

# Analysis of the Human Oral Microbiome of Smokers and Non-Smokers Using PCR-RFLP and Ribotyping

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## Abstract

Recent advances in the field of microbial and medical ecology emphasize the critical role played by oral bacteria in the delicate dynamic equilibrium of human health and disease, creating the need to define the bacterial communities associated with healthy and non-healthy conditions and to capture shifts in community structure germane to diagnosis. Employing PCR-RFLP of the 16S rDNA gene from metagenomes and plate-wash (cultured) bacteria of oral wash from 10 volunteers, this study evaluated the stability of oral bacteria in healthy subjects and documented community shifts in smokers. Sequence analysis of selected 16S gene amplicons cloned with the Gene Hunter PCR-Trap vector and pCR 4-TOPO cloning kits was conducted to determine the bacteria identity and diversity indices of the two groups. Ribopatterns generated by the restriction enzymes *HaeIII* and *Sau3AI* were significantly ( $p < 0.05$ ) more distinct compared to *AluI* using the GelCompare II software cluster analysis. A stable core of bacteria DNA fingerprint was detected in all healthy subjects, and remained unchanged over the study period of 3 months. Signature bands (1500 bp with *HaeIII*) in smokers and in non-smokers (800 bp and 700 bp with *Sau3AI*) were evidently suggesting the presence of potential biomarkers of healthy and non-healthy states. There was no significant difference in the DNA fingerprints of cultured and metagenomic extracts. The genera *Xanthomonas*, *Streptococcus* and phylum Candidatus occurred in large numbers in both groups, however, a major shift in composition with the dominance of gram-negative bacteria in smokers compared to healthy subjects was quite remarkable. Taxonomic diversity in smokers was quite high, including members of the genera *Rothia*, *Synechococcus*, *Neisseria*, *Thiomargarita* and *Pyrobaculum*. These data highlight the presence of a stable core microbiome amidst a wide diversity, identify a distinct smokers' cluster and open the way for the search for potential biomarkers for specific diseases.

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## Keywords

**Oral Microbiome, PCR-RFLP, 16S rDNA Sequence, Restriction Enzymes**

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### 1. Introduction

The human oral microbiome is the most studied human microflora, due to the fact that it is easily sampled and is strongly associated with important oral infectious diseases such as tooth decay (dental caries), gum disease (periodontitis) and other diseases such as cardiac disease and cancers (pancreatic gastrointestinal and oral) [1]-[9]. The relationship of oral bacteria and development of these diseases tend to persist after taking into account factors like smoking, alcohol consumption, socioeconomic status etc.

Among the 700 bacterial species found in the oral cavity, most are commensal bacteria and are required to keep equilibrium in the mouth ecosystem although some of them play a key role in the development of oral diseases such as dental caries, periodontal disease [7] [10]-[12] and even oral/pharyngeal cancers like oral squamous cell carcinomas [8] [9]. In the United States, the primary risk factors of these diseases are tobacco smoking, and elevated levels of alcohol consumption [13]. A large body of evidence show that 42% of periodontitis in the United States is attributable to tobacco smoking [14], and numerous studies have reported a critical role for smoking in increasing the risk for developing extensive and severe forms of this disease [14]-[18]. Normal oral flora activate the tobacco smoke nitrosamine, nitrosodiethylamine (NDEA), to its carcinogenic (IARC, Group 2A), adduct-forming hydroxylated product [19]-[21]. This in turn increases the risk of oral cancer. Other potential risk factors include diet, human papillomavirus (particularly HPV16), and various oral factors, including oral hygiene [22]-[24]. Studies have also shown that smoking alters the microbial signatures of the oral cavity, with a decrease in the commensal population and a concomitant increase in pathogens [25] [26].

People usually become aware of the presence of these microbial communities within the body when the balance between the microbiota and the host is lost, and disease is manifest [27]. As is often the case, there is much more knowledge of the human microbiome at the state of disease than the grossly limited understanding of the healthy-state microbiome. In order to adequately diagnose and treat disease at an early and reversible stage, a thorough description of the commensal microbiome associated with health is necessary. It is also essential to understand the microbial community changes that accompany the early stages of periodontitis and dental caries that would allow diagnosis and treatment before the appearance of periodontal pockets, dental hard tissue loss and even oral cancer. It is therefore necessary to search for potential shifts in the oral microbiota of not only a healthy status, but that of transitioning health in smokers and actual disease state. The analyses of 16S genes from the DNA directly extracted from environment, metagenomic DNA, and cultures are being used to study the diversity of microorganisms in various environments. Over the last two decades, the cloning and sequencing of 16S genes amplified directly from environments like human skin, oral cavity and intestine through metagenomic approaches demonstrated that microbial diversity is far more extensive than we ever imagined from culture-based studies. One of the most common microbial typing methods is PCR-Restriction fragment length polymorphism of the amplified 16S gene and it represents the variations associated with restriction sites only [28]-[30].

The aim of this study was to evaluate the potential of PCR-RFLPs combined with sequencing of the 16SrDNA gene and computer assisted pattern analysis in profiling the bacterial community in smokers and non-smoking healthy individuals. The intent was also to capture any changes in the bacterial profile in the two groups over time (3 months apart) and identify predictive patterns and/or biomarkers indicative of changing oral health.

