BIOSCIENCES BIOTECHNOLOGY RESEARCH ASIA, June 2024. Vol. **21**(2), p. 671-687

Dominance of *Prevotella* Species in Tobacco Consumers: A Metagenomic Preliminary Study

Harshitkumar J. Savalia[1], Nimisha Patel[1]*, Krishna Mohan Singh[2], Manan Patel[2], Purva Gohil[3], Apurvasinh Puvar[3] and Chaitanya G. Joshi[3]

[1]Parul Institute of Applied Sciences and Research, Parul University, Vadodara, Gujarat, India.
[2]Molecular and Cytogenetics Department, Sterling Accuris Diagnostics, Ahmedabad, Gujarat, India.
[3]Gujarat Biotechnology Research Centre (GBRC), Gandhinagar, Gujarat, India.

http://dx.doi.org/10.13005/bbra/3255

(Received: 30 April 2024; accepted: 05 June 2024)

The human mouth cavity provides valuable clinical information about both oral and overall well-being. Tobacco has an impact on the oral microbiome, which is connected to a range of systemic disorders. Global studies are examining tobacco usage and other factors, including bacteria's role in oral cancer. Although there have been studies examining the connection between tobacco and the oral microbiota using 16S rRNA amplicon sequencing, there is a lack of investigations application of metagenomic sequences. we investigate, a total of 64 samples were obtained from each of the three categories: Tobacco chewers, Tobacco smokers and Normal healthy individuals. The samples were processed in duplicates. Total 10gbp data was generated with more than 90% good quality sequences and were used in downstream analysis. Metagenomic analysis was carried out using QIIME 2-2022.2 using the default parameters. An investigation was conducted to compare the prevalence of 18 species of Prevotella, which are found in high abundance in tobacco chewers (0.004%) as well as tobacco smokers (0.0017%) with compare to normal healthy persons (0.0008%). Total 15 species are absent in healthy individuals but present in tobacco consumers, while 30 species are found to be highly prevalent in tobacco chewers 12 species are highly abundant in tobacco smokers. Streptococcus was the most prevalent genus found among all the samples.

Keywords: Metagenomics; Oral Bacterial diversity; Tobacco Chewers; Tobacco Smokers.

Joshua Lederberg invented the word “microbiome” to describe “To refer to the ecological community of commensal, symbiotic, and pathogenic microorganisms that practically coexist in our bodies and are largely disregarded as risk factors for disease.”[1] All of the bacteria and their genes that inhabit the human body are collectively referred to as the “human microbiome”. Oral microbiomes are microorganism genomes found in oral cancer. When compared to other biomarkers from the host, the oral microbiota is regarded as an ideal biomarker for oral tumor. Anaerobes have a niche that is created by aerobic bacteria. In oral cancer, more than 700 different bacterial species can be found. In order to maintain equilibrium, mutualistic and pathogenic bacteria collaborate during coevolution. Bacterial organisms have a stable habitat at the 37 °C temperature in the oral cavity and pH 6.5 to 7.5 of saliva. The use of saliva is important for the diagnosis of oral disorders as

*Corresponding author E-mail: nimisha.patel@paruluniversity.ac.in

This is an Open Access article licensed under a Creative Commons license: Attribution 4.0 International (CC-BY).
Published by Oriental Scientific Publishing Company © 2024

well as the examination of their progression.[2] The microbiota receives nutrients from saliva and is kept hydrated. Oral biofilms created by aerobic and anaerobic bacteria stop their environment from changing.[3] In comparison to eukaryotic cells, human bodies contain a greater number of prokaryotic organisms. These prokaryotic visitors conduct several biological processes that humans would be unable to execute on our own and safeguard us against pathogenic germs. Scientists believed that the sequencing of the human genome would be sufficient to comprehend the underlying causes of human function and disease in the early 1990s, but the analysis of the human genome was merely a basic overview of the genetic makeup of our bodies.[4] Many microorganisms, including bacteria, viruses, and fungus, live in the oral cavity and may have a role in the development and spread of oral cancer.[5] The most common phyla in the adult human oral cavity, according to the Human Oral Microbiome Database (HOMD), are *Proteobacteria, Firmicutes, Fusobacteria, Bacteroidetes and Actinobacteria.* In the taxonomic hierarchy of the human oral microbiome, as of March 2020, the HOMD listed 1,567 genomes and 784 distinct bacterial species (HOMD, www.homd.org). The total volume of oral microorganisms is around 1011 bacteria/mL. The most frequent genus found in the oral cavity is *Streptococcus*, which is then followed by *Porphyromonas, Haemophilus, Leptotrichia, Propionibacterium, Prevotella, Veillonella, Staphylococcus*, and *Treponema*. [1,6,7] Compared to other body locations, oral microbiota has the lowest beta diversity but the largest alpha diversity, and there aren't many differences in the makeup of the oral cavity microbiota between different people.[37] Smoking in persons leads to a substantial increase in the possibility of developing cancer. It has been scientifically proven that tobacco smoke includes more than 60 known cancer-causing substances.[8] After drinking alcohol, ethanol can still be tasted in the tongue for hours. The uniform distribution of absorbed alcohol in body fluids causes the amounts of ethanol in saliva and blood to decline at the same pace. However, it is unknown how alcohol consumption alters the oral microbiome. It has been hypothesized that bacterial overgrowth in those with bad dental hygiene may enhance the production of microbial compounds with potential cancer-causing properties. The microbial synthesis of cancer-causing acetaldehyde from ethanol is of particular relevance in this context.[9] Tobacco may affect the progression of a disease by changing the microbial populations in the oral cavity.[10,11] *Prevotella* species have been linked to the emergence of inflammatory conditions and oral cancer. The development of diseases may be facilitated by smoking-associated dysbiosis of the salivary microbiome in cigarette smokers, particularly higher abundance of *Prevotella* and *Megasphaera* species.[12] Bacteria play a crucial role in oral carcinogenesis through suppression of apoptosis, activation of cell proliferation, promotion of invasion, chronic inflammation, and generation of carcinogens.[13] The development of knowledge about many pathogenic microorganisms and the abundance of information about the diseases that these organisms cause have improved the full genome sequencing investigations of many of these organisms.[14] Metagenomics has revolutionized microbiology research and opened up a window for examining previously unknown world of micro-organisms and their diversity, according to a report by the United States National Research Council committee, "The New Science of Metagenomics: Revealing the Secrets of Our Microbial Planet".[15] In the past ten years, a number of studies have examined the variations in oral bacteria linked to OSCC (Oral squamous cell carcinoma) from new angles, and their analyses have revealed both similarities and differences. A comparison was made between the DNA of oral microbiome obtained from cancer patients and healthy volunteers using 16S rRNA amplicon sequencing. The results revealed significant connections between oral squamous cell carcinoma (OSCC) and oral bacteria. Microbiological diagnostics involve the identification of pathogens in clinical samples to advise the management and treatment of infections in patients.[16] Traditional microbiology diagnostic methods include microorganism cultivation, serology for the detection of pathogen-specific antibodies or antigens, and polymerase chain reaction (PCR) for the identification of microbial nucleic acids. These methods aid in the discovery and description of infectious agents in clinical samples.[17] Metagenomics has grown in popularity as a method of microbiological study with the advancement of DNA sequencing technologies. A metagenomic method has been

employed in several recent research to investigate the functions of microbes in oral disorders.[18]

The objective of this study is to compare the bacterial diversity between those who consume tobacco and those who do not consume tobacco, as well as to identify the dominant species in tobacco consumers.

MATERIAL AND METHODOLOGY

Sample collection

The study included 64 male patients or volunteers. 28 volunteers chewed tobacco, 28 volunteers smoked tobacco, and 8 volunteers were healthy. All samples were run in duplicates. This study excluded patients and volunteers with diabetes, hepatitis, HIV, and autoimmune illnesses who were taking antibiotics. The Sterling Hospital Ethics Committee of Sterling Hospital Ahmedabad, Gujarat, India (SHEC/HS/OC-Study/235-2022) accepted this study for research and recruiting of oral cancer patients and healthy, long-term tobacco chewing and smoking volunteers. Each patient gave written consent before sample collection.

DNA Isolation

HIMEDIA Sterile Cotton Swabs (PW003) screw-capped polypropylene tube, cotton bud with polypropylene stick, individually packaged were used to take buccal and gum swabs. Swabs were kept at 4 °C in 500 µL 1x PBS Solution (M1452-500G, Phosphate Buffered Saline, pH 7.2) until use. The collected samples were vortexed at 2000 rpm for 10 minutes at 37 °C. DNA isolation was carried out by making minor modifications and combining the procedures provided by the manufacturer, using the QIAamp DNA Mini Kit (Catalog no: 51304). 50 ìL elution buffer was used to elute DNA samples. Until PCR amplification, the DNA was stored frozen at 20 °C. Spectrophotometric analysis of DNA purity and yield was performed using a BioTek EPOCH2 microplate reader. The ratio of absorbance at 260 and 280 nm (A260/A280) was used to evaluate the purity of DNA.

