

Article

Exploratory Assessment of Oral Microbiome Dysbiosis Associated with Chronic Smokeless Tobacco Use: A Sibling-Based Study

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Abstract

Smokeless tobacco (SLT) consumption is a significant risk factor for oral dysbiosis and oral cancer in South Asian countries, including India. However, its impact on oral microbial ecology, functional restructuring, and sex-specific microbial responses remains unclear. The present study aimed to evaluate tobacco-associated oral microbiome dysbiosis across different subject groups (addicted male/AM, addicted female/AF, non-addicted male/NAM, and non-addicted female/NAF) using a controlled sibling-based pooled temporal representative framework. To reduce short-term temporal variability and generate representative oral microbiome profiles, oral gargle samples were collected daily for seven days and pooled within each study group prior to DNA extraction and 16S rRNA gene sequencing. Proteobacteria, Firmicutes and Bacteroidetes were the dominant phyla in all samples. Addicted users showed a higher Firmicutes/Bacteroidetes ratio, fewer cultivable species, and more pathobionts, along with lower Shannon diversity in tobacco addiction and a partial separation in beta diversity between addicted and non-addicted samples. The enrichment of *Streptococcus*, *Gemella*, *Neisseria*, *Veillonella*, *Acinetobacter*, and *Pseudomonas* in tobacco-associated microbiomes and *Comamonas*, *Chryseobacterium*, *Delftia*, *Flavobacterium*, and related taxa in non-addicted individuals were consistently identified by all three analyses, that is, differential abundance, STAMP, and SIMPER. A shared core oral microbiome and unique “tobacco-associated” microbial signatures were observed. Otype mapping showed that the transition in the microbial community to a *Streptococcus*-dominated (S-type) dysbiotic state was driven by tobacco addiction, introducing a novel conceptual framework, the Tobacco-Associated Dysbiosis Index (TDI), which showed higher values in addicted participants. Furthermore, functional prediction analysis revealed increased enrichment of ammonia oxidation, dehalogenation, sulfate reduction, nitrite reduction, chitin degradation, and xenobiotic metabolism pathways, highlighting microbial adaptation towards detoxification. In summary, chronic exposure to smokeless tobacco is linked to ecological imbalance, pathobiont enrichment, loss of microbial diversity, functional dysbiosis, and metabolic reprogramming, suggesting descriptive ecological patterns that may be relevant to chronic inflammation and oral carcinogenesis.



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Keywords: smokeless tobacco; oral microbiome; oral dysbiosis; orotype transition; tobacco consumption

1. Introduction

The human oral cavity contains a rich and diverse microbiome comprising over 700 microbial species across various environments (e.g., saliva, tongue, gums, and teeth) that play a significant role in maintaining oral health. Large-scale studies, such as the Human Microbiome Project, have explored and identified the core taxonomy of oral microbiota in healthy individuals [1]. This microbial community, including bacteria, fungi, and viruses, coexists with host and environmental tissues and can greatly influence the development of a healthy microbiome [2,3]. However, an imbalance in this microbial community can lead to various oral pathologies, including periodontitis, dental caries, oral cancer, and systemic diseases. This alteration in the oral microbial community is called oral dysbiosis, which is an indicator or precursor of various oral diseases. Oral dysbiosis is mainly caused by food habits, lifestyle, and poor oral hygiene [4–6].

Alterations in the microbial community can occur due to factors such as alcohol use, smoking, diet type, and different lifestyle conditions [7–9]. Recent studies have shown an increase in the use of tobacco in different parts of the world, which can lead to different health consequences [10]. Nearly 80% of the 360 million people who consume smokeless tobacco worldwide live in South and Southeast Asia [11]. In India, tobacco is mainly utilized in two forms: smoked and smokeless tobacco (SLT). SLT consumption is a major health problem in India and South Asia, and oral cancer due to tobacco accounts for one of the highest proportions of cancer cases in this region. India alone is responsible for oral cancer cases, which are mainly due to the high consumption of smokeless tobacco products, such as gutkha, khaini, zarda, and betel quid with tobacco [12]. According to the Global Adult Tobacco Survey, India 2016–2017, almost 30% of all Indian adults consume tobacco, and this form of consumption is the most common type of tobacco usage [13]. In India, almost 37% of the lip and oral cavity cancer burden is attributed to the use of smokeless tobacco and areca nut chewing [14].

The main components of tobacco, such as nicotine, nitrosamines, and other reactive molecules, can modify the physical and chemical environment of the oral cavity [15,16]. The chemical constituents of both smoked and smokeless tobacco can alter the pH, oxygen levels, and immune function in the oral cavity. This alteration can lead to the growth of opportunistic microbes and other pathogens while depleting the commensal microbes present in a healthy oral microbial community [16–18]. These environmental changes can also trigger functional metabolic drift in the oral microbiome, affecting xenobiotic degradation, nitrogen metabolism, inflammatory response, and carcinogenic microenvironmental changes [19,20].

Microbial changes related to tobacco smoking have also been linked to systemic consequences, including cardiometabolic diseases, via microbiome-induced metabolic disturbances, such as altered lipid metabolism and inositol degradation [15]. In addition to its association with oral squamous cell carcinoma (OSCC), smokeless tobacco chewing is also associated with esophageal, gastric, pancreatic, colorectal, and hepatocellular cancers, especially in South Asian populations, where chronic tobacco chewing is highly prevalent [21]. Another mechanism by which tobacco-related oral dysbiosis can promote chronic inflammation, nitrosative stress, epithelial injury, and carcinogenic progression is through ecological and metabolic changes in the microbiome [22]. Cigarette smoking could induce a relatively acute, more aggressive oral ecological dysbiosis with combustion-related

oxidative stress and oxygen deficiency, whereas smokeless tobacco use could induce a more chronic, chemically adaptive, and metabolically specialized oral dysbiosis with chronic mucosal exposure to tobacco-specific carcinogens [23]. Thus, it is vital to investigate long-term smokeless tobacco users to understand the progressive oral microbial dysbiosis patterns and chronic ecological compositional differences associated with tobacco exposure.

Beyond health applications, the oral microbiome plays a significant role in forensic applications. Microbial patterns have been used to identify individuals based on lifestyle characteristics. The microbial community is shaped by a person's lifestyle and habits, which further helps in identifying individuals based on lifestyle-associated microbial communities [24,25]. Oral microbiome studies have also recently identified distinct oral microbial communities, or "orotypes" as they are known, which are dominated by *Streptococcus* (S-type), *Prevotella* (P-type), or *Neisseria* (N-type), and are highly associated with oral hygiene, smoking, oral inflammation, and diet [26]. Although multiple studies have reported the influence of smoking on the oral microbiome, there are relatively fewer studies on smokeless tobacco, especially in specific geographical regions. Given the widespread use of smokeless tobacco in India, investigating its effects on the oral microbiome is important [27,28]. This is one of the first studies to combine orotype ecology, the Tobacco-Associated Dysbiosis Index (TDI), comparative restructuring analysis, and functional metabolic drift assessment in smokeless tobacco users.

Therefore, the current study sought to assess oral microbial dysbiosis in a controlled sibling-based sampling design of addicted and non-addicted male and female participants. Along with the orotype compositional redistribution, the Tobacco-Associated Dysbiosis Index (TDI), functional metabolic drift, and ecological restructuring with chronic smokeless tobacco exposure were also analyzed. We hypothesized that chronic exposure to smokeless tobacco would be related to taxonomic dysbiosis, ecological shift to *Streptococcus*-dominated oral ecosystem states, and functional metabolic drift compared to non-addicted controls.

2. Materials and Methods

2.1. Study Design and Sample Grouping

This exploratory research was conducted to examine the differences in oral microbiome composition between smokeless tobacco users (addicted) and non-users (non-addicted). Oral samples were collected from four healthy individuals, forming an exploratory case series ($n = 1$ individual per subgroup: AM, AF, NAM, and NAF). Due to the small sample size, the observed data were interpreted as descriptive baseline tendencies rather than generalizable group-level biological effects. The smokeless tobacco user group consisted of one tobacco-addicted male (AM) and one tobacco-addicted female (AF); similarly, the non-user group consisted of one non-addicted female (NAF) and one non-addicted male (NAM). To reduce the influence of host genetic background, diet, and environmental exposure on oral microbiome composition, addicted and non-addicted participants included in the study were selected from sibling pairs belonging to the same family. This study design helped maintain relatively comparable genetic and lifestyle conditions between the study groups. Because each subgroup contained a single participant, all results are presented as descriptive observations from individual profiles and were not subjected to inferential statistical testing. These findings should be interpreted as hypothesis-generating and require validation in larger, independent cohorts.

2.2. Sample Collection and Processing

The first mouth rinse in the morning using sterile 10 mL N-saline (0.85 g sodium chloride dissolved in 1 L of sterile distilled water) (unstimulated oral gargle samples) was collected daily over a week continuously from the participants under sterile condi-

tions through standardized procedures to reduce temporal variability (Figure 1). Samples were collected from one male participant with a 10-year history of chronic smokeless tobacco consumption (specifically utilizing standard commercial chewing tobacco blends, averaging 3–5 applications daily) and his wife, who shared the same usage pattern. At baseline selection, all four individuals presented with basic self-reported functional oral health and reported no painful active dental caries, active periodontal bleeding, or visible oral mucosal lesions during the sampling window.

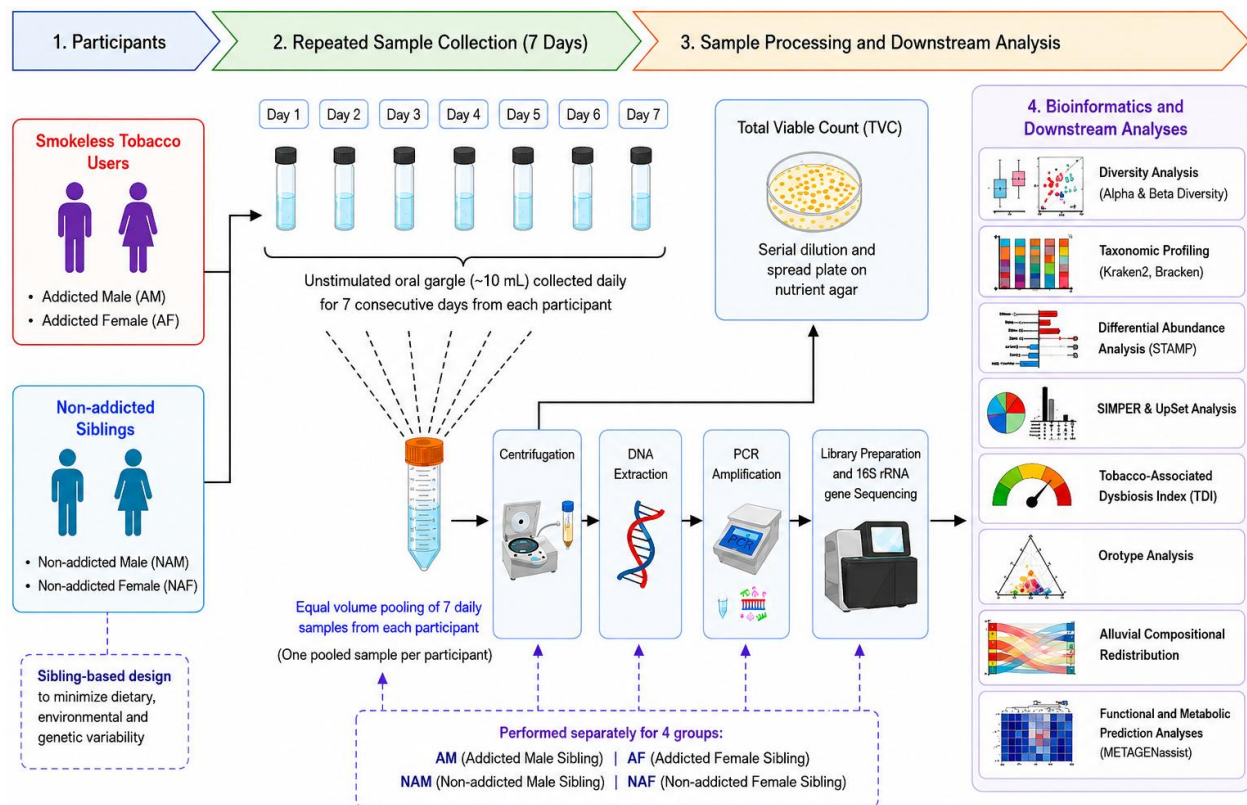


Figure 1. A schematic illustration of the sample collection, processing, and metagenome analysis workflow.

Oral gargle samples from younger siblings were collected as a control (non-addicted to tobacco). The collected samples were pooled separately and concentrated before downstream processing. Pooling was performed before DNA extraction to obtain a representative oral microbiome profile for each participant and was not intended to evaluate the temporal microbial succession. The participants were instructed not to brush their teeth and to refrain from eating and drinking for at least one hour prior to sample collection. Participant details, such as age, medications, diseases, diet, and fasting status during sampling, were also recorded. The samples were collected in sterile collection tubes and immediately stored at 2 °C until downstream processing. The criteria for participation were no history of antibiotic use in the past three months and no chronic diseases. The “non-addicted” sibling controls were validated as clear lifetime non-users of tobacco with no history of consistent passive smoke exposure or active oral mucosal disease.

2.3. Sample Physicochemical Characterization and Total Viable Count

The collected samples were tested for pH, turbidity, and microscopic analysis before metagenomic analysis. The total viable count (TVC) in 1 mL of the collected samples was determined using nutrient agar plates to estimate the cultivable microbial load in oral gargle samples from addicted and non-addicted participants. Serial dilution and spread

plate techniques were used, followed by incubation under standard laboratory conditions to count the colonies.