### 2. Materials and Methods

#### 2.1. Human Oral Wash Samples

Oral wash samples were collected in compliance with IRB and IBC approved protocol (IRBNET ID # 388951-2) and clinical consent was obtained from the volunteers. The study was carried out in the Microbial Biotechnology laboratory of the Charles E Schmidt College of Science at Florida Atlantic University, Davie, Florida.

Samples were collected from 5 healthy individuals above the age of 18 years. These individuals were antibi-

otic free; were not addicted to smoking for at least 3 months preceding the study; and adhered to their normal oral hygiene for 2 weeks before enrolment in the study. Samples were also collected from 5 smokers for comparison. The demographic information, periodontal parameters and smoking status for each group are summarized in **Table 1**. Oral wash samples were prepared by providing subjects with a 25 ml of bottled drinking water for oral gargle first thing in the morning. The sample is then collected in a 50 ml sterile Falcon tube that was provided by the lab. Samples were frozen at  $-20^{\circ}\text{C}$ . They were also asked to complete a questionnaire for reference.

#### a) Direct extraction of metagenomes from the oral wash

Samples were thawed by incubating at room temperature for 15 - 20 mins and 20 ml of the sample were transferred in 15 centrifuge tubes (1.5 ml) and centrifuged at 10,000 rpm for 10 mins in an aerosol-tight microcentrifuge (Eppendorf 5424R) to collect bacterial pellets. The pellets were used for direct extraction of the metagenomes. Total bacterial genomic DNA from oral wash samples was isolated using the Qiagen Dneasy Blood and Tissue DNA purification kit (Qiagen, Valencia, CA). In brief, the pellets were suspended in Phosphate Buffer Saline and washed twice by spinning at 15,000 rpm for 10 mins per wash. 200  $\mu\text{l}$  of enzymatic lysis buffer was added to the final pellet and incubated at  $37^{\circ}\text{C}$  for 1 - 2 hours. A 25  $\mu\text{l}$  aliquot of a proteinase K (Qiagen, Valencia, CA) and 200  $\mu\text{l}$  of AL buffer were added and incubated at  $56^{\circ}\text{C}$  for 30mins. 200  $\mu\text{l}$  of 96% - 100% ethanol was added to the sample and mixed by vortexing. The mixture was then be transferred in a QIAmp Mini Spin column with silica membrane for the adsorption of bacterial DNA Samples were washed with AW1 and AW2 buffer which were amended with alcohol as prescribed by manufacturer and the DNA was eluted using the 100  $\mu\text{l}$  of the AE buffer.

#### b) Genomic DNA extraction from plated oral wash samples

A sub-set (1.5 ml) dissolved in Phosphate Buffered Saline (PBS) of each pellet from “a” above was plated (100  $\mu\text{l}$ ) on Brain Heart Infusion Agar and incubated aerobically for 48 hours at  $30^{\circ}\text{C}$ . Two milliliters of sterile PBS was added to the agar culture plates to resuspend all visible growth into slurry. The community of cultivable bacteria was then transferred to microfuge tubes and centrifuged at 10,000 rpm for 10 mins to collect the bacterial pellets.

The quality and quantity of DNA samples were measured at 260 nm and 280 nm using a Nanodrop 2000C Spectrophotometer (Nanodrop products, DE, USA) respectively.

## 2.2. PCR Amplification and PCR Conditions

PCR was performed using the Mastercycler Nexus PCR Systems (Eppendorf North America, NY, USA). The bacterial 16S rDNA was amplified using the universal primer 1492 Reverse (5'GGTTACCTTGTTACGACTT-3') and the primer 27 Forward (5'-AGAGTTTGATCCTGGCTCAG-3') that targets the V1-V9 region of the 16S rRNA gene. Reaction mixtures were incubated for 4 mins at  $94^{\circ}\text{C}$  for denaturation, followed by 35 cycles consisting of 1 min at  $94^{\circ}\text{C}$ , annealing for 30 s at  $45^{\circ}\text{C}$ , and extension for 2 mins at  $72^{\circ}\text{C}$ . PCR product (approximately 1500 bp) will be confirmed by electrophoresis in 1% agarose (w/v) gel at 120 V for 30 mins. 10  $\mu\text{l}$  of the amplified PCR product will be utilized for restriction digest.

## 2.3. Restriction Fragment Length Polymorphism (RFLP)

PCR-amplified bacterial 16S rDNA was digested using 3 restriction enzymes: *HaeIII*, *AluI* and *Sau3A* (1U), ac-

**Table 1.** Demographic information, periodontal and smoking status of participants.

	Non-smokers n = 5	Smokers n = 5
Gender (M; F)	2; 3	3; 2
Age	27.6 $\pm$ 3.36	35.8 $\pm$ 12.5
Cigarette consumption (#)	--	13.4 $\pm$ 2.19
Duration of smoking (years)	--	19 $\pm$ 13.03
Periodontal health status	Self-recognized healthy	Gingivitis (n = 1)
Diet	Non-vegetarian	Non-vegetarian

cording to the manufacturer's instructions (New England Biolabs, Beverly, Mass.) [29]. The DNA fragments were separated by gel electrophoresis on a 3% (w/v) low melting agarose gel run in 1X TAE (Tris Acetate-EDTA, pH-8) buffer. The RFLP ribopattern profile were digitally captured and recorded by means of the FOTO/Analyst<sup>®</sup> Investigator/FX System using the PC Image Acquisition Software (Fotodyne Incorporated, Hartland, WI).