PCR and Sequencing

A particular primer pair of the V3-V4 region unique to the bacterial 16S rRNA gene was used to amplify DNA samples. The reaction process was performed at 95 °C for 5 min, followed by 32 cycles at 95 °C for 1 min, 58 °C for 50 secs, and 72 °C for 1 min, with a final extension at 72 °C for 7 min. For the PCR, reactions were set up for a total volume of 25 ìL, containing 1 ìL each primer (final concentration 5 pM), 12.5 ìL 2x KAPA HiFi HotStart Ready Mix, 90-100 ng sample DNA was used, 1 ìL BSA (Bovine serum albumin) for increases PCR yields. For high yields also performed nested PCR for from Previous PCR Product at 95 °C for 5 min, followed by 15 cycles at 95 °C for 1 min, 58 °C for 50 secs, and 72 °C for 1 min, with a final extension at 72 °C for 7 min. The size of the PCR result was verified by running DNA samples via a 2 % agarose gel electrophoresis. PCR products were purified with 20 mL of AMPure XP (BECKMAN COULTER; Catalog No. A63881) in accordance with the manufacturer's protocol and eluted into 50 ml of 10 mM Tris, pH 8.5.[19]

Library preparation and sequencing

Illumina adapters were attached to the PCR products using Nextera XT Index Kit V2 (Illumina®, USA-Catalog No. 15052164). After purification, the quantity of dsDNA was determined using Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific®, USA -Catalog No. 2438348) with Qubit Fluorometer 2.0. Particular to the size (~ 450 bp) purification was carried out by using the E-Gel CloneWell II agarose gels, which come with two comb systems. The eluted PCR products were purified with AMPure XP beads (Beckman Coulter, Switzer-land) with a ratio of 0.9X as recommended with the user manual. Quantification was performed using the Qubit dsDNA HS Assay Kit with the Qubit 2.0 Fluorometer once the purification process was completed. The library was pooled at 4nM and final loading concentration on flowcell was 100pM and sequencing was carried out on a NovaSeq 6000 SP 500 cycle system using 250X2 bp chemistry.[19]

Amplicon sequence analysis

Prinseq lite was used after a Perl script to process the raw readings.[20] The quality value of 50 was chosen for the data filter, and 5 nucleotides were trimmed from the both sides. A filtered data was analyzed using the program QIIME 2–2022.2 with its default parameters before the data was analyzed. This was done unless it was specifically stated otherwise. Denoising and demultiplexing the reads were both accomplished with the assistance of DADA2 pipelines. The greengenes2 database was utilized in order to carry out the taxonomic study.[20–22] To conduct a complete statistical,

functional, and integrative analysis of microbiome data, MicrobiomeAnalyst 2.0 was used.[23–25]

RESULTS AND DISCUSSION

The FastQC tool was crucial in assessing data quality for downstream analyses, removing sequence artifacts and trimming 5 nucleotides from both sides to ensure good quality. Per Sequence Quality Scores are a set of scores associated with each base (nucleotide) in a DNA sequence to represent the quality or reliability of the nucleotide (Figure 1). Per Sequence GC Content measures guanine and cytosine nucleotides in DNA sequences, providing insights into structural and functional properties, with an average of 53% (Figure 2). Total 10 Gbp raw data obtained and 9 Gbp data is good quality which is 90 %. This trimmed good quality data used for downstreme analysis.

The rarefaction curve indicated that the graph has reached the sampling size and sequencing depth that are sufficient to observe the total diversity of the microbiota because it has achieved these levels. At the sequencing depth of 10000 reads, the alpha rarefaction curve obtained a plateau for all of the samples. **(Figure-3)**

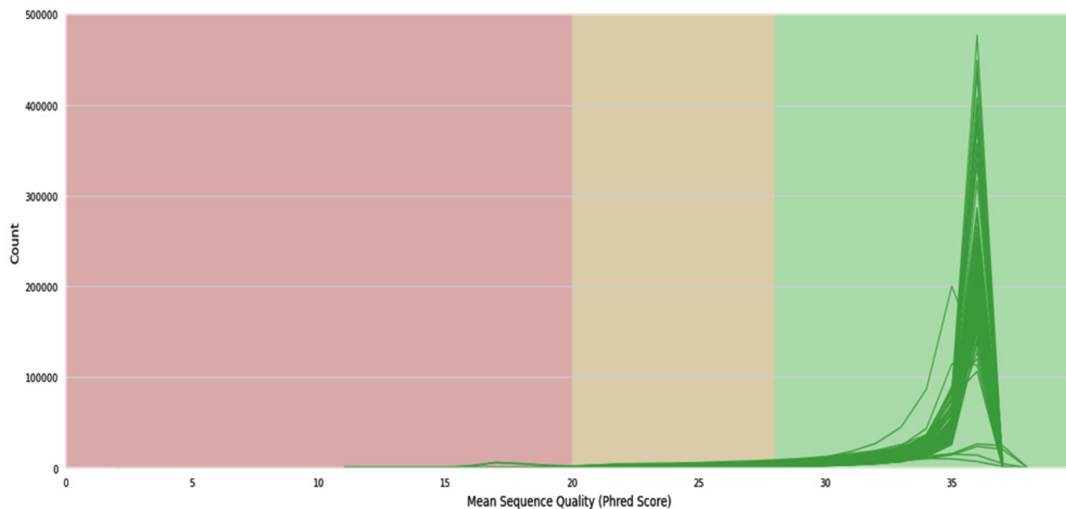

Fig. 1. Per sequence quality score

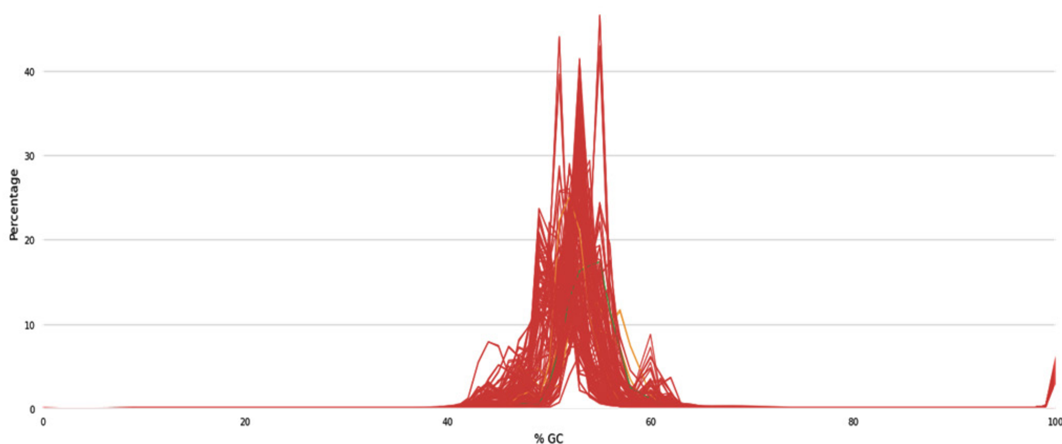

Fig. 2. Per sequence GC content

Alpha diversity refers to the level of species diversity within a specific habitat or ecosystem. In our study F-value of 10.06 and a p-value of 0.00016755 indicate that there is significant variation in alpha diversity between the groups being compared (e.g., tobacco consumers and normal healthy individuals). Alpha diversity is a measure of biodiversity within a single sample. The null hypothesis is rejected and it is determined that there is a significant difference in alpha diversity between the two groups based on the low p-value, which suggests that this difference is unlikely to be the result of random chance. This finding corresponds with the 2020 study conducted by Rituja Saxena et al. to evaluate the impact of smokeless tobacco use on oral microbiome in healthy and oral cancer patients in 2022.[26] **(Figure-4)**

Beta diversity, in comparison, measures the level of diversity among different ecosystems or communities. As per our study indicates that there are notable variations in beta diversity among persons who chew tobacco, those who smoke tobacco, and healthy individuals. There may be significant differences between the groups being compared, as indicated by a higher F-value (6.5689), which indicates that the variation within and between groups is greater. The study found that tobacco use status explained 0.1772 of the variance, a p-value of 0.001 shows that the results are unlikely to be observed if the groups are not different. A p-value below 0.05 is statistically significant, indicating that the groups may differ. This result is same as the previous study of Assessing the Effect of Smokeless Tobacco Consumption on Oral Microbiome in Healthy and Oral Cancer Patients by Rituja Saxena et. al. in 2022.[26] **(Figure-5)**

In our study we found that Healthy Persons have a higher abundance of *Bacteroidota*, while Tobacco Chewers and Smokers have more *Fusobacteriota*, *Furmicutes* as well as *Actinobacteriota*. Figure 6 presents a stacked bar chart showing the relative abundance of various bacterial phyla across three groups: healthy people, tobacco chewers, and smokers. Each bar represents an individual's oral microbiome composition, with different colors representing different phylum. As per figure 6 the x-axis represents individual samples, while the y-axis shows relative abundance in percentages. The chart provides a comparative visualization of the microbiome diversity and composition across the three groups, showing how tobacco use might be associated with changes in the oral bacterial community. Each group's microbiome composition is visually distinct, with different species being more or less abundant in each group.