2.4. DNA Extraction and Sequencing

To minimize short-term variations, we collected oral gargle samples from each participant daily for seven days. These samples were then combined before DNA extraction and sequencing to create a stable snapshot of each participant's microbiome. DNA was extracted from the oral samples using the HiPurA™ Water DNA Purification Kit. The quality and concentration of the extracted DNA were assessed using gel electrophoresis and Qubit to ensure sample quality for 16S rRNA sequencing. The V₃–V₄ region of the 16 S rRNA gene was amplified using polymerase chain reaction (PCR) with the universal prokaryotic primer pair 341F (5'-CCTACGGGNGGCWGCAG-3') and 785R (5'-GACTACHVGGGTATCTAATCC-3'). Amplicon libraries were prepared using the Illumina 16S Metagenomic Sequencing Library Preparation Protocol. The quantified libraries were subsequently sequenced on the Illumina MiSeq (Illumina, San Diego, CA, USA) platform using the MiSeq Reagent Kit v3 (2 × 300 bp paired-end chemistry) at Biokart India Pvt. Ltd. (Bengaluru, India). In each pooled oral microbiome sample, approximately 0.8 M raw sequence reads were generated, with reads ranging in length from 35 to 251 bp. A high merging efficiency of approximately 98% was achieved using PEAR (Paired-End reAd mergeR) to merge forward and reverse reads. The merged sequences originally ranged from 89 to 490 bp, followed by quality filtering, chimera removal, and downstream taxonomic analysis. To assess the diversity and taxonomy of oral microflora, a high rate of read merging and a high sequencing level were achieved, providing sufficient sequencing depth for downstream taxonomic analysis of the oral microbiome.

2.5. Preprocessing and Quality Control of Metagenomic Sequence Data

The total data quality of each sequencing run was analyzed for both forward and reverse reads using FastQC (version 0.12.0) [29]. Low-quality tails and other reads were trimmed using the Trimmomatic tool (version 0.39) [30]. The trimming parameters were kept the same for all samples to minimize bias. Trimming was performed at 150 bp length using SLIDINGWINDOW (4:20), with a minimum length (MINLEN) of 100 bp. FastQC was used to ensure good-quality data after removing low-quality reads. For comparative statistical and functional prediction analyses using Statistical Analysis of Metagenomic Profiles (STAMP) (version 2.1.3) and METAGENassist, paired-end reads were merged using Paired-End reAd mergeR (PEAR version 0.9.8) before downstream processing [31–33].

2.6. Taxonomic Classification and Diversity Analysis

The taxonomic classification of the samples was performed using Kraken2 (version 2.0.8-beta) against a standard reference database. The abundance of taxonomic groups was estimated using Bracken (version 3.0.1) to improve the abundance accuracy at phylum, family, genus, and species levels with a read length parameter of 150 bp (-r 150) and classification level set to phylum (-l P), family (-l F), genus (-l G), and species (-l S) [34,35].

Downstream microbiome analysis was performed in RStudio using the R statistical software (version 4.5.0) [36]. The microbial diversity and community composition were also analyzed using the R statistical environment (version 4.5.0) with relevant packages including vegan (version 2.7.3), ggplot2 (version 4.0.2), tidyr (version 1.3.2), ggrepel (version 0.9.8), tidyverse (version 2.0.0), UpSetR (version 1.4.0), and dplyr (version 1.1.4). Alpha diversity was calculated using the Shannon index to estimate species richness and evenness, and the non-parametric Wilcoxon rank-sum test was used to analyze differences between smokeless tobacco users and non-users. Beta diversity was estimated using Bray–Curtis dissimilarity, and ordination was visualized using principal coordinate analysis (PCoA). Statistical analy-

sis for beta diversity was performed using permutational multivariate analysis of variance (PERMANOVA) to evaluate changes in community composition between groups using Bray–Curtis dissimilarity with 999 permutations in the vegan package. Comparative analyses were performed between the smokeless tobacco user and non-user groups. Differential abundance was explored using the log2 fold change, and STAMP was used for comparative species-level taxonomic profiling. Similarity percentage (SIMPER) analysis was also used to identify the taxa that contributed most strongly to group-wise dissimilarity.

2.7. Tobacco-Associated Dysbiosis Index

The Tobacco-Associated Dysbiosis Index (TDI) was created using genus-level relative abundance profiles derived from the Kraken classification of 16S rRNA sequencing data to estimate tobacco-associated ecological imbalance within the oral microbiome. The TDI was computed as follows:

$$TDI = \frac{(\sum \text{Pathobiont associated genera abundance} - \sum \text{Commensal associated genera abundance})}{\sum \text{Total classified abundance}}$$

Seven genera, each with several different species, exhibited increased abundance in association with tobacco, and seven genera of comparatively commensal/environmental species were selected for the construction of indexes: pathobiont-associated genera (including *Pseudomonas*, *Streptococcus*, *Acinetobacter*, *Enterobacter*, *Veillonella*, *Neisseria*, and *Gemella*) and commensal-associated genera, i.e., *Flavobacterium*, *Chryseobacterium*, *Delftia*, *Comamonas*, *Azospira*, *Dechloromonas*, and *Arcobacter*. These 14 genera were preselected based on their established roles in independent, external peer-reviewed literature evaluating tobacco stress and oral mucosal dysbiosis (e.g., *Pseudomonas*, *Streptococcus*, and *Veillonella* as opportunistic pathobionts and *Flavobacterium* and *Comamonas* as environmental commensals) [23,37]. Rather than serving as a universal diagnostic marker, the Tobacco-Associated Dysbiosis Index (TDI) is presented here as an exploratory proof-of-concept metric for mapping community-level shifts in this specific sibling case series. A higher TDI was considered indicative of pathobiont enrichment, ecological imbalance, and tobacco-associated dysbiosis of the oral microbial ecology, whereas a lower TDI represented a relatively balanced oral microbial ecology.

2.8. Orotypes Analysis, Ecological Mapping Visualization, and Ternary Orotypes Mapping

To explore how tobacco exposure shapes the broad ecological landscape of the mouth, community profiles were interpreted using the oral orotype classification framework established by Yamauchi et al. [26]. Given the pilot nature of our small sample size, we chose not to apply unsupervised clustering algorithms, distance metrics or strict threshold parameters. Instead, we used a direct mapping strategy centered on the three principal ecological indicator genera of the mouth: *Streptococcus* (S-type), *Prevotella* (P-type), and *Neisseria* (N-type). To assess the internal ecological balance of the three core genera associated with the three orotypes, the abundance of *Streptococcus*, *Prevotella*, and *Neisseria* was normalized to a sum of 100% per sample, and all remaining oral genera were excluded from the normalization calculation. Therefore, orotype assignment was based on the dominant normalized abundance of *Streptococcus*, *Prevotella*, and *Neisseria*. No predefined abundance threshold was applied; instead, each sample was assigned to the orotype represented by the highest normalized relative abundance of the three indicator genera. The proportional normalized abundance was calculated using the following equation:

$$\text{Normalized abundance(\%)} = \frac{(\text{Genus abundance})}{\text{Streptococcus abundance} + \text{Prevotella abundance} + \text{Neisseria abundance}} \times 100$$

Alluvial diagrams were used solely as descriptive graphical summaries of the normalized proportional composition among pooled microbiome profiles and were not intended to infer temporal microbial transitions. In addition, ternary maps (orotype maps) were created to examine the relative distribution of addicted female (AF), addicted male (AM), non-addicted female (NAF), and non-addicted male (NAM) samples in the *Streptococcus* (S-type), *Prevotella* (P-type), and *Neisseria* (N-type) ecological spaces. To aid in the visual ecological interpretation of the data, illustrative arrows connecting the four pooled profiles, conceptual orotype dominance regions, and Tobacco-Associated Dysbiosis Index (TDI) annotations were added. Accordingly, the orotype analyses presented in this study should be interpreted as descriptive ecological mapping within an established classification framework, rather than as unsupervised microbial community clustering.

2.9. Phenotype-Based Functional and Metabolic Prediction Analyses

Phenotypic and metabolic prediction analyses of functional and metabolic potential were performed using METAGENassist (version 2) based on taxonomic abundance profiles obtained from 16S rRNA gene sequencing data [32]. The functional categories obtained by METAGENassist are representational predictions of metabolic functions based on taxonomic composition and should be considered as proxies of potential ecological function, not of gene content, gene expression, enzymatic activity, or metabolic flux [38]. Thus, these predictions are based on ecological potential and do not necessarily represent the actual metabolic activities of the microbes. Functional categories from the predicted microbial functional attributes, environmental adaptation, nutrient cycles, and xenobiotic degradation were comparatively analyzed between tobacco-addicted and non-tobacco-addicted male and female microbiome samples from the oral cavity to assess tobacco-associated functional dysbiosis and metabolic drift. Alluvial flow visualization diagrams were then created to show the enrichment, depletion, and relative stability of predicted functional categories under chronic smokeless tobacco exposure, using CSV files exported from METAGENassist.

3. Results

3.1. Sample Metadata, pH, Turbidity, and Total Viable Count Analysis

The pooled samples of addicted male and female participants were more acidic than those of their non-addicted siblings. The turbidity of all four samples was due to the presence of food particles, and microscopic observation suggested the presence of tobacco particles in addicted males and females. Total viable count analysis showed that the cultivable microbial counts were comparatively high in the non-user groups and relatively low in the smokeless tobacco user groups (Table 1). The lower number of cultivable microorganisms in tobacco-addicted individuals may reflect changes in the oral microbial environment and ecological balance associated with tobacco use.

Table 1. Participants' details, samples' pH, turbidity, and total viable count of cultivable oral microbiota among tobacco-addicted and non-addicted individuals.

Sample	Age of Participants (Years)	Diet Pattern	Any Other Disease/Medication	Fasting Before and During Sampling	pH	Turbidity/Microscopic Observation	TVC (CFU/mL)
AM	47	Non-vegetarian	No	No	6.1–6.25	Turbid	198×10^8
NAM	42	Non-vegetarian	No	No	6.8–6.9	Slight Turbid	215×10^8
AF	45	Non-vegetarian	No	No	6.2–6.4	Turbid	48×10^8

Table 1. Cont.

Sample	Age of Participants (Years)	Diet Pattern	Any Other Disease/Medication	Fasting Before and During Sampling	pH	Turbidity/Microscopic Observation	TVC (CFU/mL)
NAF	35	Non-vegetarian	No	No	6.9–7.1	Slight Turbid	53×10^8

3.2. Sequencing Data Summary

The oral samples sequenced using the 16S rRNA sequencing method produced a high number of reads for all four participants in the study. After the raw read quality assessment with FastQC and the preprocessing steps in the form of trimming and sorting, high-quality reads were saved for downstream taxonomic analysis. For comparative statistical and functional analyses, paired-end reads were merged using PEAR. All sequencing reads were normalized to a fixed read length of 150 bp before taxonomic classification to follow the same protocol and minimize bias.

The read counts obtained for AF and AM were 49,735 and 62,111, respectively, after trimming. Likewise, for NAF and NAM, 1,509,493 and 94,619 reads were generated. A high per-base sequencing quality was observed in all samples, with most reads exhibiting Phred quality scores above Q30. During trimming, adapter contamination was eliminated and low-quality terminal bases were removed, enhancing the overall quality of the data for microbiome profiling. High-quality reads were then processed and taxonomically classified using Kraken2, and abundance was estimated using Bracken to analyze the microbial community.

3.3. Taxonomic Composition

The microbial profiling at the phylum level across all samples showed that Proteobacteria, Firmicutes, and Bacteroidetes were the predominant phyla, whereas Pseudomonadaceae, Streptococcaceae, Enterobacteriaceae, Flavobacteriaceae, and Comamonadaceae were the most abundant families (Supplementary Figures S1 and S2). Relatively higher Firmicutes/Bacteroidetes (F/B) ratios were found in addicted female (AF: 3.5) and addicted male (AM: 6.27) samples, while lower and comparatively balanced F/B ratios were found in non-addicted female (NAF: 0.32) and non-addicted male (NAM: 0.7) samples. Tobacco-associated oral dysbiosis and microbial ecological imbalance were suggested by a higher F/B ratio in addicted individuals, and a relatively stable oral microbial environment was reported by the comparatively balanced F/B ratios in NAM and NAF individuals.

The taxonomic composition of oral microbiome samples showed significant variability in the composition of the microflora among all participants, with a few predominant genera observed among the most abundant taxa. The most common bacterial genera found in all samples at the genus level were *Pseudomonas*, *Streptococcus*, *Salmonella*, *Acinetobacter*, *Aeromonas*, *Comamonas*, *Enterobacter*, *Escherichia*, *Prevotella*, and *Chryseobacterium*.

In tobacco-associated samples, AF showed a relatively higher abundance of *Pseudomonas* (approximately 68 percent), with the relative abundance of *Streptococcus* and *Prevotella* decreasing. Conversely, a more heterogeneous microbial composition was observed in the AM sample, with *Streptococcus* as the predominant genus (approximately 37 percent), followed by *Acinetobacter*, *Pseudomonas*, and *Enterobacter*.

Samples of non-tobacco showed a relatively uniform distribution of microbes. *Pseudomonas* (approximately 32%), followed by *Prevotella*, *Comamonas*, *Chryseobacterium*, *Enterobacter*, and *Streptococcus*, were mostly reported in NAF samples (Figure 2). Similarly, the NAM sample exhibited a fairly diverse microbial profile, with *Streptococcus* (approximately 19%) and *Pseudomonas* (approximately 12%) being the most abundant genera.