## 2.4. Sequencing the 16SrRNA gene

A small number of plasmid colonies of PCR products were transformed into chemically competent *E. coli* cells using the Gene Hunter PCR-Trap vector and pCR 4-TOPO high copy number vectors (Invitrogen, Carlsbad, CA, USA) according to manufacturer's manual. 500  $\mu$ ls of each transformation were plated on LB plates containing 50  $\mu$ g/ml tetracycline and were grown overnight. Cells from 10 colonies picked from selective LB agar plates were used to create a library and were dispatched for sequencing. Sequencing reactions were performed on an ABI GeneAmp<sup>®</sup> 9700 thermal cycler (Applied Biosystems) using v3.1 Big Dye kit according to the manufacturer's standard protocol of 25 cycles, followed by purification using Performa<sup>®</sup> gel filtration short plates (Edgemo, Gaithersburg, MD, USA). The sequencing runs were performed using POP7 polymer on 36-cm capillaries in an ABI 3730 DNA sequencer (Applied Biosystems) and were analyzed by ABI Sequencing Analysis software v5.1 and KB Basecaller (both from Applied Biosystems) capable of sequence quality value (QV) prediction on each base call. Sequences were analyzed by BLAST on the RDB II database and the HOMD. Diversity indices were also calculated for PCR-RFLP analysis: the Shannon-Wiener index ( $H'$ ) was calculated from  $H' = -\sum P_i \ln P_i$  where  $P_i$  is the proportion of the phylotypes.

## 2.5. Analysis of the 16S rDNA Fingerprint Generated by RFLP

An image of the RFLP gel was captured by Fotodyne Imager as mentioned above. The banding patterns were examined to identify the commonalities and differences (which is presumed to represent the pattern for the bio-marker strain) among the 2 different study groups. This was achieved by using the Gel Compare II (Applied Maths, Kortrijk, Belgium) software to analyze the 16s rDNA fingerprint. This software searches for discriminative bands between selected groups of patterns and also searches for unique and common bands within selections. To obtain an objective comparison and normalization of the bands on different slab gels, a molecular weight marker was included in each gel at least two times. Digitized images were normalized and combined [31]. A similarity matrix was created by using the Dice similarity coefficient [32] [33]. For the same slab gels similarity matrix was created using the Pearson correlation coefficient using the densitometry curve created by the fingerprints. Similarities were displayed graphically as a dendrogram. For band comparison, a band position tolerance value of 0.8% was allowed to compensate for misalignment of homologous bands due to technical imperfections. The unweighted pair group method using average linkages (UPGMA) [31] was used to cluster the patterns.

## 3. Results and Discussion

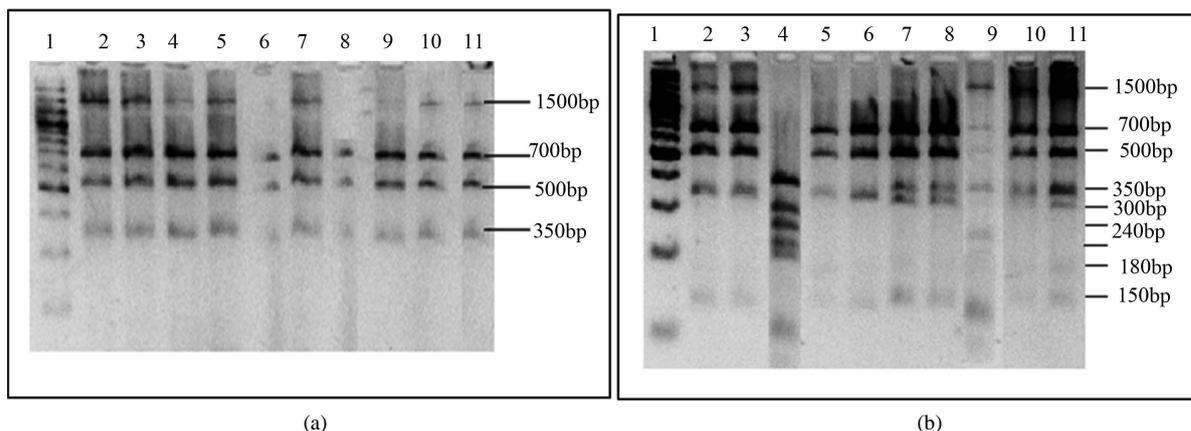
### 3.1. PCR-RFLP Analysis

DNA isolates from direct oral wash and cultured samples of ten subjects (5 healthy and 5 smokers) 3 months prior and post yielded 40 samples in total. All 40 samples were digested with the selected endonucleases for restriction analysis. The three enzymes generated distinctive PCR-RFLP finger prints in cultured and metagenomic subsets. The ribopattern for the cultured and metagenomic subset were also diverse. The analysis and comparison of restriction profiles obtained with the digestion of the amplified 16SrRNA gene sequences allowed the identification of a set of appropriate frequent-cutter endonucleases. Restriction enzyme *HaeIII* and *Sau3AI* was found to give the clearest and most discriminatory profiles for PCR-RFLP patterns compared to *AluI*.