There are 220 species found during this study *Prevotella saccharolytica, Prevotella baroniae, Prevotella marshii, Prevotella micans, Prevotella maculosa, Shuttleworthia satelles, Peptostreptococcus stomatis, Prevotella scopos, Prevotella salivae, Treponema C maltophilum, Fretibacterium fastidiosum, Neisseria oralis* this all species are highly abundant in tobacco smokers and chewers with compare to normal healthy individuals. A stacked bar chart representing the relative prevalence of several bacterial species in three groups healthy individuals, tobacco chewers, and smokers is shown in Figure 7.

The LDA score, which refers to linear discriminant analysis, is a quantitative measure of the amount to which microorganisms contribute to the distinguishing of classes. It is possible to determine whether a feature is more effective in distinguishing between the groups that are being compared by examining its LDA score. As per this findings *Patescibacteria* are found highly abundant in the group Tobacco smokers in comparisons to tobacco chewer and healthy individuals. *Fusobacteriota*, *Actinobacteriota*, *Campyalobacterota*, were found highly abundant in the group Tobacco chewers. *Patescibacteria* were found to be highly abundant in Tobacco smokers. *Firmicutes* was highly abundant in both tobacco chewers as well as tobacco smokers Figure 8 represents a bar graph with microbiological characteristics on the y-axis and linear discriminant analysis (LDA) scores on the x-axis.

Different groups, such as tobacco chewers, tobacco smokers, and healthy persons, are represented by different colors on the dendrogram provided in figure 9. The samples were clustering together according to the degree of similarity between them. The scale at the bottom of the branching pattern indicates the level of similarity or distance between clusters,

and the branching pattern itself demonstrates how closely connected the samples are to one another. (Figure 9)

The figure 10 describes a heatmap that presents data on various types of bacteria found in samples from different groups of people, including tobacco smokers, tobacco chewers, and normal healthy persons for control. We found number of bacteria species across a wide range of samples, detailing the abundance of each bacterium in the context of patient and sample types. The heatmap likely visualizes the comparative abundance of these bacteria across different patient groups, showing variations in microbial presence or absence. (Figure 10)

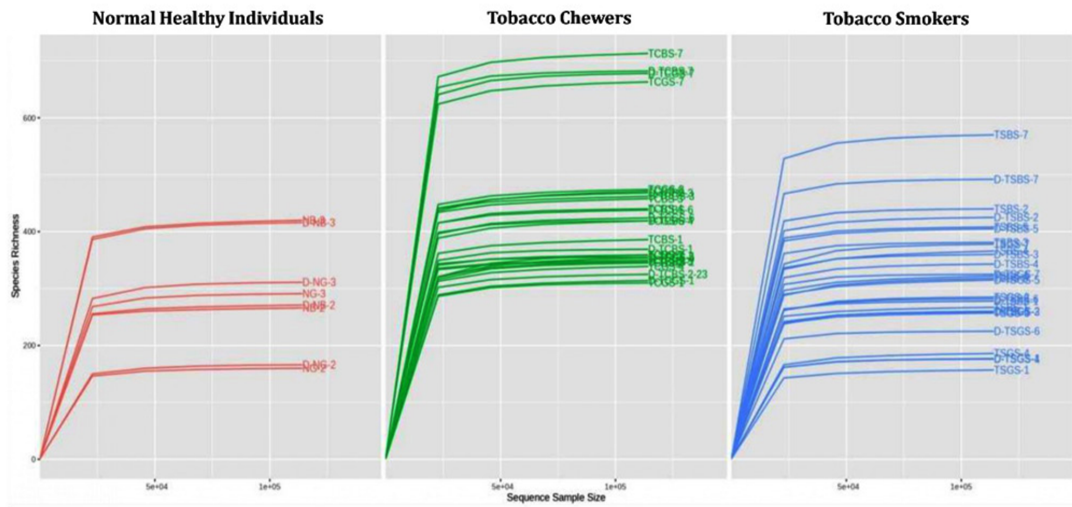

Fig. 3. The alpha rarefaction curve was created using the observed features of all samples at a depth of 10000 reads

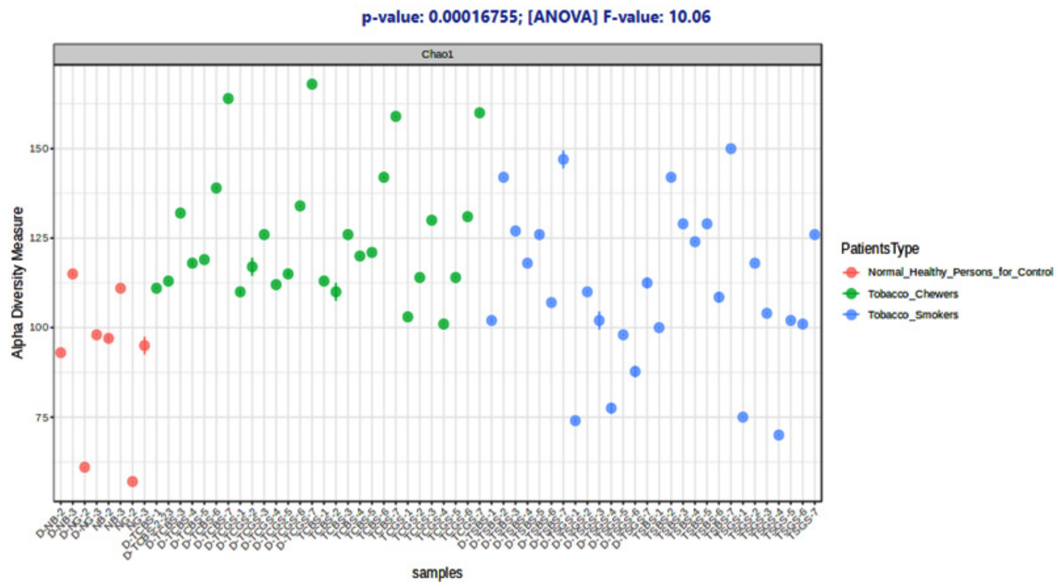

Fig. 4. Alpha diversity

The number of species that we found was 220, and among those, 53 species were shown to be extremely prevalent in tobacco consumers in comparison to healthy individuals. A total of 15 species were not found in healthy individuals, but they were found in extremely large numbers in tobacco chewers and smokers. (Table 1)

Actinomyces spp. were identified in tobacco chewers (0.00025%) and smokers (0.00014%). According to research by Valour and other scientists, *Actinomyces spp*. is the cause of *Actinomycosis*, an uncommon chronic disease. [27] *Campylobacter curvu* was identified in smokers (0.000075%), tobacco chewers (0.0015%) and Normal healthy individuals (0.0%). Medical literature has documented occasional cases of extra oral abscesses, namely in the oral region, in some *Campylobacter spp.* Rice, Tarrand, and Han's research from 2005.[28]

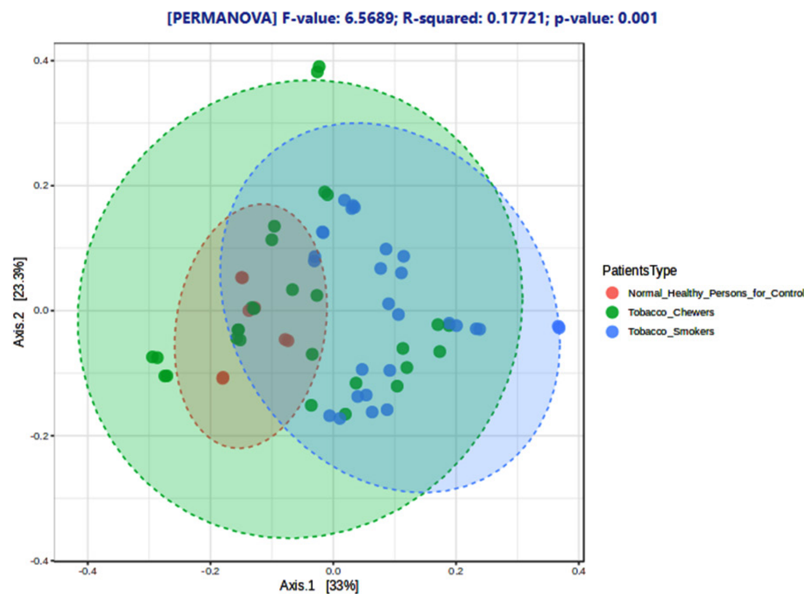

Fig. 5. Beta Diversity

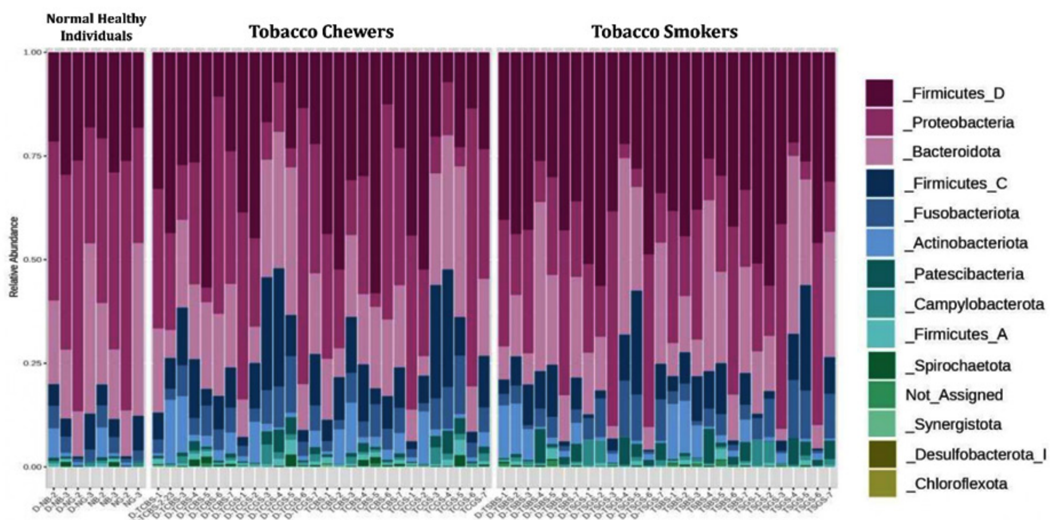

Fig. 6. A stacked bar chart of Phylum level abundance profiles across samples

We observed that tobacco chewers had 0.00018%, tobacco smokers had 0.00014%, and normal, healthy people had 0.0%. E. Veras and other researchers identified *Lancefieldella Spp.* as a novel discovered pathogen in periodontitis in 2023.[29] As per to the our results, the prevalence of *Leptotrichia spp.* was found to be 0.00095% in tobacco chewers, 0.00031% in tobacco smokers, and 0% in healthy normal people.