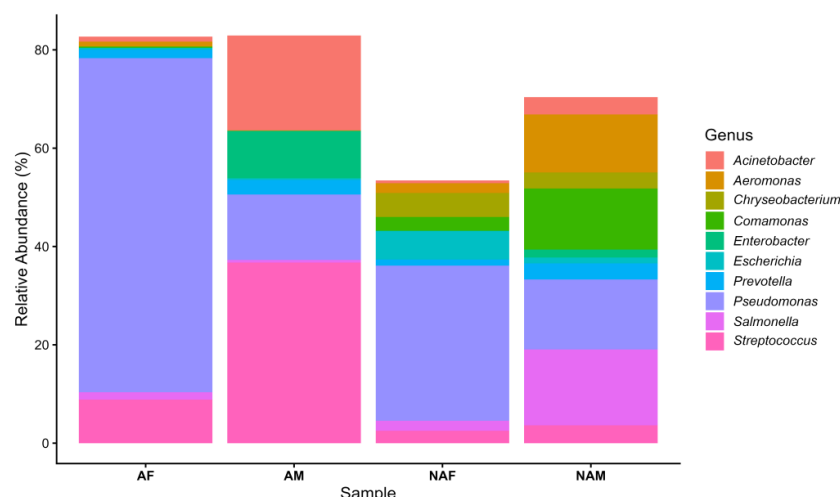


Figure 2. Relative abundance of the top 10 bacterial genera identified across the pooled salivary microbiome profiles. The top 10 genera were filtered and ranked based on their mean global relative abundance percentages across all sequence reads, with lower-abundance taxa omitted from visualization. The data represent descriptive pilot snapshot profiles from a single baseline cohort ($n = 1$ per subgroup) to highlight broad directional trends. Subgroups: AF (addicted female), AM (addicted male), NAF (non-addicted female), and NAM (non-addicted male).

The most common taxa identified at the species level were *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Streptococcus agalactiae*, *Klebsiella pneumoniae*, *Escherichia coli*, and *Aeromonas veronii*, with significant changes in their abundance patterns among the participants (Figure 3).

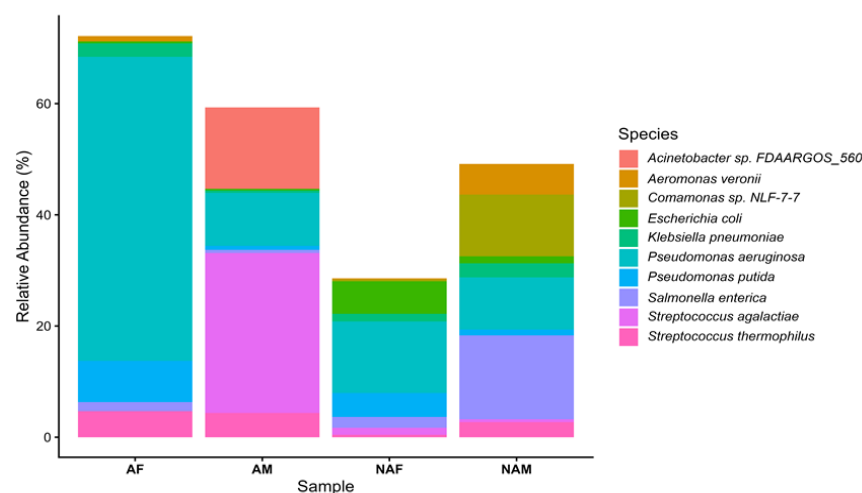


Figure 3. Relative abundance of the top 10 bacterial species identified in pooled salivary microbiome profiles. The top 10 species-level categories were isolated based on the cumulative database read mappings. These assignments represent exploratory taxonomic tendencies from a pilot case-series dataset rather than absolute species-level identification, owing to the resolution constraints of short V₃–V₄ 16S rRNA gene amplicons. Subgroups: AF (addicted female), AM (addicted male), NAF (non-addicted female), and NAM (non-addicted male).

In general, tobacco-related samples seemed to be characterized by a stronger presence of particular taxa, specifically *Pseudomonas* and *Streptococcus*, and other enriched pathobiont genera, whereas non-tobacco-related samples were characterized by a more uniform distribution of microbial communities.

3.4. Alpha Diversity

The Shannon diversity index of alpha diversity analysis showed evident differences in microbial evenness and richness between the tobacco and non-tobacco user groups (Figure 4). Shannon diversity values were significantly higher in the non-tobacco group, with a range of approximately 3.0–3.3, indicating that the microbial richness and evenness of oral samples in non-tobacco users were higher. Conversely, the tobacco-related group had a lower Shannon diversity index of approximately 1.6–2.2, indicating lower microbial diversity in tobacco users.

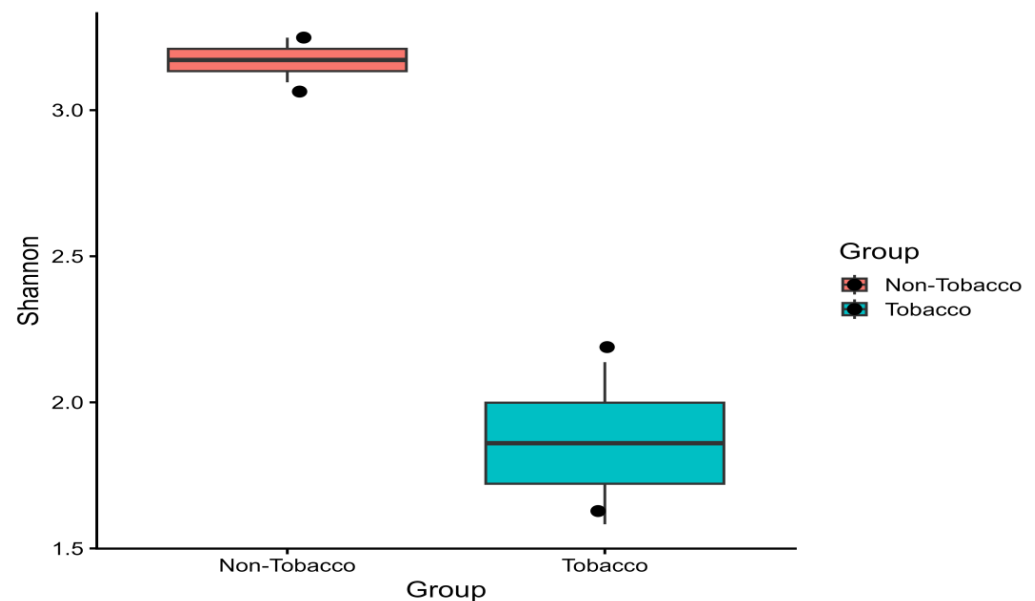


Figure 4. Shannon alpha diversity index of oral microbiome samples comparing smokeless-tobacco users and non-users.

Overall, the lower Shannon index in tobacco users shows a tendency towards diminished microbial complexity and potential dominance of fewer taxa in the oral microbiome of tobacco users. Although the observed pattern could not be statistically proven because of the small sample size, tobacco exposure may be involved in changes in oral microbial diversity. Although trends were observed, the interpretation should be considered exploratory, given the limited number of biological subjects.

The results were statistically compared using the Wilcoxon rank-sum test, which showed that the difference in Shannon diversity between the two groups was not statistically significant ($W = 4$, $p = 0.333$). However, alpha diversity was lower among tobacco users, indicating a tendency towards reduced complexity and dominance of fewer taxa in the oral microbiome of tobacco users.

3.5. Beta Diversity

Beta diversity analysis using Bray–Curtis dissimilarity and plotted with principal coordinate analysis (PCoA) showed that the oral samples had differences in the composition of the microbial communities (Figure 5). The ordination plot showed a distinct spatial distribution of samples, indicating differences in the microbial composition between tobacco users and non-users.

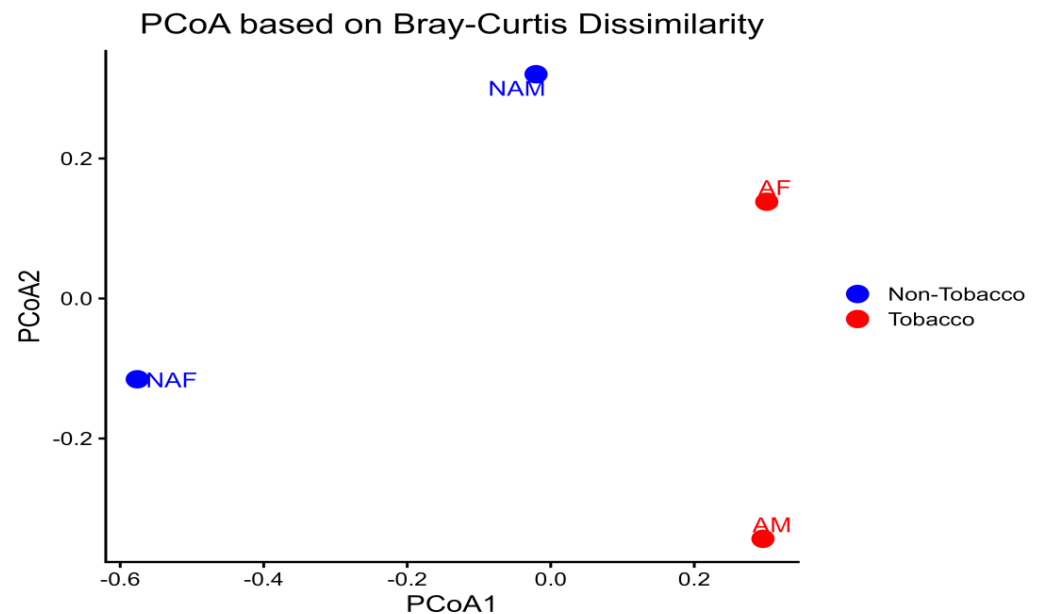


Figure 5. Principal coordinates analysis (PCoA) based on Bray–Curtis dissimilarity illustrated beta diversity patterns between smokeless tobacco users and non-users. Subgroups: AF (addicted female), AM (addicted male), NAF (non-addicted female), and NAM (non-addicted male).

The tobacco-addicted samples (AF and AM) were positioned closer to each other along the primary ordination axis (PCoA1), suggesting a relatively similar overall community composition. However, they were separated along the vertical axis (PCoA2). With only two participants in the tobacco addiction category ($n = 1$ per subgroup), this separation cannot be interpreted as a structural biological feature of tobacco use. Instead, it most likely reflects individual-level biological variability that cannot be disentangled in a study of this magnitude. Although the sibling-based study design and shared household environment were intended to minimize variability arising from shared dietary habits and common environmental exposures, residual differences related to individual host factors, including age, genetics, and other unmeasured biological variables, cannot be excluded.

Similarly, the two non-tobacco reference profiles did not cluster close to each other on the chart. Because each subgroup represents a single person ($n = 1$), this distance cannot be used to measure group-scale variation; rather, it highlights personal baseline differences and normal background noise between individuals.

Therefore, the relative positions of individual samples in ordination space should be interpreted as descriptive visualizations of ecological similarity rather than as evidence of statistically distinct microbial clusters. Nevertheless, tobacco-exposed and non-tobacco samples exhibited partial separation in ordination space, suggesting an association between tobacco exposure and oral microbial community composition. However, this observation should be regarded as exploratory and hypothesis-generating rather than confirmatory because of the limited sample size of the study. An exploratory PERMANOVA analysis yielded an effect size of $R^2 = 0.415$ for tobacco exposure; however, the result was not statistically significant ($p = 0.333$). Accordingly, the PERMANOVA results should be interpreted descriptively rather than inferentially, and larger longitudinal cohorts are required to determine whether the observed ordination patterns are reproducible and statistically robust at the population level.

3.6. Differential Abundance Analysis

A log₂ fold change was used to determine the taxa with their relative enrichment in addicted and non-addicted male and female participants (Figure 6). Some genera showed notable descriptive differences in abundance patterns across the two profiles.

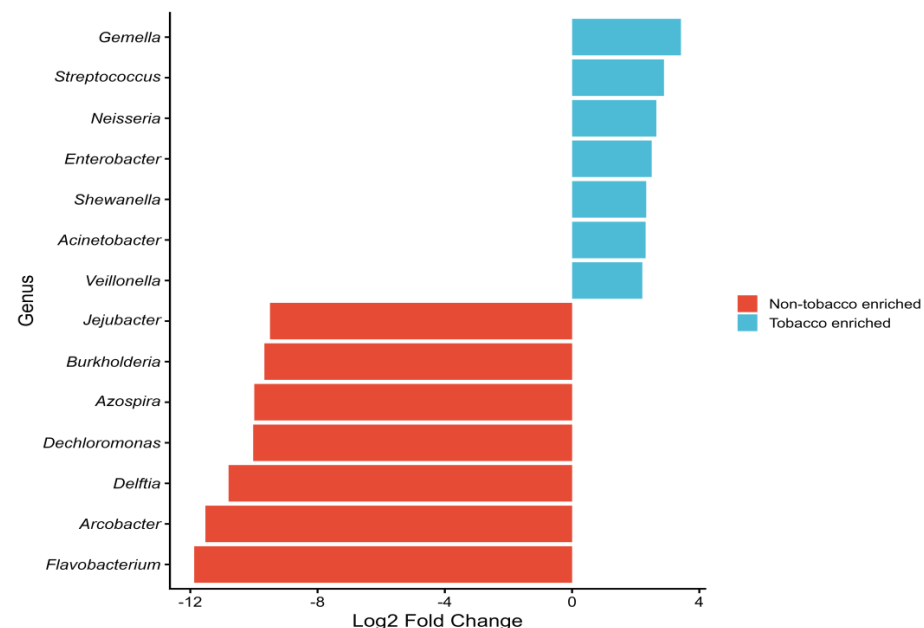


Figure 6. Log₂ fold change in top differentially abundant genera between tobacco-addicted and non-addicted groups.