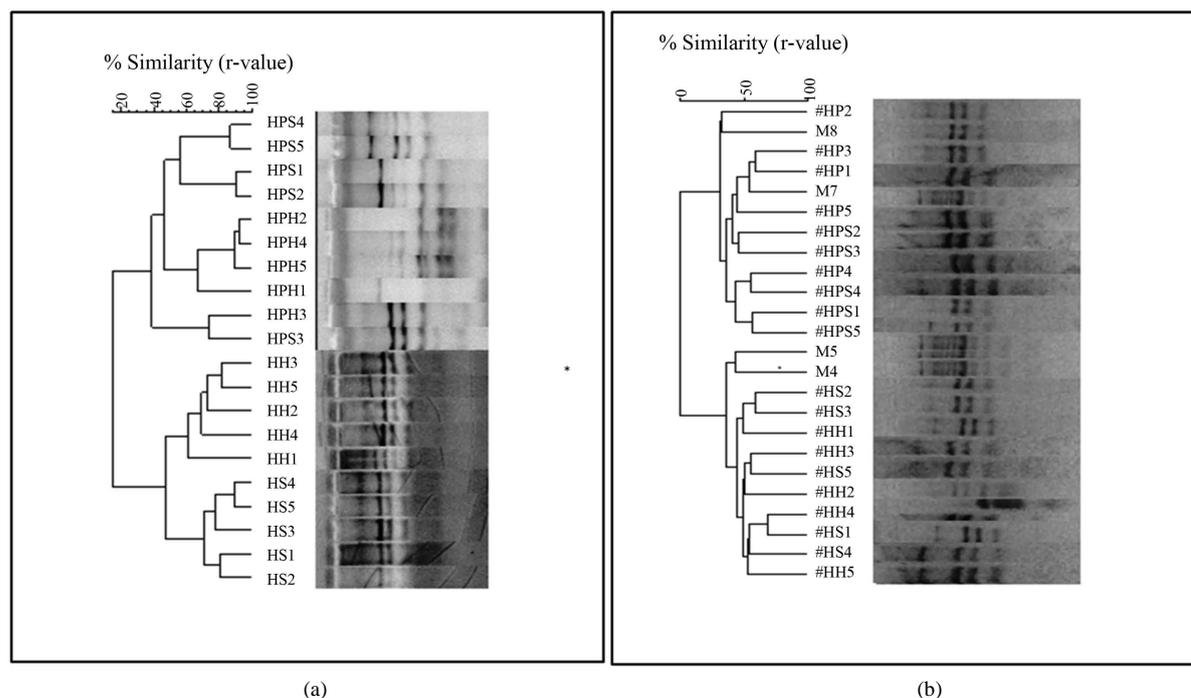
PCR-RFLP ripo pattern using *HaeIII* of initial samples for Healthy non-smokers and smoker yielded 3 well resolved stable bands of 700 bp, 500 bp and 350 bp in both cultured and metagenomic subsets (**Figure 1(a)** & **Figure 1(b)**). A fourth band (1500 bp) of lighter intensity was seen in three smokers (Smoker #3, Smoker #4 and #5) and 2 non-smokers (Healthy #1 and #2) in the cultured subset. In the same subset four smokers (Smoker #1, 2, 4 and 5) also displayed a unique 300 bp band which was absent in the other subjects (**Figure 1(b)**). The restriction patterns of the amplified 16S rDNA were used for cluster analysis by UPGMA. Analysis of the clus-

ter revealed that both healthy non-smoker and smoker groups were tightly grouped together in both cultured and metagenomic sets at a linkage level of ( $S_D$ ) 70%. However, it aligned one healthy subject (Healthy #3) with the smokers in the cultured subgroup (**Figure 2(a)**).

Post 3 months analysis showed the presence of three similar ubiquitous bands as was observed during the initial data analysis, affirming the existence of a core group in all the samples. This outcome suggested that this core group is well established and does not change over time. The 1500 bp fragment which was more prominent in smokers was intensified in the healthy subjects, Healthy #1 and #2. Clustering data of the cultured subset



**Figure 1.** RFLP analysis of HaeIII digest of the PCR product containing V1-V9 hypervariable region from metagenomic isolates (a) and cultured plate wash isolates (b) (post 3 months) separated on 3% agarose gel. (a) (b) Lane 1: Molecular weight marker (100 bp), Lanes 2 - 6: Healthy Non-smokers, Lanes 7 - 11: Smokers.



**Figure 2.** Dice-UPGMA Cluster Analysis of composite dataset for metagenomes and cultured bacterial ribotype using HaeIII. (a) Initial sample analysis: HH-Metagenomic isolates from healthy non-smokers, HS-Metagenomic isolates from smokers, HPH-Cultured plate wash isolates from healthy subset, HPS-Cultured plate wash isolates from cultured smoker subset; (b) Post 3 months sample analysis: #HH-Metagenomic isolates from healthy non-smokers, #HS-Metagenomic isolates from smokers, #HPH-Cultured plate wash isolates from healthy subset, #HPS-Cultured plate wash isolates from cultured smoker subset. On the scale  $r$  values are expressed as percentage. Clustering of Healthy #3 with smoker #5 ( $r = 60\%$ ).

showed that the smokers were clustered together but the healthy groups were scattered with Healthy #1, #2 and #3 falling out of the cluster. In the metagenomic subset the healthy samples showed similarities with the smokers (Healthy #1 with Smoker #2, Healthy #4 with Smoker #1 and Healthy #3 with Smoker #5) (**Figure 2(b)**).

