smoking group.

Comparison of Microbial Communities Between Smoker Subgroups

To investigate the microbial features distinguishing the small, distinct smoker cluster from the rest, we stratified the smoker group into two subgroups: “Smoker-I” (distinct cluster) and 'Smoker-II' (remaining smokers). Beta diversity analysis using Bray-Curtis dissimilarity demonstrated significant differences in overall microbial community composition between “Smoker-II” and Smoker-I (PERMANOVA, R^2 = 0.466, F = 11.35, p = 0.001). This strong effect size indicates that microbial communities in these smoker subgroups are compositionally distinct, highlighting heterogeneity within smokers.

In order to identify the microbial community distinct between these two groups (Smoker-I vs Smoker-II), we compared the bacterial genus among the two groups using Mann-Whitney U Test with multiple comparison correction using false discovery rate (FDR). We identified 64 genera that were differentially abundant between the Smoker-I and Smoker-II groups (adjusted p < 0.05). A heatmap illustrating the abundance patterns of these genera revealed a clear microbial distinction between the two smoker groups (**Figure 4**), suggesting that different levels of smoking exposure may drive shifts in the oral microbiome composition. Smoker-I individuals showed enrichment of Gram-negative genera such as *Aeromonas*, *Klebsiella*, *Enterobacter*, *Yersinia*, *Salmonella*, *Serratia*, *Vibrio*, and *Campylobacter*. Additionally, rare genera such as *Nocardia*, *Gordonia*, and *Rhodococcus* were observed at higher relative abundance compared to other smoker groups. These distinct microbial signatures highlight the potential influence of varying smoking behaviors or exposure intensities on

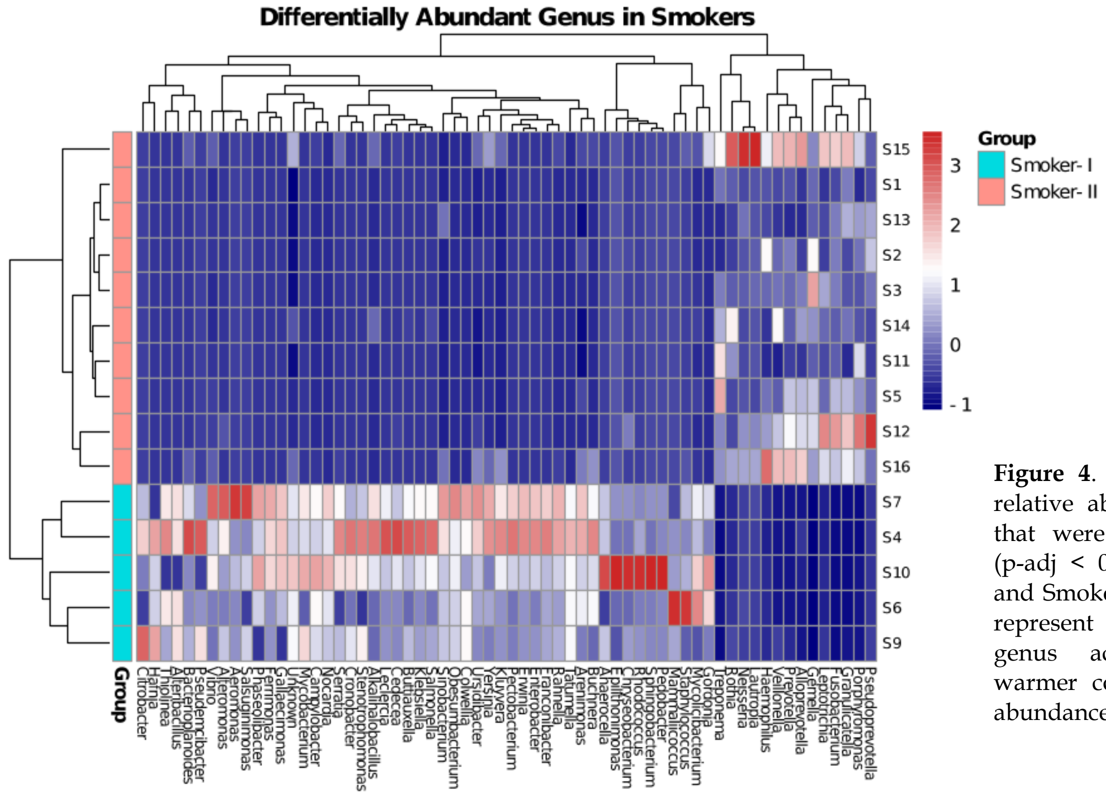

Figure 4. Heatmap showing the relative abundance of 64 genera that were significantly different (p-adj < 0.05) between Smoker-I and Smoker-II sub-groups. Colors represent the abundance of each genus across samples, with warmer colors indicating higher abundance.

the oral microbiome and underscore the importance of considering smoker heterogeneity in studies of tobacco-associated microbial dysbiosis.