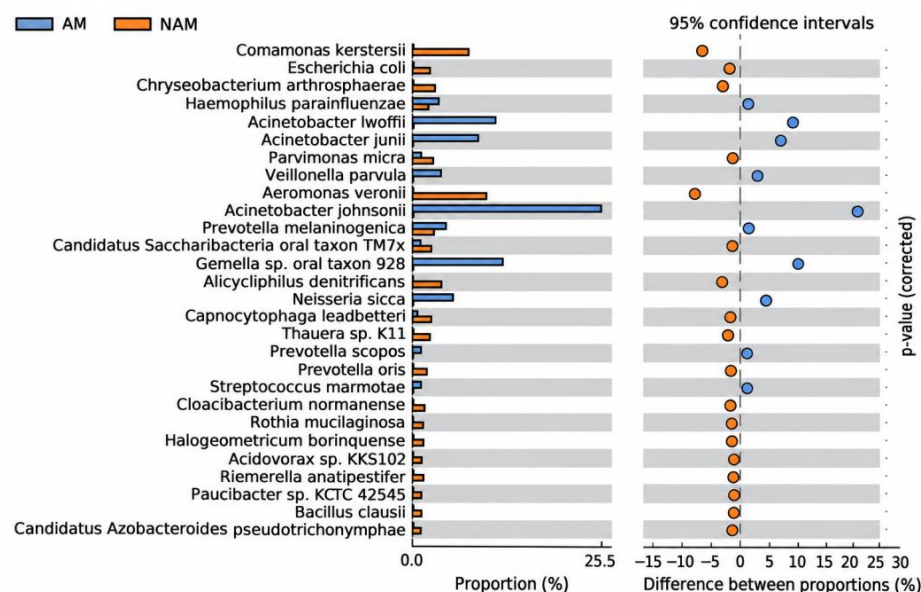
Tobacco-addicted samples were relatively enriched with genera, including *Gemella*, *Streptococcus*, *Neisseria*, *Enterobacter*, *Shewanella*, *Acinetobacter*, and *Veillonella*, as shown by positive values of log₂ fold change. *Gemella* and *Streptococcus* were the most abundant genera in the tobacco group. Conversely, non-tobacco samples were enriched in genera such as *Flavobacterium*, *Arcobacter*, *Delftia*, *Dechloromonas*, *Azospira*, *Burkholderia*, and *Jejunibacter*, which showed negative log₂ fold changes. *Flavobacterium* and *Arcobacter* were the most abundant in the non-user group.

These exploratory patterns suggest a descriptive association between tobacco use history and specific variations in the relative abundance of certain microbial taxa within the individual profiles. Nevertheless, the changes here are determined by fold change without statistical testing; therefore, the differences can only be viewed as exploratory tendencies, but not statistically tested associations.

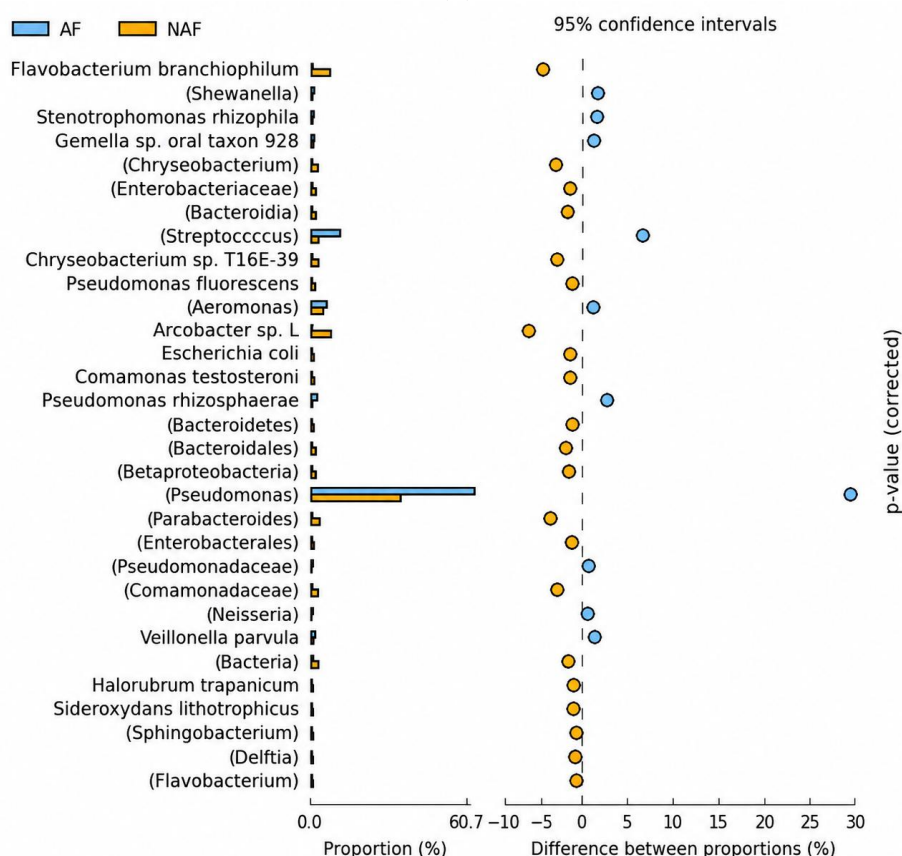
3.7. STAMP-Based Species-Level Comparative Analysis

The compositional differences between the oral microbiomes of tobacco-addicted and non-addicted males were highlighted using species-level comparative analysis of the samples based on STAMP. In an addicted male (AM), comparative profiling showed relatively high-abundant species such as *Acinetobacter johnsonii*, *Acinetobacter junii*, *Acinetobacter lwoffii*, *Gemella* sp. oral taxon 928, *Neisseria sicca*, *Veillonella parvula*, and *Prevotella melaninogenica*, indicating a dysbiotic, inflammation-prone, and biofilm-favoring oral environment (Figure 7A). In contrast, non-addicted males exhibited higher abundance of commensal and environmentally associated taxa such as *Comamonas kerstersii*, *Aeromonas veronii*, *Parvimonas micra*, *Capnocytophaga leadbetteri*, *Rothia mucilaginosa*, and *Bacillus clausii*, reflecting a relatively stable and balanced microbial ecosystem. The marked dominance of *Acinetobacter* species in addicted males highlights their potential role as key microbial signatures of tobacco-induced oral dysbiosis, with all observed differences in the proportional relative

abundance between individual profiles. We explicitly clarify that because the statistical unit of analysis in the STAMP software framework was the independent sequence read count rather than replicated participant-level individuals, the exceptionally low values originally output by the software merely reflect library-level pseudo-replication.



(A)



(B)

Figure 7. (A). STAMP-based differential analysis at the species level showed a comparative microbial distribution between addicted males (AM) and non-addicted males (NAM) oral microbiome samples. (B). STAMP-based differential analysis at the species level showed a comparative microbial distribution between addicted female (AF) and non-addicted female (NAF) oral microbiome samples.

The oral bacterial species profile in tobacco-addicted females was significantly different from that of non-tobacco-addicted females, as observed in the differential abundance analysis using STAMP. Opportunistic and biofilm-forming taxa were significantly higher in addicted females, especially *Pseudomonas*, *Stenotrophomonas*, *Neisseria*, and *Veillonella*, suggesting a dysbiotic and inflammation-prone oral environment in these women. Conversely, the non-addicted females were found to contain greater numbers of commensal and environmentally associated bacterial species, including members of the family Flavobacterium, which also included *Chryseobacterium* and members of Bacteroidetes, indicating a relatively stable microbial ecosystem in the oral cavity. *Pseudomonas* is a common member of the microbiota of these individuals, given its abundance, suggesting its potential use as a microbial biomarker for tobacco-induced oral dysbiosis (Figure 7B).

Tobacco addiction was consistently enriched by free-living opportunistic taxa such as *Pseudomonas* (addicted females) and *Acinetobacter* spp. The presence of *Neisseria* and *Veillonella* (addicted males) is a strong oral dysbiosis microbial biomarker. Because each subgroup consisted of one pooled microbiome profile, differential abundance comparisons were interpreted descriptively based on relative abundance and fold change.

3.8. SIMPER/Biomarker Candidate Analysis

SIMPER analysis was performed to determine the taxa that contributed most to the observed differences between the tobacco-addicted and non-addicted groups in terms of microbial community composition (Figure 8). The dissimilarity between the two groups, as shown by the analysis, focused on a few important genera.

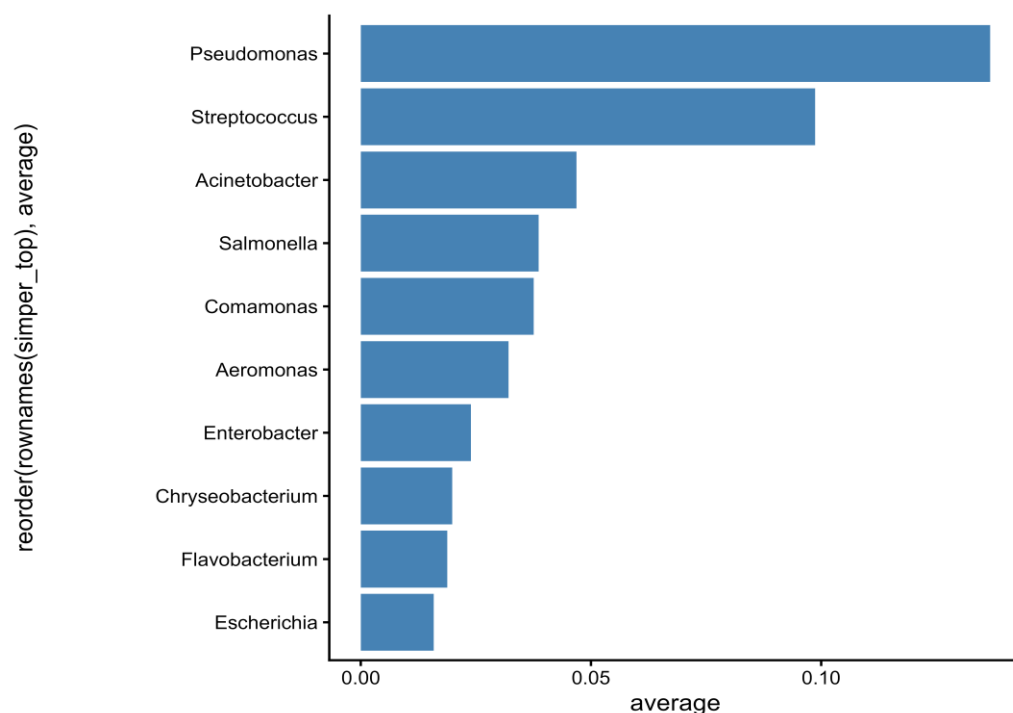


Figure 8. SIMPER analysis showing the contribution of the top 10 dominant genera to the overall similarity between tobacco-addicted and non-addicted groups.

Pseudomonas had the greatest contribution to total community dissimilarity, followed by *Streptococcus* and *Acinetobacter*. Other contributors were *Salmonella*, *Comamonas*, *Aeromonas*, and *Enterobacter*, all of which made a moderately significant contribution to the measured variation in microbial composition. Minor contributions were noted among genera, such as *Chryseobacterium*, *Flavobacterium*, and *Escherichia*.

These results imply that the variation between tobacco-addicted and non-addicted oral samples is mainly caused by the differences in the relative abundance of a small group of taxa. Taxa characterized by SIMPER analysis may be potential candidate biomarkers for characterizing microbial community changes during tobacco exposure. However, owing to the small sample size, these taxa should be treated as initial indicators that require confirmation in a larger cohort.

3.9. Core and Unique Taxa

To evaluate the core and unique taxa between the tobacco-addicted and non-addicted groups, UpSet plot analysis (Figure 9) was performed, which showed an overlap between the four samples (AF, AM, NAM, and NAF). A large proportion of taxa ($n = 247$) were shared among all sample groups, constituting a core oral microbiome shared by both tobacco users and non-users. In addition to this central core, various taxa were exclusively related to particular groups or combinations of groups.

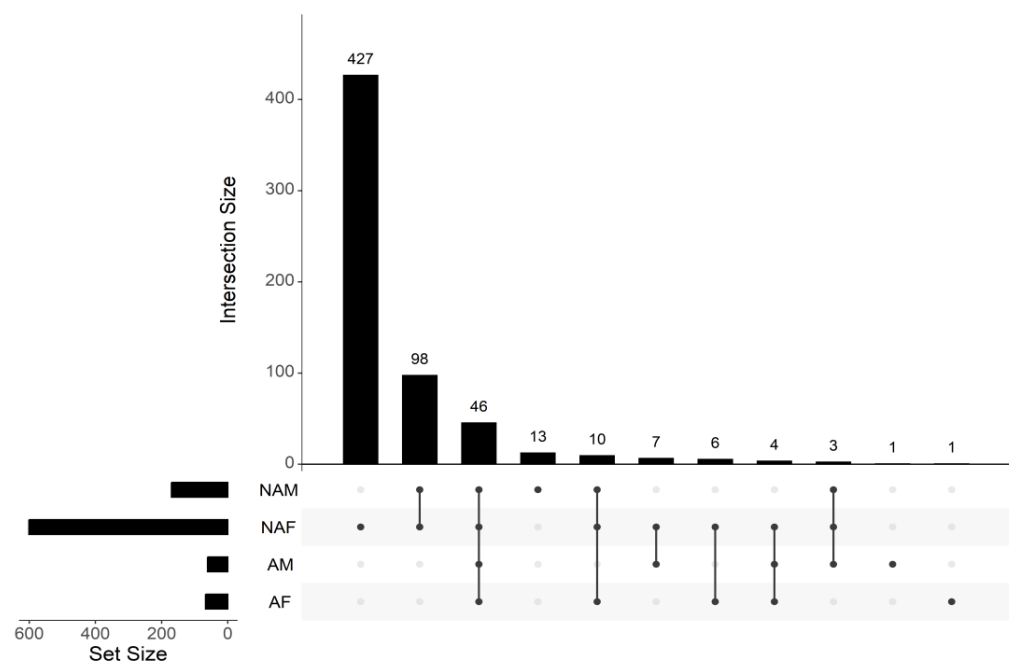


Figure 9. UpSet plot showing shared and unique taxa across AF, AM, NAF and NAM samples.