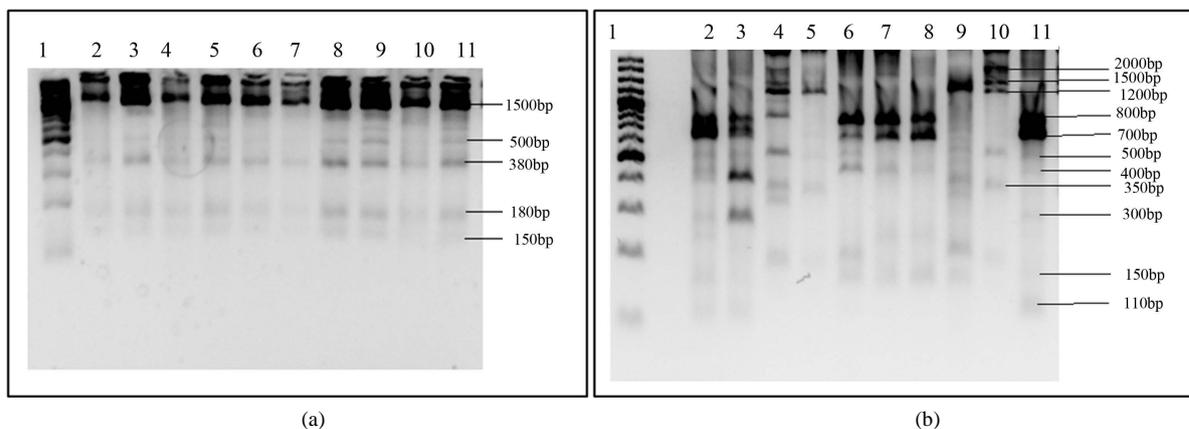
*Sau* 3AI yielded a more complex fingerprint. *Sau*3AI restriction produced 4 well-defined stable fragments (1000 bp, 380 bp, 180 bp and 150 bp) in both the subsets (**Figure 3(a)** & **Figure 3(b)**). The fingerprint of the cultured subtype showed 2 fragmented bands (800 bp and 700 bp) which were variable and becomes less intense/disappears in smokers (**Figure 3(b)**). This suggested the alteration of well-established core microbiome with changing oral health. Hence, a shift in oral microbial community was captured. These bands are critical and could serve as potential signatures of compromised oral health. Clustering pattern of the initial metagenomic isolates showed a tight clustering of smokers (**Figure 4(a)**). The healthy samples were widely distributed some showing similarity with smokers. Mention worthy were samples Healthy #1, #3 and #4 in both cultured and metagenomic subset (**Figure 4(a)**). There was no noticeable change in the ribopatterns of samples collected after 3 months (**Figure 4(b)**). Clustering of the fingerprints exhibited the similar relatedness of Healthy #1, #3 and #4 with smokers among the metagenomic subgroup ( $r > 0.60$ ). Both groups demonstrated noticeable tight clustering unlike initial pre 3 months analysis.

Alu I produced 5 fragments (1200 bp, 900 bp, 580 bp, 300 bp, 260 bp) present in both cultured plate wash and metagenomic extracts (**Figure 5(a)** & **Figure 5(b)**). A fragment of 450 bp length was distinct in smokers in the cultured plate wash category (**Figure 5(b)**). The cluster analysis based on densitometry curve grouped healthy non-smokers and smokers into closely clustered groups within the culture subset of the initial sample. The metagenome isolates however showed similar alignment of the Healthy #3 and #4 with the smokers (**Figure 6(a)**). There were no distinguishable differences in the data analysis post three months (**Figure 6(b)**).