According to a study conducted by Amer et al. in 2017, it was found that the presence of high levels of *Leptotrichia spp.* was closely associated with severe dysplasia, and this association was statistically significant ($P < 0.05$). Oral leukoplakia has been observed to be characterized by a microbiota that is altered and shares characteristics with the microbiome of colorectal cancer.[30]

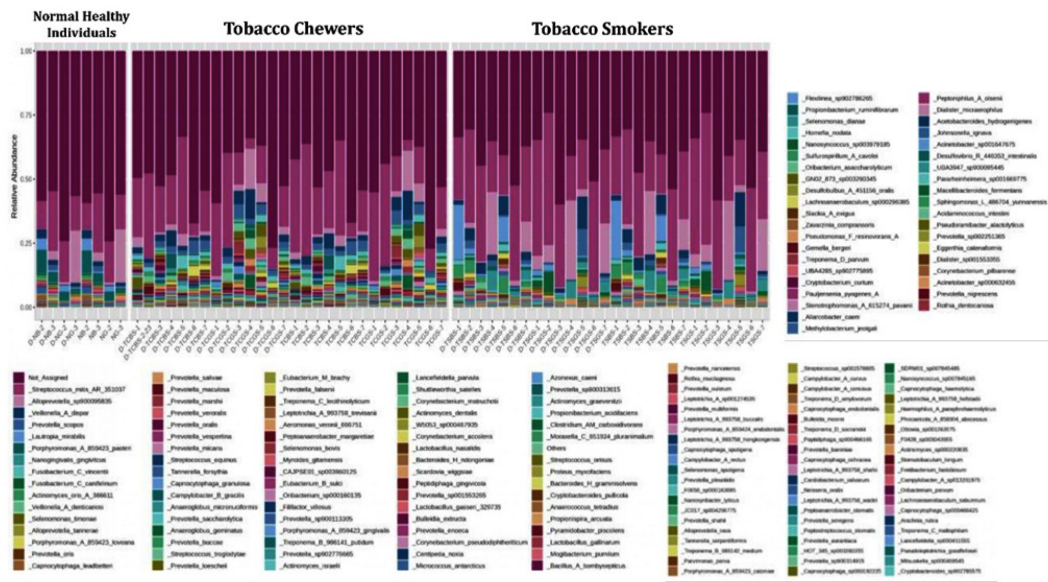

Fig. 7. A stacked bar chart of Species level abundance profiles across samples

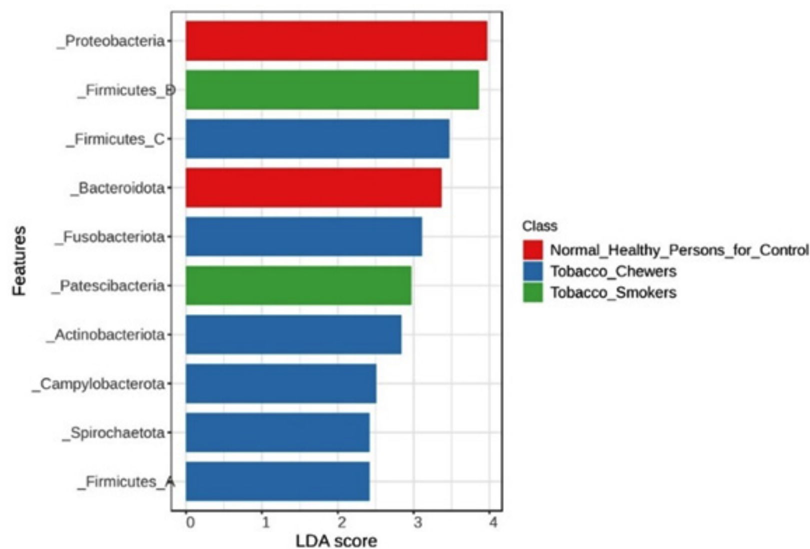

Fig. 8. Differentially abundant phylum in the Tobacco chewers, smokers and normal healthy individuals

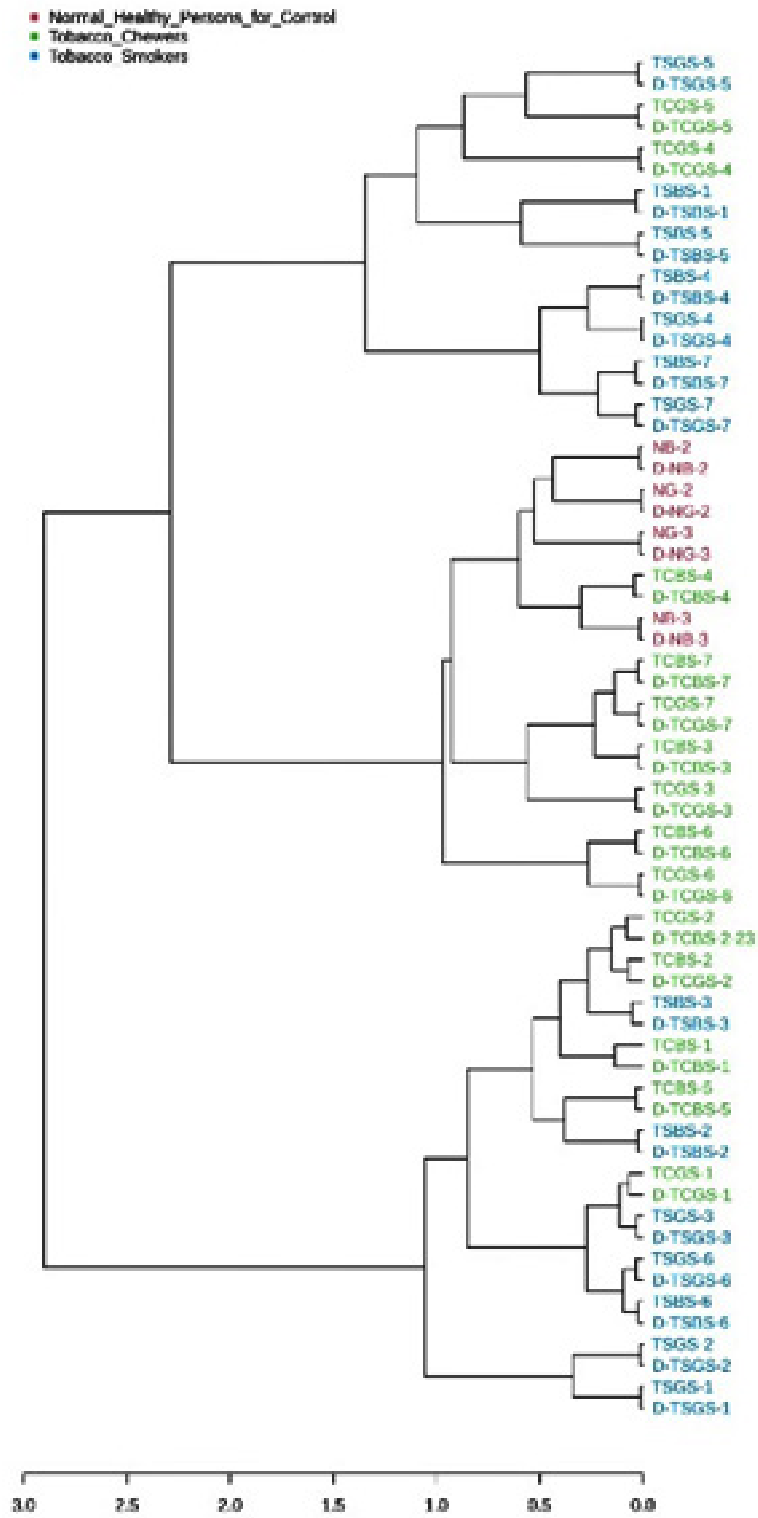

Fig. 9. A dendrogram presenting the clusters of samples annotated differently by color

Table 1. The percentage-wise abundance of tobacco chewers, smokers, and healthy people