There were more unique taxa in the non-addicted samples than in the tobacco-addicted samples, with the highest proportion of unique taxa in NAF. The tobacco-associated samples (AF and AM) had reduced distinct taxa, indicating a decrease in microbial diversity and potential homogenization of the microbial community in the presence of tobacco.

Subgroup-specific and pairwise overlap were also identified. For example, subsets of taxa were exchanged only in NAF and NAM ($n = 98$), which showed a preserved microbial signature in non-addicted samples. Similarly, the tobacco user and mixed group combinations also showed some smaller intersections, indicating a partial overlap in microbial composition.

These results suggest that although a core microbiome is preserved in individuals, exposure to tobacco can alter the occurrence and distribution of less abundant or distinct taxa, which can change the composition of the microbial community.

3.10. Tobacco-Associated Dysbiosis Index Analysis

The Tobacco-Associated Dysbiosis Index (TDI) values provided an individual descriptive overview of the four participants (Figure 10). Male (AM) and female (AF) samples

showed marked increases in TDI values of 0.8620 and 0.8231, respectively, reflecting a descriptive imbalance characterized by an overrepresentation of opportunistic pathobiont candidates and a matching reduction in common commensal genera. The relatively balanced oral microbial ecology is reflected in the much lower TDI values of non-addicted female (NAF) and non-addicted male (NAM) samples (0.1037 and 0.0466, respectively). The higher TDI in tobacco addicts is congruent with the notion of tobacco-associated oral dysbiosis, which is defined by a predominance of pathobionts, reorganization of the oral microbiome, and a decrease in microbial homeostasis. The latter also indicates that there may be a dynamic change towards dysbiotic oral flora with chronic tobacco consumption, as a difference in the gradient between addicts and non-addicts was observed.

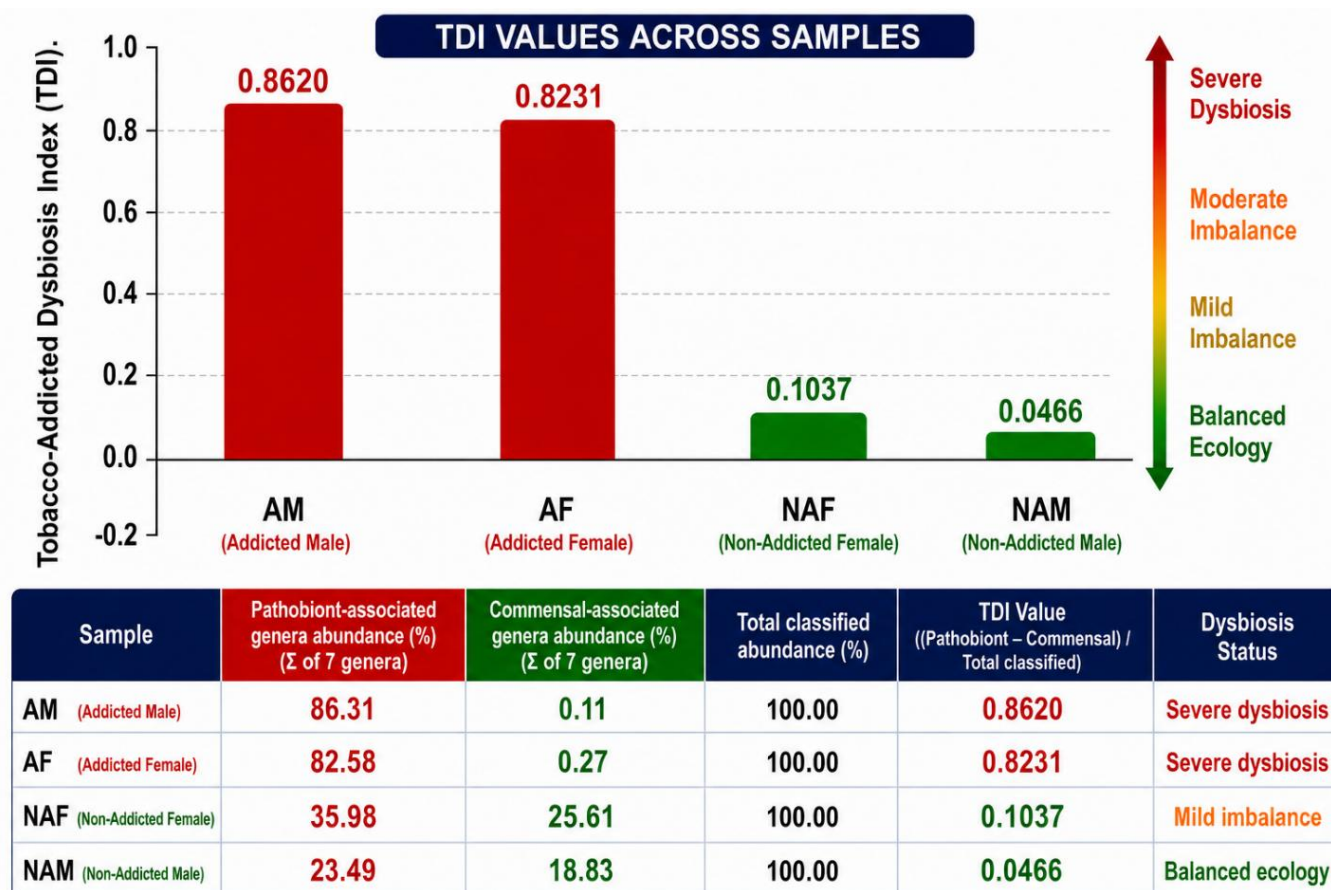


Figure 10. Tobacco-Associated Dysbiosis Index (TDI) analysis across AF, AM, NAF, and NAM samples (TDI is based on the abundance of seven opportunistic/pathobiont-associated genera, including *Pseudomonas*, *Streptococcus*, *Acinetobacter*, *Enterobacter*, *Veillonella*, *Neisseria*, and *Gemella*, and seven commensal/environment-associated genera, including *Flavobacterium*, *Chryseobacterium*, *Delftia*, *Comamonas*, *Azospira*, *Dechloromonas*, and *Arcobacter*). The calculation of TDI is provided in the columns below.

3.11. Orotypic Compositional Restructuring and *Streptococcus*-Dominant S-Type Dysbiosis

The tobacco-associated profiles showed a descriptive tendency toward *Streptococcus*-dominant (S-type) ecological states compared with non-tobacco profiles. Normalized orotypic composition showed that both addicted male (AM) and addicted female (AF) samples had strong S-type dominance, with the highest percentage of *Streptococcus*, 85.53% and 77.20% abundance in the normalized orotypic composition, respectively, along with a notable decrease in *Prevotella*-associated ecological contribution. However, relatively similar *Streptococcus*–*Prevotella* ratios were observed for non-addicted male (NAM) (50.07% vs. 48.14%) and non-addicted female (NAF) samples (41.36% vs. 46.93%), indicating a

moderate predominance of *Streptococci* with a relatively higher abundance of *Neisseria* in NAF. Directional ecological shifts from relatively balanced oral microbial communities in non-addicted groups to an S-type dysbiotic community composition in tobacco-addicted individuals were also apparent when using alluvial orotype composition analysis (Figure 11). Likewise, when AF and AM samples were mapped by ternary orotype, they fell within the S-dominant dysbiosis-associated ecological space, whereas NAF and NAM samples were in relatively transitional and ecologically balanced spaces, respectively. Additionally, progressive pathobiome restructuring was observed in tandem with the severity of dysbiosis in the ternary ecological landscape in response to chronic tobacco exposure and integration of Tobacco-Associated Dysbiosis Index (TDI) values (Figure 12). These findings suggest that in both addicted males and females, tobacco exposure may be associated with a progressive transition to an S-type dysbiotic oral microbial community dominated by acidogenic *Streptococci*, acidogenic pathobiome enrichment, and ecological imbalance.

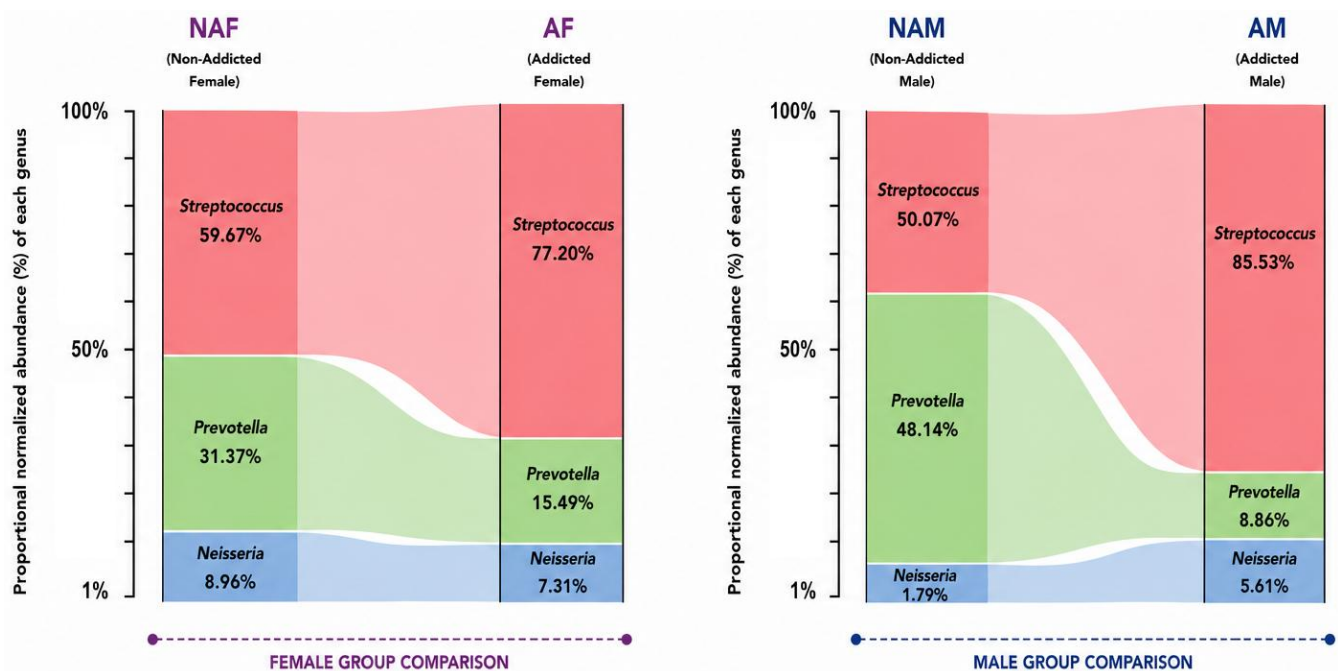


Figure 11. Comparative alluvial visualization of normalized orotype composition between addicted and non-addicted oral microbiome profiles. Flow widths represent the proportional redistribution of the three principal orotype-associated genera (*Streptococcus*, *Prevotella*, and *Neisseria*) following normalization to 100% within each individual pooled sample. The diagram provides a descriptive comparison of the relative compositional restructuring between NAF, AF, NAM, and AM profiles and should not be interpreted as a temporal trajectory or longitudinal microbial transition.

3.12. Phenotype-Based Functional and Metabolic Predictive Analysis

We used the METAGENassist platform to investigate functional dysbiosis and predict the metabolic capability of non-addicted males (NAM) and addicted males (AM), not by measuring the abundance of genes, their expression, or metabolic activity, but by determining their phenotypes derived from taxonomic composition (Figure 13) [5]. The alluvial flow analysis showed that there were several functional categories that were comparatively underrepresented in the alluvial flow of the AM microbiome than in the NAM microbiome, namely, those associated with nutrient cycling and the environment. In particular, the predicted representation of microorganisms associated with ammonia oxidation fell from 79.0% (NAM) to 53.0% (AM); the representation of microorganisms responsible for the reduction of nitrite fell from 72.5% to 47.0%; for the reduction of sulfate fell from 66.7% to 46.3%; for the oxidation of sulfur decreased from 50.7% to 18.5%; for

chitin, degradation reduced from 49.4% to 30.5%; for xylan, degradation changed from 47.7% to 18.5%; a similar dynamics was found for the degradation of aromatic hydrocarbons from 47.2% to 17.6%; and for the degradation of chlorophenols fell from 46.2% to 17.3%. Similarly, the representation of microorganisms capable of sulfide oxidation, nitrogen fixation, selenate reduction, and unknown functional groups was comparatively lower than the predicted levels (11.5% to 3.9%, 5.6% to 1.7%, 0.4% to 0.09%, and 16.1% to 7.8%, respectively). Together, these results suggest a relative decrease in the ratio of various functional groups responsible for nutrient cycling and related to the environment in the tobacco-associated oral microbiome of the participants.