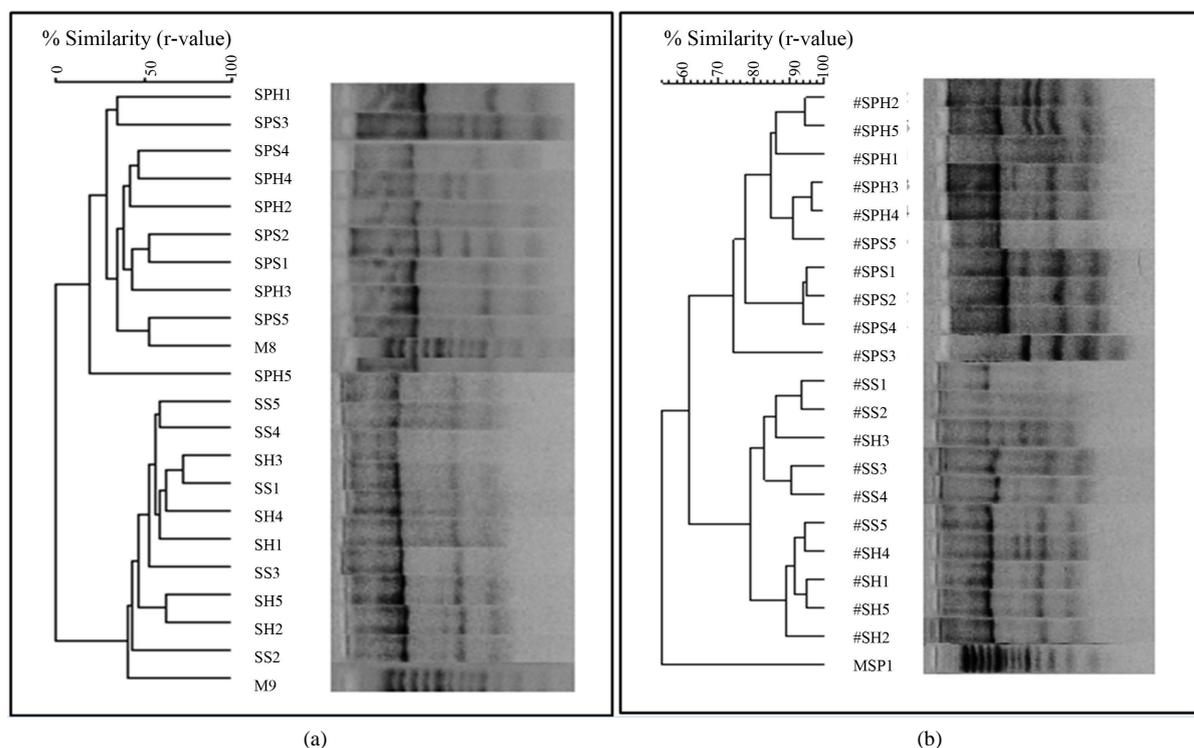
*Hae*III ribopattern for the initial sampling showed that both healthy and smokers were tightly clustered. Noteworthy, initial cluster analysis of the fingerprints produced by *Sau*3AI captured the shift in the health status of Healthy #1, #3 and #4 at an early stage. This shift was later captured by *Hae*III ribotyping in the post 3 month's analysis as mentioned earlier. This observation prompted a careful analysis of the questionnaire. It showed that Healthy #1 was a smoker for 15 years. It was evident that even though the subject quit there was a signature that persists and thereby aligning him with the smokers. It was also found that Healthy #4 was exposed to passive cigarette smoke and hence harbours a signature tax on that led to the clustering with smokers. It is noteworthy that the subjects self-identified their categories but the protocol developed in this study was able to determine the appropriate groups. This outcome correlates with the finding that every individual has a variable set of microbiome which is affected by the host's environment and lifestyle. It was however uncertain why Healthy #3 showed a similarity with smokers.

### 3.2. 16SrDNA Sequence Analysis

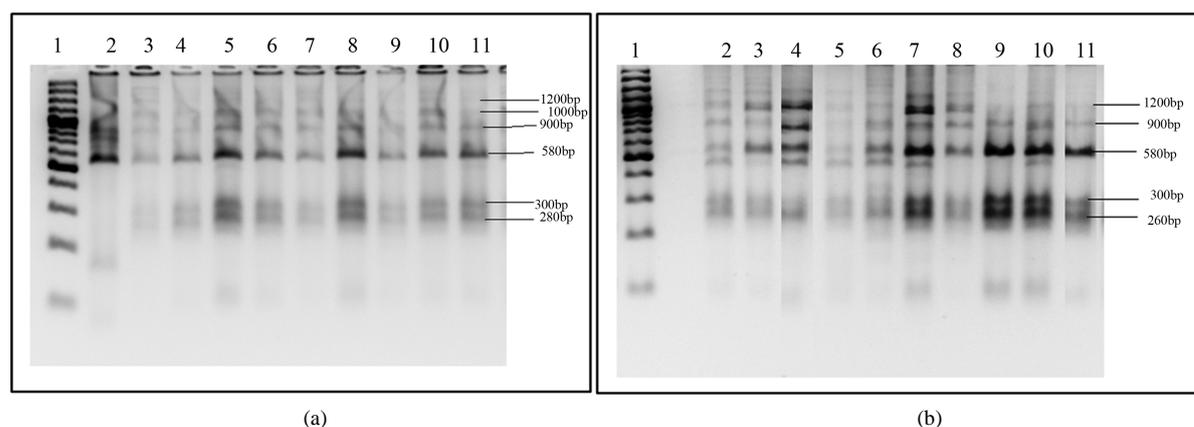
When subjected to PCR with primer pair F27/R1492 both the metagenomic and cultured plate wash isolates



**Figure 3.** RFLP analysis of *Sau* 3AI digest of the PCR product containing V1-V9 hypervariable region from metagenomic isolates (a) and cultured plate wash isolates (b) (post 3 months), separated on 3% agarose gel. (a) (b) Lane 1: Molecular weight marker (100 bp), Lanes 2 - 6: Healthy Non-smokers, Lanes 7 - 11: Smokers.