No.	Species	Normal persons	Tobacco chewers	Tobacco smokers
1	*Actinomyces sp000220835*	0.0000000	0.0002596	0.0001475
2	*Campylobacter A curvus*	0.0000000	0.0015636	0.0000755
3	*Capnocytophaga haemolytica*	0.0000000	0.0004669	0.0000105
4	*Lancefieldella sp000411555*	0.0000000	0.0001844	0.0001455
5	*Leptotrichia A 993758 shahii*	0.0000000	0.0009554	0.0003182
6	*Leptotrichia A 993758 trevisanii*	0.0000000	0.0003662	0.0003989
7	*Porphyromonas A 859423 gingivalis*	0.0000000	0.0003473	0.0001318
8	*Prevotella falsenii*	0.0000000	0.0005140	0.0000343
9	*Prevotella seregens*	0.0000000	0.0008295	0.0002711
10	*Pseudoleptotrichia goodfellowii*	0.0000000	0.0001612	0.0000441
11	*Shuttleworthia satelles*	0.0000000	0.0001350	0.0000569
12	*Streptococcus equinus*	0.0000000	0.0022637	0.0000249
13	*Streptococcus sp001578805*	0.0000000	0.0012386	0.0000000
14	*Streptococcus troglodytae*	0.0000000	0.0018131	0.0002315
15	*Treponema C lecithinolyticum*	0.0000000	0.0006566	0.0001775
16	*Neisseria oralis*	0.0000023	0.0000016	0.0011889
17	*Phocaeicola A 858004 abscessus*	0.0000034	0.0002214	0.0000873
18	*Lancefieldella parvula*	0.0000069	0.0001249	0.0001027
19	*Prevotella sp902776665*	0.0000103	0.0005075	0.0002521
20	*Myroides gitamensis*	0.0000103	0.0002361	0.0004901
21	*Porphyromonas A 859423 loveana*	0.0000114	0.0006811	0.0033100
22	*Fretibacterium fastidiosum*	0.0000160	0.0002972	0.0001135
23	*Treponema C maltophilum*	0.0000195	0.0001677	0.0000762
24	*Cryptobacteroides sp902785575*	0.0000229	0.0001504	0.0000526
25	*Treponema D socranskii*	0.0000446	0.0011833	0.0001429
26	*Tannerella forsythia*	0.0000458	0.0017399	0.0005032
27	*Selenomonas bovis*	0.0000492	0.0008367	0.0001256
28	*Peptoanaerobacter stomatis*	0.0000504	0.0010355	0.0000451
29	*Peptostreptococcus stomatis*	0.0000698	0.0010826	0.0000765
30	*Anaeroglobus geminatus*	0.0000755	0.0013498	0.0007154
31	*Nanosynbacter lyticus*	0.0000778	0.0045097	0.0005617
32	*Prevotella vespertina*	0.0000996	0.0010692	0.0012876
33	*Prevotella buccae*	0.0001167	0.0019007	0.0002152
34	*Stomatobaculum longum*	0.0001225	0.0002168	0.0000664
35	*Prevotella salivae*	0.0001373	0.0026184	0.0004378
36	*Prevotella saccharolytica*	0.0001385	0.0018334	0.0002596
37	*Prevotella baroniae*	0.0001408	0.0010964	0.0000788
38	*Prevotella marshii*	0.0005825	0.0029248	0.0004113
39	*Prevotella micans*	0.0001694	0.0025678	0.0001674
40	*Prevotella maculosa*	0.0003353	0.0027404	0.0003862
41	*Prevotella veroralis*	0.0001797	0.0017291	0.0009695
42	*Prevotella pleuritidis*	0.0002861	0.0026394	0.0028558
43	*Prevotella multiformis*	0.0002976	0.0060498	0.0009744
44	*Prevotella oulorum*	0.0003021	0.0071445	0.0007540
45	*Fusobacterium C vincentii*	0.0003445	0.0156286	0.0066651
46	*Prevotella oris*	0.0005493	0.0060612	0.0032309
47	*Alloprevotella tannerae*	0.0009693	0.0078158	0.0050165
48	*Rothia mucilaginosa*	0.0015061	0.0053023	0.0031236
49	*Veillonella A denticariosi*	0.0018368	0.0128719	0.0029448
50	*Prevotella nanceiensis*	0.0046487	0.0029189	0.0050221
51	*Prevotella scopos*	0.0069375	0.0242713	0.0145208
52	*Veillonella A dispar*	0.0175246	0.0300098	0.0299608
53	*Streptococcus mitis AR 351037*	0.1495434	0.2012523	0.2984361

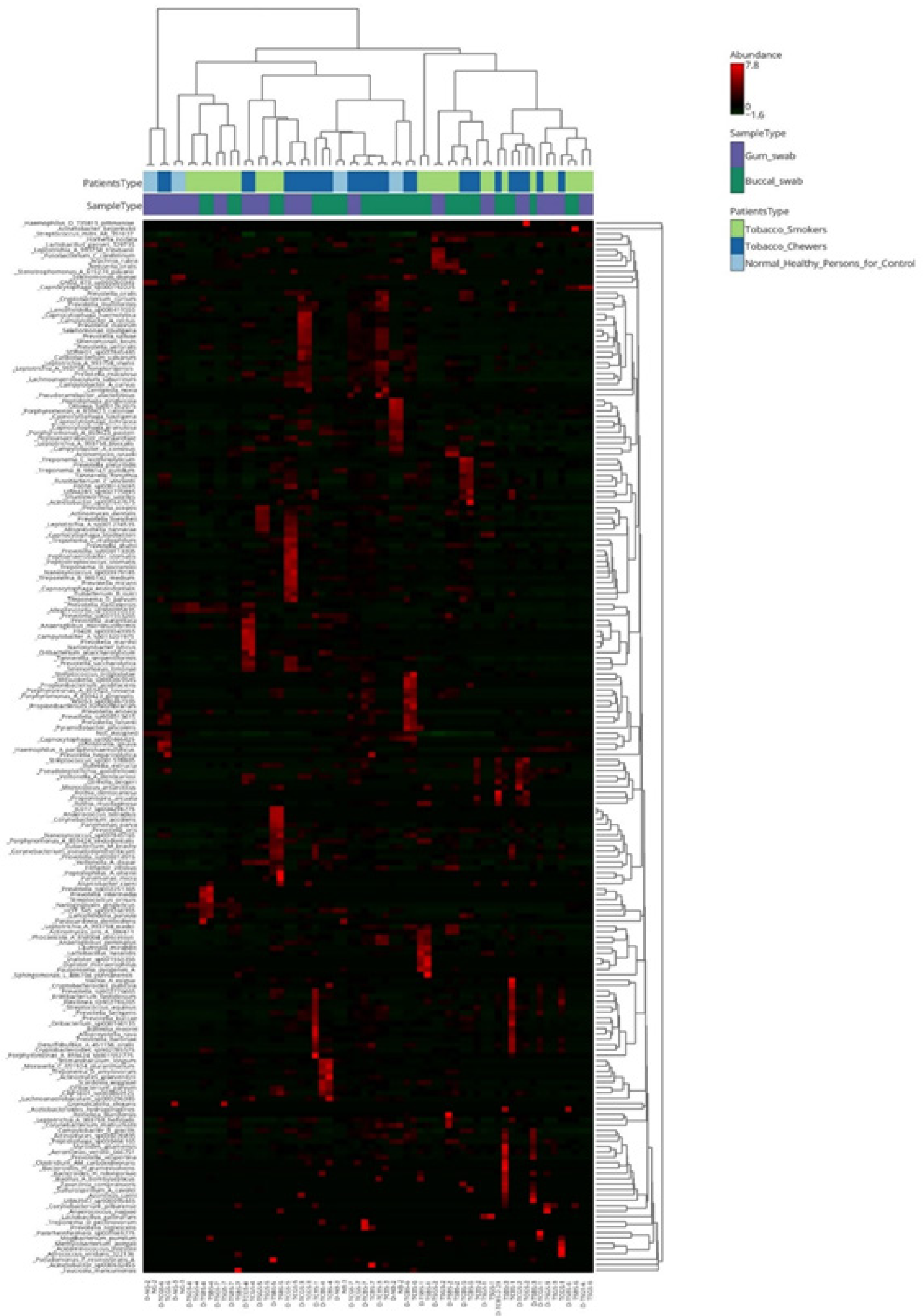

Fig. 10. A clustered heat map showing the variation of taxonomic abundance with regard to Tobacco chewers, smokers and Normal healthy individuals

Based on our data, we observed that the prevalence of *Porphyromonas gingivalis* was 0.00034% in tobacco chewers, 0.00013% in tobacco smokers, and 0.0% in those who were considered to be normal. As a result of the widespread distribution of this species in malignant oral epithelium, there is a potential connection between the bacteria and gingival squamous cell carcinoma. Furthermore, this species is an oral carcinogenic in both human and animal studies as per the J. Katz and other his colleagues in 2011.[31]