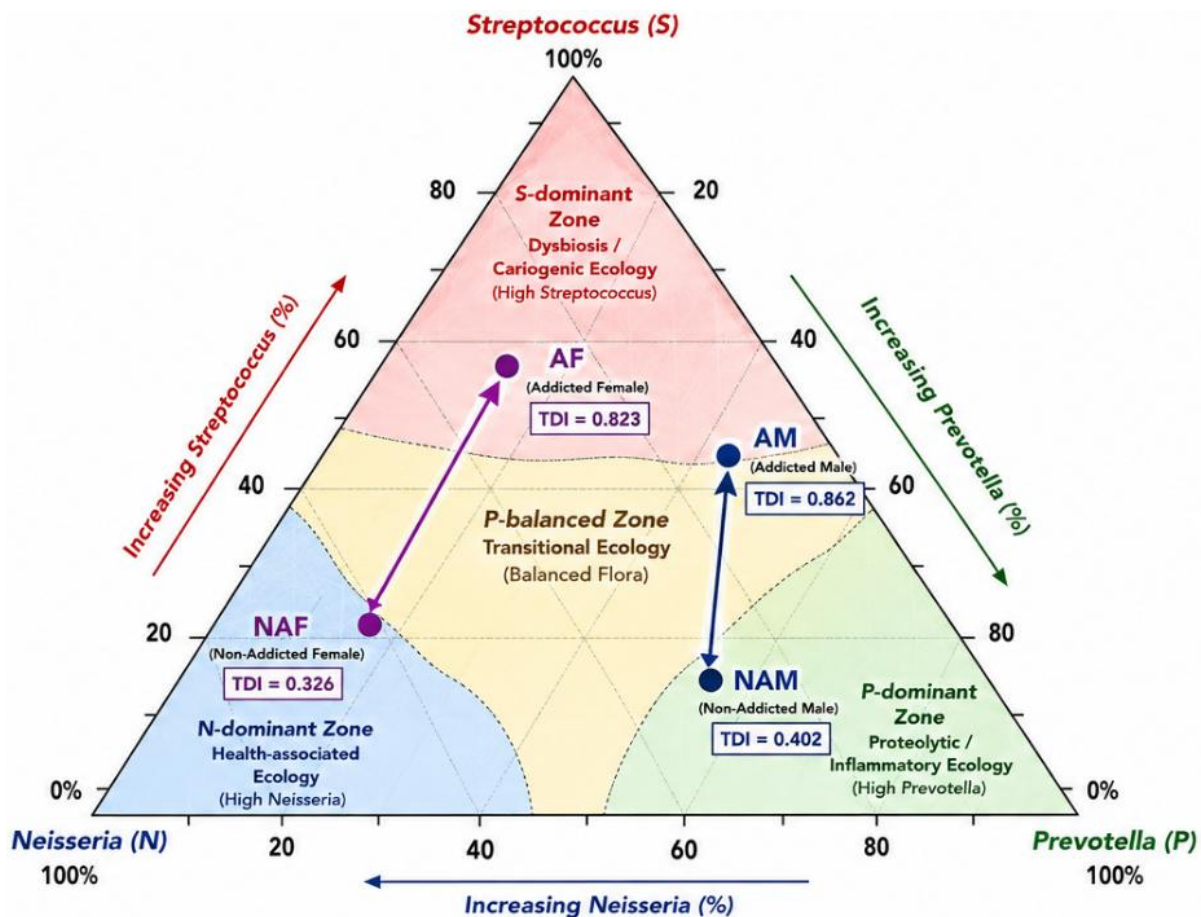


Figure 12. Ternary ecological representation of normalized oral orotype composition among addicted females (AF), addicted males (AM), non-addicted females (NAF), and non-addicted males (NAM) pooled oral microbiome profiles. Each point represents the proportional distribution of *Streptococcus*, *Prevotella*, and *Neisseria* after normalization to 100%. Directional arrows illustrate comparative compositional differences between paired addicted and non-addicted profiles for visual interpretation only and do not represent temporal microbial trajectories or longitudinal ecological transitions. The plot represents the proportional normalized abundance of *Streptococcus* (S-type), *Prevotella* (P-type), and *Neisseria* (N-type) genera. Data points position each individual profile within a two-dimensional geometric coordinate system derived from the normalized orotype framework (*Streptococcus* + *Prevotella* + *Neisseria* = 100). Closeness to any triangle apex reflects the high proportional dominance of that specific genus within the core ecosystem framework, whereas center placement indicates balanced ecology. Directional gradient vectors represent descriptive, exploratory paths linked to tobacco exposure, alongside annotated Tobacco-Associated Dysbiosis Index (TDI) values across pilot profiles (AF, AM, NAF, NAM).

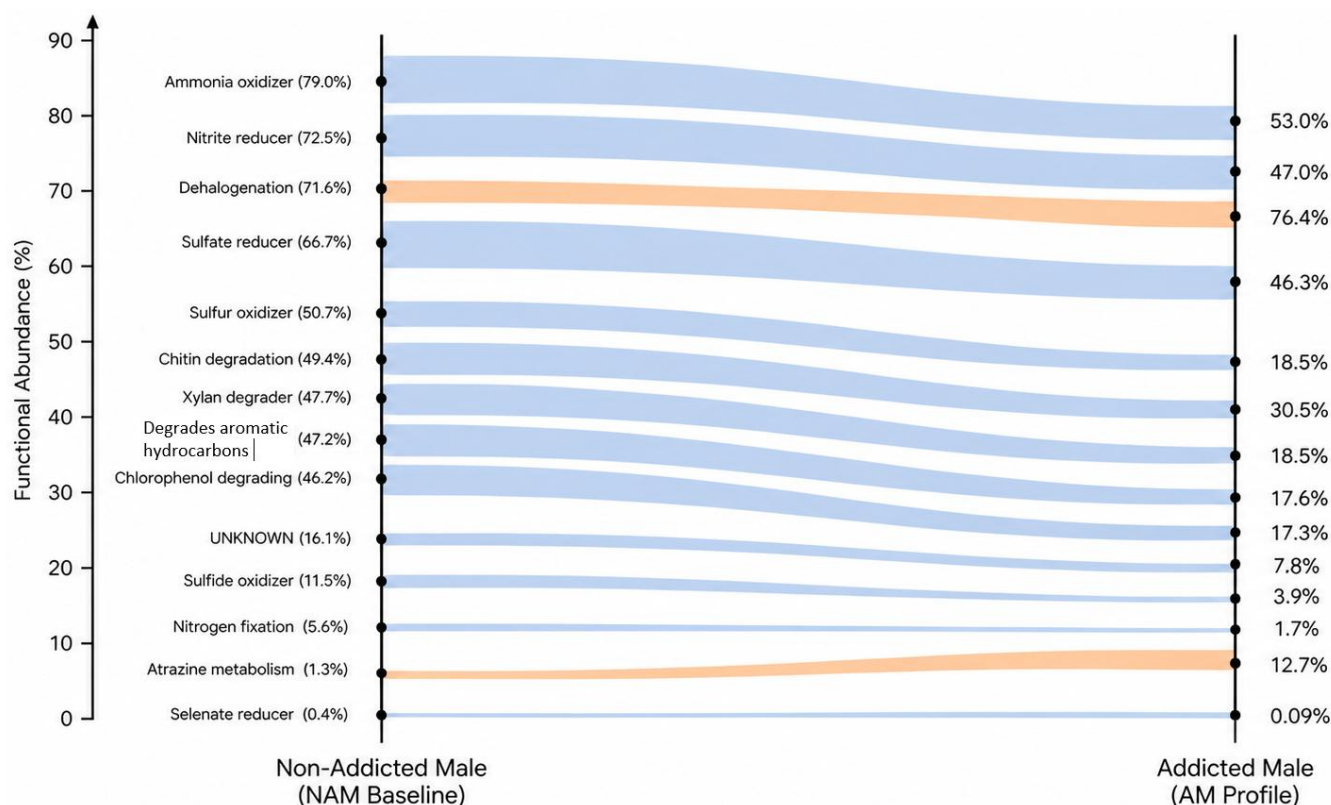


Figure 13. Alluvial flow diagram illustrating functional metabolic shifts between non-addicted male (NAM) and addicted male (AM) salivary microbiome samples predicted using METAGENassist. The flow width represents the relative functional abundance (%) of the microbial metabolic pathways. Blue streams indicate the reduction in functional pathways under tobacco exposure, whereas orange streams represent enriched functions in addicted males.

In contrast, several functional categories selected for xenobiotic transformation and chemical stress adaptation were overrepresented in addicted males. The proportion of microorganisms predicted to undergo dehalogenation increased from 71.6% in NAM to 76.4% in AM, and those predicted to undergo atrazine metabolism increased from 1.3% to 12.7%. Alluvial flow visualization further demonstrated a comparison between the broader distribution of the nutrient-cycling functions in the non-addicted oral microbiome and the relative increase in the number of predicted xenobiotic-associated functional categories in addicted males. Overall, the tobacco-associated oral microbiome showed a relatively low representation of a subset of predicted nutrient-cycling functions and a relatively high representation of predicted stress-associated and xenobiotic transformation functions (Figure 13), suggesting that the tobacco-associated oral microbiome may be ecologically restructured due to chronic use of smokeless tobacco.

These differences are predicted through taxonomic profiles derived from the 16S rRNA gene; therefore, the differences observed are only approximations of the functional potential of the microbes and need to be substantiated through the use of shotgun metagenomics, metatranscriptomics, metabolomics, or targeted biochemical assays.

Several predicted functional categories related to nitrogen cycling, sulfur metabolism, and xenobiotic transformation exhibited comparatively greater representation in the alluvial compositional redistribution between addicted females and the non-addicted female baseline. Major increases were observed in atrazine metabolism (34.7–64.8%), chitin degradation (41.4–71.2%), nitrite reduction (54.8–78.4%), dehalogenation (60.6–83.1%), sulfate reduction (56.5–76.3%), and ammonia oxidation (74.8–84.7%). The overall profile of these enriched functions suggests increased microbial resistance to detoxification, redox

regulation, degradation of complex organic compounds, and chemical stress tolerance following chronic tobacco exposure. Moreover, the degradation of aromatic hydrocarbons showed a slight increase (from 1.1% to 3.1%), further strengthening the enriched xenobiotic-associated metabolic potential in addicted females. However, there were substantial decreases in functions associated with the environment and turnover of nutrients in AF samples compared with NAF, such as sulfide oxidation (14.8–4.0%), sulfur oxidation (14.2–7.7%), xylan degradation (7.6–2.9%), nitrogen fixation (5.6–1.4%), dinitrogen fixation (3.5–0.01%), chlorophenol degradation (2.1% to 0.9%), propionate metabolism (1.4–0.06%), selenate reduction (1.4–0.1%), and stores polyhydroxybutyrate (1.4–0.03%). The proportion of the unknown functional category also fell substantially from 18.9% in NAF to 5.2% in AF, reflecting less metabolic diversity in areas of unexplored metabolic space due to ecological pressure from tobacco. In a few cases, or in cases of near-stable low-abundance pathways, cellulose degradation, lignin degradation, acid production, denitrifying activity, and naphthalene degradation functions showed only minor changes, indicating little involvement of these functions in the comparative ecological reorganization (Figure 14).

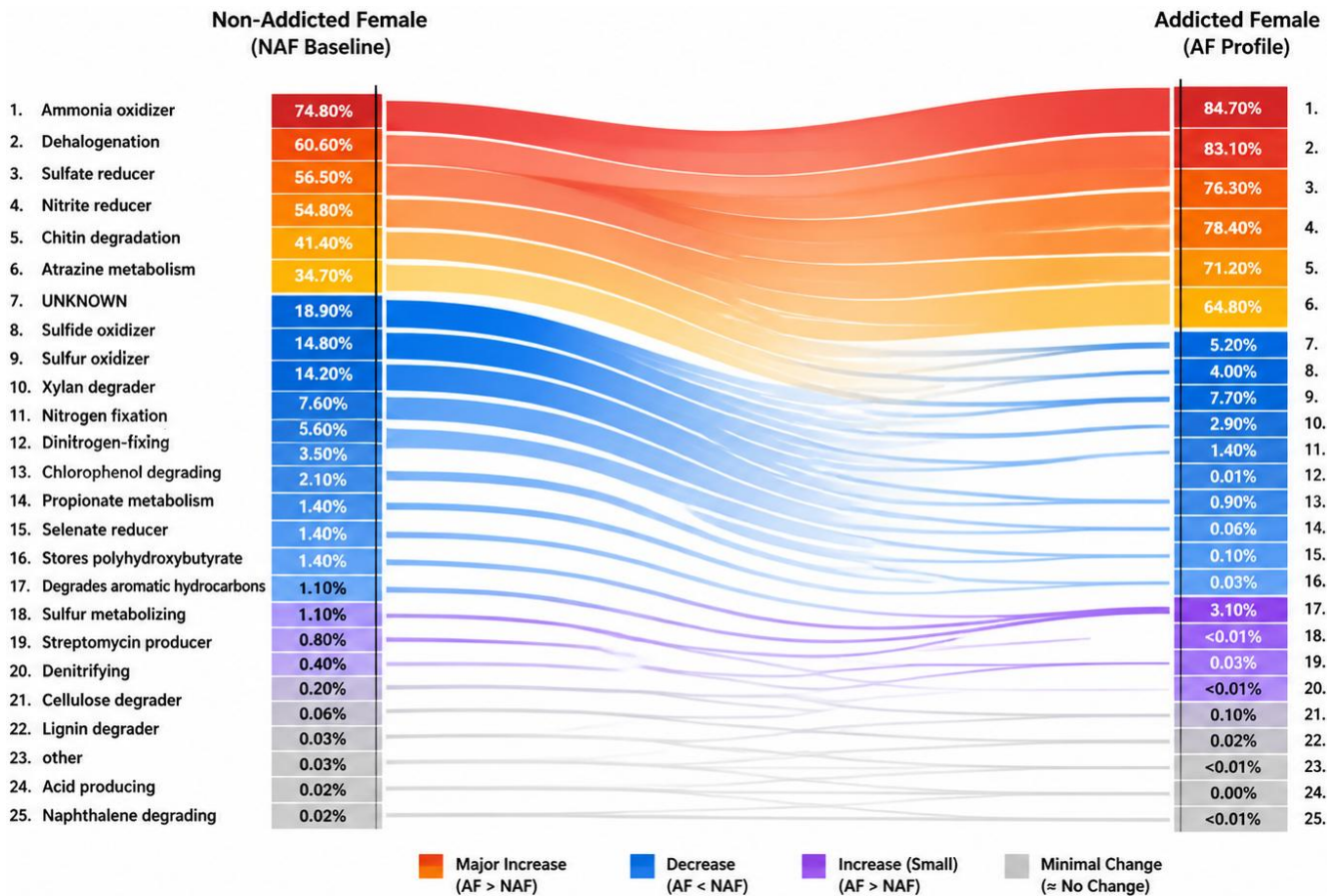


Figure 14. Alluvial flow diagram illustrating functional metabolic shifts between non-addicted female (NAF) and addicted female (AF) oral microbiome samples predicted using METAGENassist. The flow width represents the relative abundance (%) of the microbial metabolic pathways. Orange/red streams indicate major enrichment of metabolic functions under tobacco exposure, blue streams represent reduced functions in addicted females, purple streams indicate minor increases, and gray streams denote minimal or negligible changes.