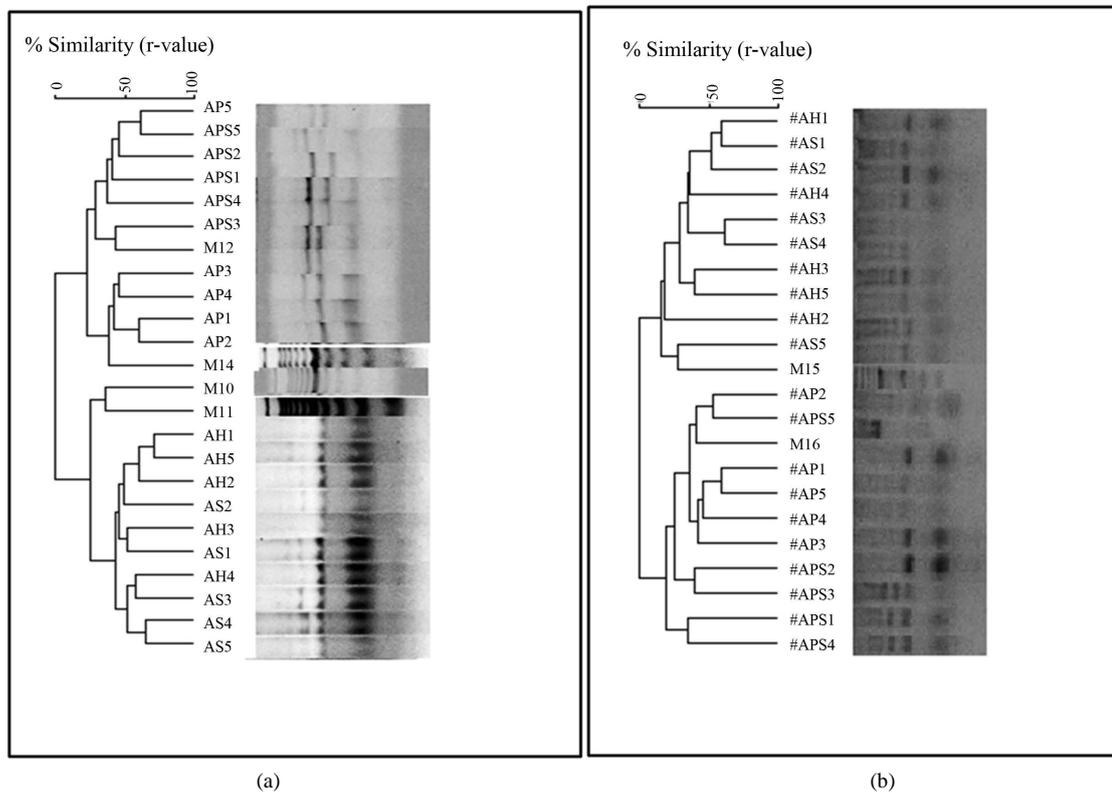
**Figure 4.** Dice-UPGMA Cluster Analysis of composite dataset for metagenomes and cultured bacterial ribotype using Sau3A1. (a) Initial sample analysis: SH-Metagenomic isolates from healthy non-smokers, SS-Metagenomic isolates from smokers, SPH-Cultured plate wash isolates from healthy subset, SPS-Cultured plate wash isolates from cultured smoker subset; (b) Post 3 months sample analysis: #SH-Metagenomic isolates from healthy non-smokers, #SS-Metagenomic isolates from smokers, #SPH-Cultured plate wash isolates from healthy subset, #SPS-Cultured plate wash isolates from cultured smoker subset. On the scale r values are expressed as percentage. Clustering of Healthy #1, #3 and #4 with smokers ( $r > 60\%$ ).



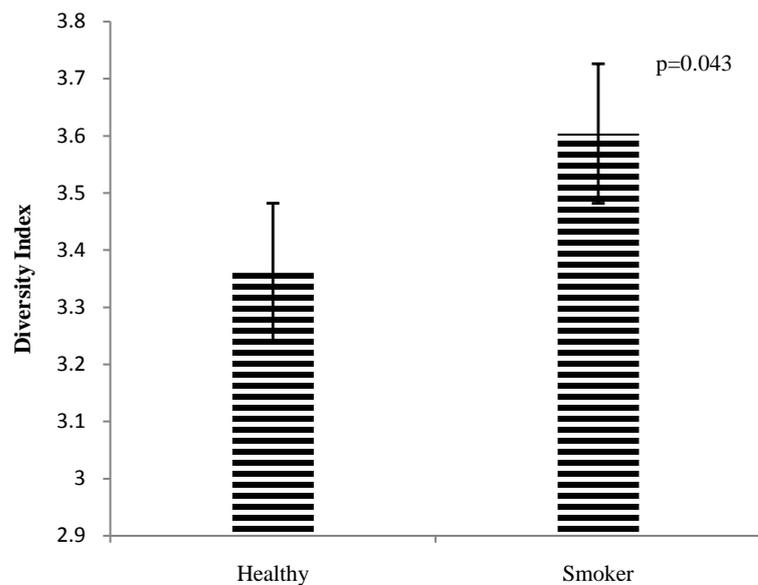
**Figure 5.** RFLP analysis of Alu I digest of the PCR product containing V1-V9 hypervariable region from metagenomic isolates (a) and cultured plate wash isolates (b) (post 3 months) separated on 3% agarose gel. (a) (b) Lane 1: Molecular weight marker (100 bp), Lanes 2 - 6: Healthy Non-smokers, Lanes 7 - 11: Smokers.

yielded a 1300 bp product. Sequencing data for the amplified PCR in Healthy non-smokers and smoker showed that the microbial diversity was significantly high in smokers than in non-smokers ( $p = 0.04$ ) (Figure 7).

Comparing healthy with smokers we observed a shift in the composition of oral microbiota composing a well-defined transition from gram-positive to gram-negative dominated community in smokers. Staphylococcus and gram-negative genera like *Neisseria*, *Thiomargarita*, *Granulicatella*, and *Synechococcus* were more preva-



**Figure 6.** Dice-UPGMA Cluster Analysis of composite dataset for metagenomes and cultured bacterial ribotype using Alu I. (a) Initial sample analysis: AH-Metagenomic isolates from healthy non-smokers, AS-Metagenomic isolates from smokers, AP-Cultured plate wash isolates from healthy subset, APS-Cultured plate wash isolates from cultured smoker subset; (b) Post 3 months sample analysis: #AH-Metagenomic isolates from healthy non-smokers, #AS-Metagenomic isolates from smokers, #AP-Cultured plate wash isolates from healthy subset, #APS-Cultured plate wash isolates from cultured smoker subset. On the scale r values are expressed as percentage.