Prevotella falsenii was found 0.00051% in tobacco chewers, 0.000034% in tobacco smokers, and 0.0% in those who were considered to be normal. *Prevotella falsenii* were isolated from tissue that was tumorous according to Chocolatewala, Chaturvedi, and Desale (2010).[32] We observed that tobacco chewers had 0.00016%, tobacco smokers had 0.00004%, and not observed in normal patients of *Pseudoleptotrichia goodfellowii* Microorganisms that are normally found in the oral communities of humans. The work of Yabuuchi et al. (2022) reported. [33]

Streptococcus troglodytae with abundance of 0.0022% in tobacco chewers, 0.000024% in tobacco smokers and absent in normal healthy persons. This was new species that was isolated from the oral cavities of chimpanzees, also known as Pan troglodytes. Which was previously discovered by Okamoto et al. in 2016.[34]

Neisseria oralis was found highly abundant in tobacco smokers (0.00118%) but in normal and tobacco chewers it was found 0.00002%. *Neisseria oralis* was found in the oral cavity of humans in extremely large numbers in the year 2015 research by Liu, Tang, and Exley.[35] According to our results, the prevalence of *Phocaeicola A 858004 abscessus* was found to be 0.00022% in tobacco chewers, 0.000087% in tobacco smokers, and 0.000003% in healthy normal people. This species previously isolated from the saliva of a person who was receiving treatment for squamous cell carcinoma in the mouth in 2023 by of Jiung-Wen and other his collegues.[36]

Yip and other researchers found that *Porphyromonas loveana* can harm the immune response, which in turn allows for the advancement of disease in animals and we highly found in tobacco smokers (0.00331%), tobacco chewers (0.00068) and very less present in normal healthy persons (0.00001%).[37] In our research, tobacco chewers had 0.00029%, tobacco smokers had 0.00011%, and normal people had 0.00001% of *Fretibacterium fastidiosum*. Vartoukian et al. have isolated this novel genus and new species from human oral cavities in 2013.[38]

In our study, we observed varying levels of *Treponema C maltophilum* and *Treponema D socranskii* species among tobacco chewers, tobacco smokers, and normal control subjects. The prevalence of *Treponema C maltophilum* was found to be highest among tobacco chewers, followed by tobacco smokers and normal controls. Specifically, tobacco chewers exhibited a prevalence of 0.000167%, while tobacco smokers had 0.000076%, and normal controls had 0.000019%. Similarly, the prevalence of *Treponema D socranskii* was highest among tobacco chewers at 0.00118%, followed by tobacco smokers at 0.00014%, and normal controls at 0.000044%. The results of this study are consistent with the studies conducted by Wyss et al. (1996), indicating that these spiral-shaped bacteria are linked to serious health conditions such as syphilis and periodontal diseases. The greater occurrence of these species among those who use tobacco, namely those who chew it, may suggest a possible connection between tobacco intake and a heightened susceptibility to these illnesses.[39]

According to our study, those who chew tobacco had a prevalence of 0.00173% of *Tannerella forsythia*, while tobacco smokers had a prevalence of 0.00050%. In comparison, individuals who do not consume tobacco had a prevalence of 0.000045% of *Tannerella forsythia*. This is Gram-negative anaerobe, a periodontal pathogen, is a significant focus for disease treatments according to Bloch et al. (2019).[40]

Our findings indicate that the occurrence of *Selenomonas bovis* was 0.00083% among individuals who chew tobacco, 0.00012% among tobacco smokers, and 0.00004% among healthy individuals without any tobacco-related habits. *Selenomonas bovis* is linked to both chronic periodontitis and periodontal health. Purnima Kumar's 2005 work.[41] In our study we found that *Peptoanaerobacter stomatis* present 0.0010% in tobacco chewers, 0.00004% in tobacco smokers, and 0.00005% in healthy normal people which was previously isolated from human sub gingival plaque by sizova and other reserchers in 2015.[42]

Prevotella scopos and *Peptostreptococcus stomatis* species were found in different amounts in tobacco chewers, smokers, and healthy control persons in our investigation. When compared to normal controls, tobacco chewers and smokers had the highest prevalence of *Prevotella scopos*. To be more precise, the prevalence of tobacco chewers was 0.0242%, that of tobacco smokers was 0.0145%, and that of normal controls was 0.0069%. Similarly, tobacco chewers had the highest prevalence of *Peptostreptococcus stomatis* (0.00108%), followed by tobacco smokers (0.000076%) and normal controls (0.000069%). These results are consistent with earlier studies conducted in 2011 and 2006 by Downes and Wade, who reported that both species were newly isolated from human oral cavities.[43,44]

According to the findings of our research, the highest prevalence of Anaeroglobus geminatus was found in tobacco chewers (0.00134%), followed by tobacco smokers (0.00071%) and normal controls (0.00007%) who had the lowest prevalence. This was a putative pathogen that was discovered relatively recently, and it has the potential to play a role in the microbial shift that is associated with periodontitis. lately, this pathogen was discovered. The deterioration of periodontal tissues by inflammatory processes is the defining characteristic of periodontitis, which ultimately results in the loss of healthy teeth. 2017 research carried out by Bao and colleagues.[45]

Our data showed that the prevalence of *Nanosynbacter lyticus* was 0.0045% in tobacco chewers, 0.00056% in tobacco smokers, and 0.00007% in normal healthy individuals. This particular strain of *Nanosynbacter lyticus* had a very small genome and only a few metabolic pathways. There was recently isolated by Hendrickson and colleagues in 2024 from the oral cavity.[46] In our investigation, we found that the prevalence of *Prevotella buccae* in tobacco chewers was 0.00190%, while the prevalence in tobacco smokers was 0.00021%, and the prevalence in normal persons was 0.00011%. These findings were previously isolated from people who suffer from chronic periodontitis by Sharma et al. 2023.[47]

As per our study tobacco chewers had 0.00261% which was higher than tobacco smokers (0.00043%) and normal healthy persons (0.00013%) of *Prevotella salivae* which was new species that have been previously isolated from the oral cavity of humans by Sizova et al. in 2013.[48] *Prevotella multiformis* had high prevalent in tobacco chewers (0.0060%) than the tobacco smokers (0.00097%) and normal persons (0.00029). This was a new species that was isolated from the human subgingival plaque in 2005, Sakamoto and other reserchers.[49] The obligate anaerobes, such as *Prevotella oris*, were among the most prevalent members of the bacteriome associated with OSCC.[50]

The presence of *Alloprevotella tannerae* was found in tobacco chewers (0.00781%), smokers (0.00501%), and healthy individuals (0.0009%). Moore and colleagues in 1994 reclassified *Prevotella tannerae* as *Alloprevotella tannerae*, which is a Gram-negative, anaerobic bacilli that was isolated from the oral cavity of humans.[51]

In our experiment, we detected varying quantities of *Veillonella A denticariosi* and *Rothia mucilaginosa* species in tobacco chewers, smokers, and healthy control subjects. *Veillonella A denticariosi* was more common in tobacco chewers and smokers than in normal controls. More specifically, tobacco chewers made up 0.0128% of the population, tobacco smokers made up 0.0018%, and normal controls made up 0.0029%. among a similar vein, the highest prevalence of *Rothia mucilaginosa* was found among tobacco chewers (0.0053%), followed by smokers (0.0031%) and healthy controls (0.0015%). These findings are in line with previous research done in 2020 by Amer and colleagues, who stated that *Veillonella A denticariosi* is a novel species that has been isolated from Rothia mucilaginosa and human carious dentine. Rothia mucilaginosa isolates are found in patients with oral leukoplakia diagnoses.[52]

Prevotella nanceiensis new species discovered in the oral cavity of humans through isolation. 2007 research by Alauzet et al.[53] Individuals who chewed tobacco had a presence of 0.2%, whereas those who smoked tobacco had a presence of 0.3% with regard to the substance. On the other hand, the prevalence of *Streptococcus mitis* was reported to be under 0.1% in healthy individuals. Narikiyo and others have stated that These bacteria are responsible for the generation of inflammatory cytokines, which in turn influence the progression of the disease, which in turn

contributes to the development of esophageal cancer. When there is a drop in the quantity of these bacteria, there is a corresponding decrease in the chance of sickness. 2004 study that was carried out.[54]

Shuttleworthia satelles Downes et al. in 2002, *Prevotella baroniae* and *Prevotella marshii* Downes et al. in 2005, *Prevotella maculosa* Downes et al. in 2007, *Prevotella micans* Downes et al. in 2009, *Prevotella saccharolytica* Downes et al. in 2010. All discovered a novel genus and a new species that were isolated from the oral cavity of humans. As per Table- 1 in our study the tobacco consumers had high prevalent of all species with compare to normal.[55–59] The smoker's oral microbiota represented a significant abundance of *Veillonella dispar*.[60]

Capnocytophaga haemolytica, Prevotella seregens, Streptococcus sp001578805, Treponema C lecithinolyticum, Lancefieldella parvula, Prevotella sp902776665, Myroides gitamensis, Prevotella veroralis, Prevotella pleuritidis, Prevotella oulorum, and Fusobacterium C vincentii. These species are present in significantly higher levels in tobacco consumers compared to healthy individuals. No additional information has been found regarding the presence of this species in individuals who consume tobacco.