Overall functional analysis based on the presence of specific organisms may indicate a metabolic shift from a relatively balanced and metabolically diverse oral ecosystem in NAF to a metabolically undiverse, detoxification-oriented, stress-adapted, and dysbiosis-related metabolic state in AF samples. These results suggest that tobacco exposure in females may

lead to a functional “simplification” of the oral microbiome and enrichment of pathways related to xenobiotic degradation, chemical stress adaptation, and ecological dysbiosis in females. The function predicted here may appear speculative based on computational data.

Functional profiling identified distinct metabolic dysbiosis in the oral microbiomes of both addicted males and females, mainly in the pathways of xenobiotic degradation and detoxification, including the dehalogenation, atrazine metabolism, nitrite reduction, and sulfate reduction pathways. In contrast, the non-addicted groups exhibited relatively stable and metabolically diverse ecological functions, reflecting a stable oral microbial ecosystem.

4. Discussion

4.1. Tobacco-Associated Oral Dysbiosis and Ecological Imbalance

The present study revealed changes in oral microbial ecology associated with chronic smokeless tobacco use, including decreased microbial diversity, changes in beta diversity clustering, elevated F/B ratio, and increased Tobacco-Associated Dysbiosis Index (TDI) values in tobacco addicts. The addicted male (AM) and addicted female (AF) groups had lower Shannon diversity and higher ecological imbalance than the non-addicted groups, indicating that they had less diverse stress-adapted taxa and less microbial homeostasis. These results resemble previous studies, which have shown that tobacco exposure disrupts the normal process of the oral microbiota and may lead to dysbiotic restructuring of the oral microbiota that is linked to inflammation and progression of oral disease [7–9]. To fully understand these dysbiosis shifts, it is helpful to examine how healthy oral routines maintain homeostasis. For instance, a recent systematic review outlined how specific enzyme- and protein-containing toothpastes actively support gum health and help stabilize the oral microbiome of healthy individuals. While that study highlighted the therapeutic mechanisms that maintain the oral ecosystem’s balance and protect it against inflammation, our pilot profiles captured the exact opposite effect [39]. Chronic exposure to smokeless tobacco overrides these natural equilibrium-promoting pathways, breaks down oral defenses, and creates a heavily altered, pathobiont-enriched environment. This contrast emphasizes the ongoing struggle between protective hygiene inputs and chronic chemical stress caused by tobacco. Recent studies have also suggested that smokeless tobacco users show distinct changes in oral microbial composition, an increase in *Firmicutes* abundance, and selective enrichment of opportunistic taxa in chemically stressed oral environments [23,37]. This dynamic is further supported by a comprehensive systematic review by Senaratne et al. (2024) [40], which mapped how different forms of tobacco smoke and smokeless leaves alter the structural boundaries of the oral cavity. Their evaluation suggests that smokeless tobacco products induce a chronically adaptive, specialized oral ecosystem that favors stress-tolerant, pro-inflammatory pathobionts over normal health-associated commensals. By integrating these insights, our individual pilot profiles can be viewed as localized examples of this broader macroecological shift, demonstrating how the oral environment restructures when subjected to long-term chemical stress. The increase in the F/B ratio observed in addicted groups, especially in addicted males, may indicate that it further supports ecological restructuring toward a dysbiosis-associated microbial state, with an increase in the abundance of the more stress-resistant *Firmicutes* bacteria, and that such ecological changes can lead to the progressive destabilization of microbial homeostasis in the oral cavity. Increased F/B ratios have been linked to inflammatory and dysbiotic microbial environments in the gut [41].

Tobacco exposure can cause changes in oral pH, oxygen availability, redox balance, and salivary chemistry, all of which would create an environment conducive to the growth of acid-tolerant, biofilm-forming, metabolically stress-adapted microbial communities [15]. The relative acidity of the oral cavity and the lower number of cultivable microorganisms

observed in the addicted subjects in the present study also suggest ecological stress and microbial adaptation in the chronic tobacco environment. Tobacco use has been associated with similar responses that lead to a pro-inflammatory oral microbiota, enrichment of opportunistic genera, and depletion of beneficial commensals, contributing to oral dysbiosis and susceptibility to oral diseases [42]. Moreover, loss of microbial diversity and the shift toward Firmicutes dominance have also been associated with carcinogenic and chronically inflammatory oral microbiomes, and it is possible that the continual use of smokeless tobacco can gradually alter oral microbial ecology toward a dysbiotic and disease-favoring condition [43]. The enrichment of opportunistic families (e.g., *Pseudomonadaceae*, *Enterobacteriaceae*, and *Streptococcaceae*) in addicted families may also be due to the selective adaptation of chronic tobacco exposure stress-tolerant and biofilm-associated microorganisms under chemically altered oral ecological conditions [23,43].

Collectively, these findings suggest that smokeless tobacco exposure is associated not only with changes in microbial abundance but also with ecological restructuring across multiple layers, including taxonomic dysbiosis, orotype transition, pathobiont enrichment, and functional metabolic adaptation in the oral microbiome. This was confirmed by UpSet plot analysis of oral microbial communities, which shared core microbiota and tobacco-associated unique microbial signatures between addicted and non-addicted individuals, suggesting that the oral microbial community is selectively restructured by long-term tobacco exposure.

4.2. Sex-Specific Taxonomic Restructuring and Opportunistic Pathobiont Enrichment

Comparative taxonomic profiling showed distinct sex-specific microbial restructuring patterns in participants with chronic tobacco addiction. The oral ecology of AM samples was mainly enriched with *Streptococcus*, *Gemella*, *Acinetobacter*, *Neisseria*, and *Veillonella*, which reflects an acidogenic and biofilm-promoting state of oral ecology associated with inflammation [44–46]. In contrast, addicted female (AF) samples showed high enrichment for *Pseudomonas* as well as *Stenotrophomonas*, *Neisseria*, and *Veillonella*, all signifying the enrichment of environmental resilient and stress-tolerant taxa that selectively adapted under the ecological stress of the tobacco environment. *Streptococcus* species are well known to proliferate in nicotine-rich environments [47] and drive the acidogenic and caries-promoting states associated with oral inflammation. *Veillonella* acts as an accessory pathogen that metabolizes lactic acid produced by *Streptococcus*. Furthermore, *Stenotrophomonas* and *Pseudomonas* species drive a pro-inflammatory microenvironment that allows opportunistic pathogens to colonize tobacco smokers through biofilm production [48,49]. Dominance of different genera was found in addicted males and females, with the common pathogenic genus *Pseudomonas* and accessory pathogens such as *Veillonella*. Therefore, all these pathogenic taxa lead to an increase in opportunistic pathobionts and taxa associated with dysbiosis [23,50]. SIMPER analysis also showed that a small number of genera, including *Pseudomonas*, *Streptococcus*, and *Acinetobacter*, accounted for the majority of the differences in microbial composition between the addicted and non-addicted groups, indicating that tobacco-induced oral dysbiosis might involve selection for a group of specific adaptive pathobionts instead of a widespread shift in the entire microbial community.

In contrast, the microbial compositions of the non-addicted male (NAM) and non-addicted female (NAF) samples were comparatively balanced, with a higher abundance of commensal and environmentally associated genera, such as *Comamonas*, *Flavobacterium*, *Delftia*, *Arcobacter*, and *Chryseobacterium*. The healthy oral microbiome is remarkably uniform at the genus level between individuals and is diverse in composition, influenced by host specificity, specific anatomic sites in the mouth, and diet [51]. Considering the dynamic and environment-dependent nature of the oral microbiome, the use of individual microbial taxa

as universal markers may be limited [52]. Hence, the Tobacco-Associated Dysbiosis Index (TDI) from the present study may be more ecologically sound, as it incorporates both the enrichment of pathobionts and the depletion of commensals to define tobacco-associated oral dysbiosis. The proposed TDI is conceptually similar to previously developed microbial dysbiosis indices, that is, the Subgingival Microbial Dysbiosis Index (SMDI), which focuses on community-level ecological imbalance and pathobiont enrichment, instead of relying on a single microbial biomarker for dysbiotic characterization [53]. Furthermore, the current results suggest that tobacco-induced dysbiosis is likely a result of ecological restructuring, in which several interacting microbial taxa play a role, a phenomenon that is complex and polymicrobial in nature under chronic tobacco exposure [22]. High TDI concentrations also provide preliminary evidence of the progressive enrichment of pathobionts and ecological imbalance due to tobacco exposure in addicted groups. The enrichment of *Streptococcus*, *Neisseria*, *Veillonella*, and *Acinetobacter* has been linked to tobacco exposure, oral dysbiosis, oral potentially malignant disorders, and oral cancer progression, respectively [23,37,44]. Together, these results indicate that long-term use of smokeless tobacco leads to descriptive differences observed between the male and female participants' microbial community, enhanced opportunistic pathobionts, and a dysbiosis-associated transitional oral ecological shift. The TDI proposed in this study should be considered a preliminary ecological index that requires validation in larger and independent cohorts. Interestingly, several genera selected a priori for TDI construction were independently identified by STAMP and SIMPER analyses as major contributors to tobacco-associated community dissimilarity, providing internal consistency between the proposed ecological index and observed microbial restructuring. However, the composite nature of the TDI may provide greater ecological robustness than the reliance on single microbial biomarkers in the highly dynamic oral environment. The currently proposed TDI is a hypothesis-generating ecological metric and not a confirmed biomarker. It needs to be tested for reproducibility, diagnostic performance, and generalizability in larger multicenter cohorts and external validation datasets in future studies.

4.3. Orotypes Ecological Mapping and *Streptococcus*-Dominant Dysbiosis

Recent developments in oral microbiome ecology have revealed that oral microbial communities can exist in different ecological states, or "orotypes", dominated by different bacterial genera, including *Neisseria*, *Prevotella*, and *Streptococcus* [26]. The oral microbiomes in the current study showed ecological restructuring of the homeostasis of communities toward a *Streptococcus*-enriched dysbiotic community with a decrease in various environment-balanced and commensal-associated genera, especially in the addicted male (AM) and addicted female (AF) groups. The ecological shift was larger in the addicted groups than in the non-addicted controls, indicating that chronic exposure to smokeless tobacco may lead to selective community adaptation and decreased community evenness. Smoking, oral hygiene habits, and dietary habits have recently been linked to similar orotype transitions and changes in oral health [54]. Recent orotype studies further suggest that S-type oral ecological states are linked to less favorable oral health, lower salivary flow rates, and metabolic-syndrome-related outcomes when compared to *Neisseria*-dominant oral ecological states [26]. A *Streptococcus*-dominated ecological configuration was found in addicted subjects, which might indicate the presence of an acidogenic and biofilm-promoting oral ecology [26]. *Streptococcus mutans* is a well-known oral pathogen associated with the development of dental caries [53]. As early colonizers and well-known biofilm-forming species, the ecological overrepresentation of *Streptococcus* species may contribute to altered microbial interactions, ecological destabilization, and dysbiosis-associated inflammatory diseases. In contrast, the non-addicted groups showed a relatively more

balanced distribution of microorganisms, with a higher proportion of microorganisms of commensal and environmental types, indicating that the oral ecological equilibrium remained preserved. Altogether, these findings suggest that tobacco-associated conditions can result in an orotype ecological drift towards the development of dysbiotic, less complex microbial community structures, defined by the presence of opportunistic adaptive taxa.