Figure 4. Heatmap showing the relative abundance of 64 genera that were significantly different (p-adj < 0.05) between Smoker-I and Smoker-II sub-groups. Colors represent the abundance of each genus across samples, with warmer colors indicating higher abundance

Discussion

Our study characterizes the oral microbiome landscape of smokers and non-smokers, revealing considerable overlap in microbial composition between the groups (**Figure 3A-E**). While this suggests a broadly similar microbial community, relatively sample size and low sequencing depth may have limited our power to detect subtle or low-abundant taxa differences. Despite this, we identified a small subset of smokers (Smoker-I) that formed a distinct cluster, suggesting a variation within the smoking group itself (**Figure 3F**). This divergence encouraged further investigation into the microbial differences between Smoker-I and the remaining smokers (Smoker-II), revealing 64 differentially abundant genera (**Figure 4**). Importantly, these results were not confounded by sequencing depth, as read counts were comparable across groups. This supports the biological relevance of the observed microbial distinctions between Smoker-I and Smoker-II.

The distinct microbial signature of Smoker-I compared to Smoker-II suggests that the effect of smoking is not uniform on the oral microbiota. This heterogeneity in microbial profile among smokers maybe due to variability in smoking behaviors which includes frequency, duration, or the type of tobacco products. Smoker-I individuals exhibited an enrichment of Gram-negative bacteria such as *Aeromonas*, *Klebsiella*, *Enterobacter*, *Yersinia*, *Salmonella*, *Serratia*, *Vibrio*, and *Campylobacter*, consistent with previous studies indicating that smokers are prone to colonization by Gram-negative organisms [36–38]. Enrichment of gram-negative bacteria in the oral cavity have been associated with increase in risk of infection like pneumonia, and periodontal diseases [39,40]. In fact, the presence of genera such as *Klebsiella* [41,42], *Campylobacter* [43], and *Fusobacterium* [44] are often linked to periodontal disease or pathogenicity. This underscores the potential health implications of the observed microbial shifts. Thus, distinct microbiome profiles within smokers may correspond to differential health risks.

The enrichment of genera such as *Yersinia*, *Salmonella*, *Cronobacter*, *Nocardia*, *Gordonia*, *Rhodococcus*, *Sphingobacterium*, *Chryseobacterium*, and *Franconibacter* in Smoker-I is concerning. Many of these genera are uncommon in healthy oral environment and are often associated with opportunistic infections, or healthcare settings [45–54]. Their presence suggests possible microbial dysbiosis and potential immunocompromise in this subgroup. Additionally, it could also indicate

frequent healthcare visits or other underlying health conditions, further distinguishing Smoker-I individuals from the broader smoking population.

These findings emphasize the complexity of smoking-related alterations in the oral microbiome and suggest that not all smokers are microbiologically alike. The emergence of a distinct microbial profile in a subset of smokers point to potential underlying differences in behavior, exposure, or health status that warrant further investigation. Our study did not categorize the smoking groups by specific factors such as the exact duration of smoking (years), daily frequency of smoking (cigarette/day) or type of tobacco used. These limitations, including the small sample size, restrict our ability to uncover the nuanced relationship between smoking exposure and microbial response. Furthermore, this study did not account for the potential sex differences, as only male participants were included. Therefore, future studies should comprise a larger and more diverse group of smokers and incorporate smoking metrices to increase resolution of smoking-associated microbial shifts. Such approach could offer new insights into how microbial shifts reflect or contribute to broader health trajectories in smokers.

Conclusion

Our findings highlight significant heterogeneity in the oral microbiome among smokers, with a distinct subgroup (Smoker-I) exhibiting microbial signatures associated with dysbiosis and potential health risks. These results emphasize the need to move beyond treating smokers as a uniform group and instead consider microbial profiling as a tool to identify at-risk individuals.

Author's Contribution

Competing Interest

There are no competing interests.

Funding

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Ethical Approval and Consent

This research received ethical approval (Proposal ID No: 2080/081 231) on 09 June 2023 from the institutional review board of the Yeti Health Science Academy which is registered with NHRC.

Verbal and written consent were taken from all the participants who volunteered to give their buccal swab sample.

Data Availability

The raw fastq files have been deposited and publicly available from Open Science Framework. https://doi.org/10.17605/OSF.IO/Y2PRV.

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