CONCLUSION

A conclusion can be reached regarding the correlation between tobacco use and specific bacterial species, microbial abundance, and diversity. The findings, which are elaborated upon in this study, reveal significant variations in the diversity of alpha and beta microorganisms between individuals who use tobacco and those who are healthy. These differences suggest that the oral cavity microbiota of tobacco consumers is distinct. This modification could increase the risk of developing oral diseases, such as oral cancer. Total 53 species were more prevalent in individuals who consume tobacco compared to normal. A total of 15 species are absent in healthy individuals but are present in individuals who consume tobacco, whereas 30 species are highly frequent among individuals who chew tobacco. 12 species are present in large numbers among individuals who smoke tobacco. Among individuals who use tobacco, the presence of 18 species of *Prevotella* is significantly higher in those who chew tobacco (0.0040%) and smoke tobacco (0.0017%) compared to individuals who do not consume tobacco and are in good health (0.0008%). This study highlights the importance of understanding the role of the oral microbiome in both maintaining good health and causing diseases. They offer fresh insights into how behavioral choices, such as tobacco smoking, can have a major impact on microbial ecosystems and potentially increase the likelihood of developing diseases. This metagenomics study provides valuable insights into the impact of tobacco use on the oral microbiome. It enhances our understanding of the complex relationship between lifestyle factors and microbial communities in the human body. The research provides a significant contribution to the field by discovering unique microbial patterns that are associated with tobacco usage. Acquiring this knowledge improves our understanding of how the microbiome affects oral health and illness, which in turn helps us develop more precise interventions and treatments.

ACKNOWLEDGEMENT

The authors would like to express their sincere gratitude to Sterling Accuris Diagnostics-Ahmedabad and Gujarat Biotechnology Research Centre (GBRC)-Gandhinagar for providing the essential laboratory facilities necessary to complete this research. In addition, we would like to express our gratitude to the subjects and Sterling Hospitals-Ahmedabad for permitting us to collect samples for this investigation.

Conflict of Interest

We certify that we have no conflict of interest.

Funding Source

There is no funding Sources.

Authors' Contribution

Dr. Krishna M. Singh2 and Dr. Manan Patel2: Provide the essential laboratory facilities for DNA isolation and quantification. Ms. Purva Gohil3, Dr. Apurvasinh Puvar3 and Dr. Chaitanya G. Joshi3: Provide the laboratory facilities for NGS and 16s Metagenomics Run and analysis.

Data Availability Statement

Not applicable.

Ethics Approval Statement

Sterling Hospital Ethics Committee of Sterling Hospital Ahmedabad, Gujarat, India. (Ref. No. SHEC/HS/OC-Study/235-2022)

REFERENCES

1. Dewhirst FE, Chen T, Izard J, et al. The human oral microbiome. *J Bacteriol*. 2010;192(19):5002-5017. doi:10.1128/JB.00542-10
2. López-Jornet P, Hynninen JN, Parra-Perez F, Peres-Rubio C, Pons-Fuster E, Tvarijonaviciute A. The Role of Salivary Biomarkers in Monitoring Oral Health in Patients with Implants and Periodontitis. *Appl Sci*. 2024;14(2). doi:10.3390/app14020927
3. Chattopadhyay I, Verma M, Panda M. Role of Oral Microbiome Signatures in Diagnosis and Prognosis of Oral Cancer. *Technol Cancer Res Treat*. 2019;18. doi:10.1177/1533033819867354
4. Avila M, Ojcius DM, Zlem Yilmaz O¨. The Oral Microbiota: Living with a Permanent Guest. *DNA Cell Biol*. 2009;28(8):405-411.
5. Kusama K, Inoue H, Miyazaki Y, Kikuchi K, Sakashita H, Ochiai K. Microorganisms and cancer of the oral cavity. *Integr Cancer Sci Ther*. 2016;3(4):510-515. doi:10.15761/icst.1000200
6. Huse SM, Ye Y, Zhou Y, Fodor AA. A core human microbiome as viewed through 16S rRNA sequence clusters. *PLoS One*. 2012;7(6):1-12. doi:10.1371/journal.pone.0034242
7. Sender R, Fuchs S, Milo R. Revised Estimates for the Number of Human and Bacteria Cells in the Body. *PLoS Biol*. 2016;14(8):1-14. doi:10.1371/journal.pbio.1002533
8. Vakilzadeh MM, Khayami R, Daneshdoust D, et al. Prevalence of tobacco use among cancer patients in Iran: a systematic review and meta-analysis. *BMC Public Health*. 2024;24(1). doi:10.1186/s12889-024-18594-8
9. Homann N, Tillonen J, Rintamäki H, Salaspuro M, Lindqvist C, Meurman JH. Poor dental status increases acetaldehyde production from ethanol in saliva: A possible link to increased oral cancer risk among heavy drinkers. *Oral Oncol*. 2001;37(2):153-158. doi:10.1016/S1368-8375(00)00076-2
10. Mohammed RA, Ahmed SK. Oral cancer screening: Past, present, and future perspectives. *Oral Oncol Reports*. 2024;10(March):100306. doi:10.1016/j.oor.2024.100306
11. Khan MF, Hayhoe RP, Kabir R. Exploring the Risk Factors for Oral Cancer in Pakistan: A Systematic Literature Review. *Dent J*. 2024;12(2). doi:10.3390/dj12020025
12. Wirth R, Maróti G, Mihók R, et al. A case study of salivary microbiome in smokers and non-smokers in Hungary: analysis by shotgun metagenome sequencing. *J Oral Microbiol*. 2020;12(1). doi:10.1080/20002297.2020.1773067
13. Perera M, Al-Hebshi NN, Speicher DJ, Perera I, Johnson NW. Emerging role of bacteria in oral carcinogenesis: A review with special reference to perio-pathogenic bacteria. *J Oral Microbiol*. 2016;8(1). doi:10.3402/jom.v8.32762
14. Banerjee J, Mishra N, Dhas Y. Metagenomics: A new horizon in cancer research. *Meta Gene*. 2015;5:84-89. doi:10.1016/j.mgene.2015.05.005
15. Handelsman J, Tiedje J, Alvarez-Cohen L, Ashburner M, Cann I, DeLong E, Doolittle WF, Fraser-Liggett CM, Godzik A, Gordon JI RM. *The New Science of Metagenomics: Revealing the Secrets of Our Microbial Planet*.; 2007. doi:10.17226/11902
16. Li Q, Hu Y, Zhou X, Liu S, Han Q, Cheng L. Role of oral bacteria in the development of oral squamous cell carcinoma. *Cancers (Basel)*. 2020;12(10):1-18. doi:10.3390/cancers12102797
17. Chiu CY, Miller SA. Clinical metagenomics. *Nat Rev Genet*. 2019;20(6):341-355. doi:10.1038/s41576-019-0113-7
18. Xu P, Gunsolley J. Application of metagenomics in understanding oral health and disease. *Virulence*. 2014;5(3):424-432. doi:10.4161/viru.28532
19. Illumina. 16S Metagenomic Sequencing Library Preparation. *Illumina.com*. 2013;(B):1-28. http://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf
20. Cantu VA, Sadural J, Edwards R. PRINSEQ++, a multi-threaded tool for fast and efficient quality control and preprocessing of sequencing datasets. *PeerJ Prepr*. 2019;7(2019):e27553v1.
21. Bolyen E, Rideout JR, Dillon MR, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol*. 2019;37(8):852-857. doi:10.1038/s41587-019-0209-9
22. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. *Nat Methods*. 2016;13(7):581-583. doi:10.1038/nmeth.3869
23. Dhariwal A, Chong J, Habib S, King IL, Agellon LB, Xia J. MicrobiomeAnalyst: A web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. *Nucleic Acids Res*. 2017;45(W1):W180-W188. doi:10.1093/nar/