4.4. Tobacco-Driven Functional Dysbiosis, Metabolic Drift, and Oral Carcinogenic Potential

A clear shift in the functional states of relatively balanced metabolic functions in non-addicted groups towards detoxification-oriented and stress-adapted functional states in addicted persons was observed. Enrichment of dehalogenation and atrazine metabolism pathways and persistent nitrogen and sulfur cycling functions showed increased microbial adaptation towards xenobiotic degradation and chemical stress in addicted males [40]. Similarly, addicted females displayed significant increases in ammonia oxidation, nitrite reduction, sulfate reduction, dehalogenation, chitin degradation, and atrazine metabolism, reflecting enhanced microbial metabolic activity with chronic exposure to tobacco. In contrast, several commensal and environment-balanced functions, such as sulfur oxidation, xylan degradation, sulfide oxidation, nitrogen fixation, and chlorophenol degradation, were affected by a progressive decrease in the addicted groups.

The results suggest the possibility of “functional dysbiosis” in oral microbial communities, which involves the metabolic specialization of commensal microorganisms to cope with chemically challenged oral ecology. Previous studies have shown that tobacco exposure can affect oral microbial ecology by inducing oxidative stress, reducing oxygen levels, acidifying saliva, and modulating the inflammatory response, thereby favoring microbial communities that are better adapted to survive in stressful conditions and opportunistic environments [18]. Thus, functional enrichment of xenobiotic degradation pathways could be an adaptive microbial response to tumorigenesis-inducing compounds found in tobacco, such as nitrosamines, polyaromatic hydrocarbons, and other carcinogenic compounds [9,55]. Earlier reports suggest that xenobiotic biodegradation pathways are increased in smokeless tobacco users compared to non-users [20]. Comparable functional shifts in metabolism associated with detoxification mechanisms have been observed in the oral microbiomes of smokers and microbial ecosystems under environmental stress [18,42].

Notably, the continuous increase in nitrate/nitrite reduction and redox-dependent pathways may be associated with oral carcinogenesis. Under the acidic conditions in the oral cavity, nitrite-reducing microbes may facilitate the endogenous formation of nitrosamines [56], producing carcinogenic N-nitroso compounds (NOCs), which are strongly associated with oral potentially malignant disorders (OPMODs) and oral squamous cell carcinoma (OSCC) [57]. Simultaneously, a reduction in metabolically balanced commensal functions is likely to decrease ecological resilience and cause disturbances in oral homeostasis. The observed shift from broader ecological metabolism towards a more specialized microbiome with limited detoxification functions may reflect ecological adaptation to chronic tobacco exposure and could be associated with a biological environment that favors inflammation, oxidative stress, and carcinogenic processes. Overall, these findings suggest a gradual ecological continuum in which tobacco-related microbial metabolic shifts can lead to oral microbial imbalance, dysbiosis, chronic inflammation, precancerous transformation, and eventual oral carcinogenic transformation [22]. Finally, the proposed integrated ecological–functional progression model is presented in Figure 15 to summarize the collective ecological, functional, and carcinogenic implications of the oral microbiome reorganization observed in this study in a continuum progression model from microbial imbalance and dysbiosis to oral carcinogenic transformation. The ecological–functional progression model proposed in this study corroborates earlier studies showing that tobacco-induced

changes in oral microbial flora may predispose individuals to triggering inflammatory and carcinogenic microenvironmental changes during the development of oral cancers [58].

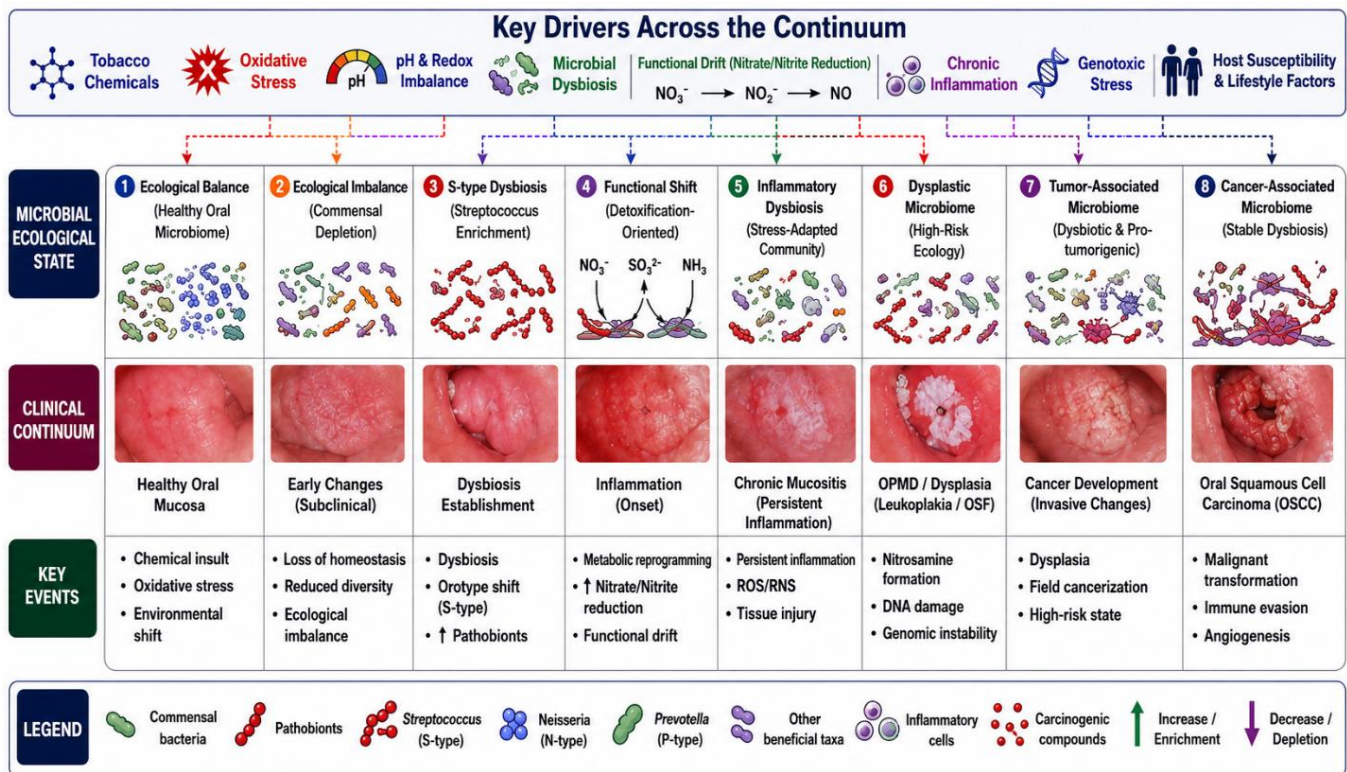


Figure 15. Proposed hypothetical ecological model based on findings from the present study and the published literature on tobacco-driven oral microbial dysbiosis and carcinogenic progression toward oral squamous cell carcinoma.

4.5. Study Implications and Limitations

The present exploratory study suggests descriptive patterns of oral microbial dysbiosis, microbial ecology restructuring, and functional metabolic drift in both male and female participants due to chronic smokeless tobacco exposure. Because seven daily samples were pooled before sequencing, the present study could not assess within-individual temporal microbiome dynamics. Accordingly, all ecological visualizations are intended as descriptive comparisons of representative pooled microbiome profiles rather than as longitudinal analyses. The tobacco-associated samples had decreased microbial diversity, changes in the ratio of Firmicutes to Bacteroidetes, enrichment of opportunistic pathobionts such as *Streptococcus*, *Pseudomonas*, *Acinetobacter*, *Gemella*, and *Veillonella*, and depletion of comparatively commensal/environment-associated genera such as *Comamonas*, *Delftia*, and *Flavobacterium*. The alpha diversity results showed decreased richness and evenness of microorganisms in both addicted groups, and the beta diversity results showed partial ecological segregation between the addicted and non-addicted groups. Tobacco-associated ecological restructuring was also confirmed using SIMPER and UpSet analyses, showing that *Pseudomonas*, *Streptococcus*, and *Acinetobacter* were important drivers of the differences among groups, with a relatively stable core bacterial community across all groups. STAMP-based differential abundance analysis also revealed that dysbiosis-associated taxa, including *Gemella*, *Acinetobacter johnsonii*, *Acinetobacter lwoffii*, *Neisseria*, and *Veillonella*, were more abundant in the tobacco-exposed groups. Chronic tobacco exposure showed a gradual shift towards *Streptococcus*-dominant (S-type) dysbiosis in orotype analyses. Phenotype-based functional prediction analysis indicated that metabolic drift, a shift in metabolic pathways,

occurred, characterized by changes in nitrogen cycling, nitrite reduction, ammonia oxidation, sulfate reduction, and pathways of degradation of xenobiotics, suggesting shifts toward an adaptation to stress-tolerant and detoxification-oriented microbial ecology. The proposed TDI also outlines a preliminary ecological model that integrates the strengths of the pathobiont enrichment pattern with the commensal depletion pattern to describe tobacco-associated oral dysbiosis. However, TDI is a preliminary ecological index that requires validation in larger studies. The small number of subjects helped limit the effect of temporal and environmental variation, while the controlled sibling-based design allowed for 7-day pooled samples over time. Overall, this study offers preliminary ecological and functional information on tobacco-associated oral dysbiosis and highlights the importance of conducting more comprehensive, large-scale, multiomic studies with consistent tobacco exposure to confirm microorganisms as potential ecological markers of orotype transitions and carcinogenesis-associated microbial progression.

We must note species-level assignments generated by k-mer tools like Kraken2 and Bracken from short-read V_3 – V_4 . The interpretation of 16S rRNA amplicons should be performed with extreme caution. Short amplicon sequences lack the polymorphic variation required to reliably differentiate closely related species within families, such as Enterobacteriaceae and Pseudomonadaceae. Consequently, the high relative abundance of species such as *E. coli*, *K. pneumoniae*, and *P. aeruginosa* observed in this study reflects broad computational taxonomic mapping rather than definitive species identification. Additionally, the substantial variance in raw sequencing depth among individual libraries (e.g., NAF vs. AF) introduces an inherent mathematical downsampling bias that can artificially skew species richness, alpha diversity, and evenness indicators, and limit rare taxon detection across individual profiles.

5. Conclusions

The present study provided a valuable close-up view of how chronic smokeless tobacco (SLT) use alters the oral microenvironment using a controlled household sibling-pair framework. By pooling samples over 7 days to wash out daily background noise, we captured stable and descriptive snapshots of each participant's oral ecosystem. Our findings suggest a clear pattern in the tobacco-addicted profiles: a higher Firmicutes/Bacteroidetes ratio, a decline in overall microbial diversity, and a notable rise in opportunistic pathobionts such as *Streptococcus*, *Acinetobacter*, *Pseudomonas*, and *Veillonella*. Ecologically, the data show a distinct shift away from a healthy, balanced state toward an acid-tolerant, biofilm-forming, and *Streptococcus*-dominant (S-type) setup. To track these changes, we introduced the Tobacco-Associated Dysbiosis Index (TDI) as a new conceptual tool; it successfully balanced pathobiont growth against commensal loss, showing a clear gap in dysbiosis scores between tobacco users and their non-user siblings. The proposed Tobacco-Associated Dysbiosis Index is exploratory and requires validation in larger independent cohorts before broader application. Furthermore, the predicted functional mapping suggests that the microbiome adapts to this chemical stress by shifting away from normal nutrient processing and heavily toward toxin degradation and nitrate/nitrite reduction.

This study was conducted with a small sample size. However, it provides a strong, hypothesis-generating baseline. The present study highlights how chronic chemical stress can drive a balanced oral ecosystem along a continuum toward a pro-inflammatory, high-risk niche in individuals. Ultimately, this exploratory study provides preliminary insights and underscores the need for larger, biologically replicated, multiomic studies to validate these microbial markers and better understand the link between smokeless tobacco exposure and oral disease progression.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/bacteria5030050/s1>, Figure S1. Relative abundance of the top 10 phyla identified across tobacco-addicted and non-addicted oral microbiome samples (AF: addicted female, AM: addicted male, NAF: non-addicted female, NAM: non-addicted male); Figure S2. Relative abundance of the top 10 families identified across tobacco-addicted and non-addicted oral microbiome samples (AF: addicted female; AM: addicted male, NAF: non-addicted female; NAM, non-addicted male).

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Institutional Review Board Statement: The study protocol was reviewed and recommended by the Departmental Research Review Committee of the Department of Biosciences, Veer Narmad South Gujarat University, Surat, India. The study involved the non-invasive collection of saliva samples from adult participants for oral microbiome analysis and did not involve any clinical intervention or treatment. According to the ICMR National Ethical Guidelines for Biomedical and Health Research Involving Human Participants (2017), collection of saliva samples is categorized as a minimal-risk procedure that does not require IEC approval as per the ICMR guidelines 2006.

Informed Consent Statement: Informed consent was obtained from all participants involved in the study. Written informed consent has been obtained from the participants to publish this paper.

Data Availability Statement: The data presented in this study are available on request from the corresponding author due to ongoing analyses. The bioinformatics and statistical analysis scripts used in this study are publicly available at <https://github.com/vmevada102/sree/blob/main/Microbiome%20analysis%20script.R> (accessed on 4 August 2026).

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Abbreviations

The following abbreviations are used in this manuscript: AF, addicted female; AM, addicted male; NAF, non-addicted female; NAM, non-addicted male; ST, smokeless tobacco; SLT, smokeless tobacco; OSCC, oral squamous cell carcinoma; TVC, total viable count; PCR, polymerase chain reaction; PCoA, principal coordinates analysis; PERMANOVA, permutational multivariate analysis of variance; STAMP, Statistical Analysis of Metagenomic Profiles; SIMPER, similarity percentage analysis; PEAR, paired-end reAd mergeR; TDI, Tobacco-Associated Dysbiosis Index; OPMD, oral potentially malignant disorder; ROS, reactive oxygen species; RNS, reactive nitrogen species.

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