gkx295

24. Chong J, Liu P, Zhou G, Xia J. Using MicrobiomeAnalyst for comprehensive statistical, functional, and meta-analysis of microbiome data. *Nat Protoc*. 2020;15(3):799-821. doi:10.1038/s41596-019-0264-1
25. Lu Y, Zhou G, Ewald J, Pang Z, Shiri T, Xia J. MicrobiomeAnalyst 2.0: Comprehensive statistical, functional and integrative analysis of microbiome data. *Nucleic Acids Res*. 2023;51(1 W):W310-W318. doi:10.1093/nar/gkad407
26. Saxena R, Vishnu Prasoodanan PK, Gupta SV, et al. Assessing the Effect of Smokeless Tobacco Consumption on Oral Microbiome in Healthy and Oral Cancer Patients. *Front Cell Infect Microbiol*. 2022;12(March):1-20. doi:10.3389/fcimb.2022.841465
27. Valour F, Sénéchal A, Dupieux C, et al. Actinomycosis: Etiology, clinical features, diagnosis, treatment, and management. *Infect Drug Resist*. 2014;7:183-197. doi:10.2147/IDR.S39601
28. Han XY, Tarrand JJ, Rice DC. Oral Campylobacter species involved in extraoral abscess: A report of three cases. *J Clin Microbiol*. 2005;43(5):2513-2515. doi:10.1128/JCM.43.5.2513-2515.2005
29. Veras EL, Castro dos Santos N, Souza JGS, et al. Newly identified pathogens in periodontitis: evidence from an association and an elimination study. *J Oral Microbiol*. 2023;15(1). doi:10.1080/20002297.2023.2213111
30. Amer A, Galvin S, Healy CM, Moran GP. The microbiome of potentially malignant oral leukoplakia exhibits enrichment for Fusobacterium, Leptotrichia, Campylobacter, and Rothia species. *Front Microbiol*. 2017;8(DEC):1-9. doi:10.3389/fmicb.2017.02391
31. Katz J, Onate MD, Pauley KM, Bhattacharyya I, Cha S. Presence of Porphyromonas gingivalis in gingival squamous cell carcinoma. *Int J Oral Sci*. 2011;3(4):209-215. doi:10.4248/IJOS11075
32. Chocolatewala N, Chaturvedi P, Desale R. The role of bacteria in oral cancer. *Indian J Med Paediatr Oncol*. 2010;31(4):126-131. doi:10.4103/0971-5851.76195
33. Yabuuchi S, Oiki S, Minami S, Takase R, Watanabe D, Hashimoto W. Enhanced propagation of Granulicatella adiacens from human oral microbiota by hyaluronan. *Sci Rep*. 2022;12(1):1-11. doi:10.1038/s41598-022-14857-9
34. Okamoto M, Naito M, Miyanohara M, et al. Complete genome sequence of Streptococcus troglodytae TKU31 isolated from the oral cavity of a chimpanzee (Pan troglodytes). *Microbiol Immunol*. 2016;60(12):811-816. doi:10.1111/1348-0421.12453
35. Liu G, Tang CM, Exley RM. Non-pathogenic neisseria: Members of an abundant, multi-habitat, diverse genus. *Microbiol (United Kingdom)*. 2015;161(7):1297-1312. doi:10.1099/mic.0.000086
36. Jiung-Wen Chen1 ,† , Chao-Jen Shih2 , Li-Wha Wu3 ,4 , Yen-Chi Wu2 , Wei-Fan Chiang5 , Yuh-Ling Chen6 JHW. Phocaeicola oris sp. nov., an anaerobic bacterium isolated from the saliva of a patient with oral squamous cell carcinoma. *Int J Syst Evol Microbiol*. 2023;73(2):005703. https://doi.org/10.1099/ijsem.0.005703
37. Yip S, Dehcheshmeh MM, McLelland DJ, et al. Porphyromonas spp., Fusobacterium spp., and Bacteroides spp. dominate microbiota in the course of macropod progressive periodontal disease. *Sci Rep*. 2021;11(1):1-10. doi:10.1038/s41598-021-97057-1
38. Vartoukian SR, Downes J, Palmer RM, Wade WG. Fretibacterium fastidiosum gen. nov., sp. nov., isolated from the human oral cavity. *Int J Syst Evol Microbiol*. 2013;63(PART2):458-463. doi:10.1099/ijs.0.041038-0
39. Wyss C, Choi BK, Schüpbach P, Guggenheim B, Göbel UB. Treponema maltophilum sp. nov., a small oral spirochete isolated from human periodontal lesions. *Int J Syst Bacteriol*. 1996;46(3):745-752. doi:10.1099/00207713-46-3-745
40. Bloch S, Tomek MB, Friedrich V, Messner P, Schäffer C. Nonulosonic acids contribute to the pathogenicity of the oral bacterium Tannerella forsythia. *Interface Focus*. 2019;9(2). doi:10.1098/rsfs.2018.0064
41. Purnima Kumar. *Molecular Analysis of Bacteria Associated with Chronic Periodontitis and Periodontal Health*. Vol 7.; 2005. http://dx.doi.org/10.1016/j.intell.2008.09.007%0Ahttp://dx.doi.org/10.1016/S0010-9452(58)80010-6%0Ahttp://pss.sagepub.com/content/17/1/67.short%0Ahttp://dx.doi.org/10.1016/j.cogdev.2013.06.002%0Ahttp://www.chabris.com/Hooven2008.pdf%0Ahttp://www.ncbi.nlm.
42. Sizova M V., Chilaka A, Earl AM, et al. High-quality draft genome sequences of five anaerobic oral bacteria and description of Peptoanaerobacter stomatis gen. nov., sp. nov., a new member of the family Peptostreptococcaceae. *Stand Genomic Sci*. 2015;10(1):1-12. doi:10.1186/s40793-015-0027-8
43. Downes J, Wade WG. Peptostreptococcus stomatis sp. nov., isolated from the human oral cavity. *Int J Syst Evol Microbiol*. 2006;56(4):751-754. doi:10.1099/ijs.0.64041-0
44. Downes J, Wade WG. Prevotella fusca sp. nov.

and Prevotella scopos sp. nov., isolated from the human oral cavity. *Int J Syst Evol Microbiol*. 2011;61(4):854-858. doi:10.1099/ijs.0.023861-0

45. Bao K, Bostanci N, Thurnheer T, Belibasakis GN. Proteomic shifts in multi-species oral biofilms caused by Anaeroglobus geminates. *Sci Rep*. 2017;7(1):1-11. doi:10.1038/s41598-017-04594-9
46. Hendrickson EL, Bor B, Kerns KA, et al. Ultrasmall epibiont Nanosynbacter lyticus strain TM7x and host bacteria transcriptional activity after initial host parasitism. *J Oral Microbiol*. 2024;16(1). doi:10.1080/20002297.2023.2287349
47. Sharma G, Garg N, Hasan S, Saffarini D, Shirodkar S. Fumarate and nitrite reduction by Prevotella nigrescens and Prevotella buccae isolated from Chronic Periodontitis patients. *Microb Pathog*. 2023;176. doi:10.1016/j.micpath.2023.106022
48. Sizova M V., Muller P, Panikov N, et al. Stomatobaculum longum gen. nov., sp. nov., an obligately anaerobic bacterium from the human oral cavity. *Int J Syst Evol Microbiol*. 2013;63(PART4):1450-1456. doi:10.1099/ijs.0.042812-0
49. Sakamoto M, Huang Y, Umeda M, Ishikawa I, Benno Y. Prevotella multiformis sp. nov., isolated from human subgingival plaque. *Int J Syst Evol Microbiol*. 2005;55(2):815-819. doi:10.1099/ijs.0.63451-0
50. Minarovits J. Anaerobic bacterial communities associated with oral carcinoma: Intratumoral, surface-biofilm and salivary microbiota. *Anaerobe*. 2021;68. doi:10.1016/j.anaerobe.2020.102300
51. Downes J, Dewhirst FE, Tanner ACR, Wade WG. Description of Alloprevotella rava gen. nov., sp. nov., isolated from the human oral cavity, and reclassification of Prevotella tannerae Moore et al. 1994 as Alloprevotella tannerae gen. nov., comb. nov. *Int J Syst Evol Microbiol*. 2013;63(Pt 4):1214-1218. doi:10.1099/ijs.0.041376-0
52. Amer A, Whelan A, Al-Hebshi NN, Healy CM, Moran GP. Acetaldehyde production by Rothia mucilaginosa isolates from patients with oral leukoplakia. *J Oral Microbiol*. 2020;12(1). doi:10.1080/20002297.2020.1743066
53. Alauzet C, Mory F, Carlier JP, Marchandin H, Jumas-Bilak E, Lozniewski A. Prevotella nanceiencis sp. nov., isolated from human clinical samples. *Int J Syst Evol Microbiol*. 2007;57(10):2216-2220. doi:10.1099/ijs.0.65173-0
54. Narikiyo M, Tanabe C, Yamada Y, et al. Frequent and preferential infection of Treponema denticola, Streptococcus mitis, and Streptococcus anginosus in esophageal cancers. *Cancer Sci*. 2004;95(7):569-574. doi:10.1111/j.1349-7006.2004.tb02488.x
55. Downes J, Munson MA, Radford DR, Spratt DA, Wade WG. Shuttleworthia satelles gen. nov., sp. nov., isolated from the human oral cavity. *Int J Syst Evol Microbiol*. 2002;52(5):1469-1475. doi:10.1099/ijs.0.02122-0
56. Downes J, Sutcliffe IC, Booth V, Wade WG. Prevotella maculosa sp. nov., isolated from the human oral cavity. *Int J Syst Evol Microbiol*. 2007;57(12):2936-2939. doi:10.1099/ijs.0.65281-0
57. Downes J, Sutcliffe I, Tanner ACR, Wade WG. Prevotella marshii sp. nov. and Prevotella baroniae sp. nov., isolated from the human oral cavity. *Int J Syst Evol Microbiol*. 2005;55(4):1551-1555. doi:10.1099/ijs.0.63634-0
58. Downes J, Tanner ACR, Dewhirst FE, Wade WG. Prevotella saccharolytica sp. nov., isolated from the human oral cavity. *Int J Syst Evol Microbiol*. 2010;60(10):2458-2461. doi:10.1099/ijs.0.014720-0
59. Downes J, Liu M, Kononen E, Wade WG. Prevotella micans sp. nov., isolated from the human oral cavity. *Int J Syst Evol Microbiol*. 2009;59(4):771-774. doi:10.1099/ijs.0.002337-0
60. Al Bataineh MT, Dash NR, Elkhazendar M, et al. Revealing oral microbiota composition and functionality associated with heavy cigarette smoking. *J Transl Med*. 2020;18(1). doi:10.1186/s12967-020-02579-3