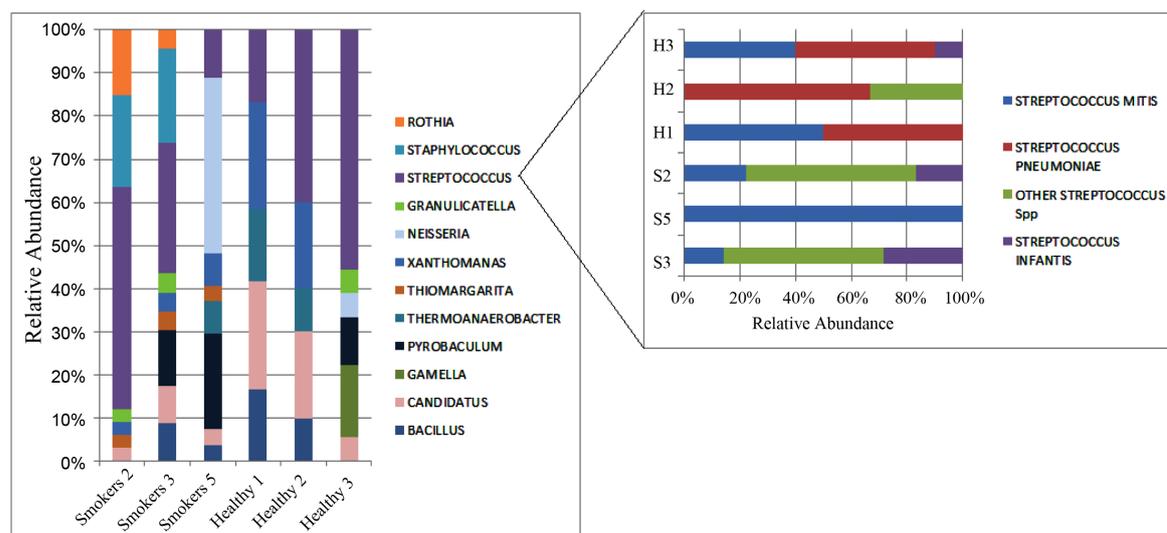


**Figure 7.** Diversity index plot of oral bacteria in healthy non-smokers and smokers. Smokers harbored a diverse array of oral microbiota (Shannon Weiner diversity index  $H' = 3.60$ ) including gram-negative genera *Neisseria* and *Thiomargarita* compared to healthy non-smokers ( $H' = 3.36$ ).

lent in smokers. Interestingly, Healthy #1 and Healthy #3 showed presence of the genera *Pyrobaculum* and *Neisseria* asserting our previous finding of a shift in the oral microbial community and a resemblance with smokers. These strains were not seen among other healthy samples. It is noteworthy that *Streptococcus mitis* which is being associated with various pancreatic diseases, including pancreatic cancer, are less abundant in healthy subjects and predominant in smokers. *Xanthomonas*, *Streptococcus* and phylum Candidatus, were also well distributed in both the groups and are probable members of the core microbiota (Figure 8). *Streptococcus sanguinis* were abundant in both groups. *Streptococcus infantis*, *Streptococcus oralis* and *Streptococcus perosis* were dominant in smokers. Interestingly, *Streptococcus pneumoniae* was abundant in healthy non-smokers however studies have shown increased pneumococcal adherence in smokers than in non-smokers. *Streptococcus oralis*, are recognized as important etiological agents of dental caries and sub-acute bacterial endocarditis and septicemia [34].

#### 4. Conclusion

The standard view of periodontitis, largely based on traditional microbiological approaches, associates the disease with the rise and damaging action of a small set of well-characterized pathogens. Identities of these pathogens are derived from the targeted 16SrRNA sequencing or from whole-metagenomic data. The strategy of combining PCR-RFLPs with sequencing of the 16SrDNA gene and computer assisted pattern analysis proved valuable in profiling the oral microbiome and detecting potential biomarkers of health and transitioning health (in smokers). The technique was effective in capturing specific polymorphisms unique to each group suggestive of a shift in bacterial community and presence of signature bands. The changing oral health of healthy subjects was captured successfully. As mentioned earlier these subjects self-identified their categories and had no such oral health concerns. However, the fingerprints grouped some of the individuals with smokers. This outcome suggests that these healthy individuals harbored signatures of periodontal bacteria similar to smokers who were successfully captured by this technique. Our study supports recent evidences which suggest that cigarette smoke changes the microbial community and causes an imbalance between the commensal group and pathogens with concomitant increase in periodontal pathogens. Enrichment of the pathogenic bacteria may be due to cellular transformation which affects colonization of the commensal group and alters the microbial signatures of these communities. This change can be instrumental in developing an early diagnostic tool if the fluxes develop into a stable reproducible signature. Our findings further open the way for the development of high throughput diagnostic



**Figure 8.** Relative Abundance of genera in Smokers and healthy non-smokers. The abundance of the genera *Candidatus*, *Xanthomonas* and *Streptococcus* among both groups are displayed in the plot. Increased abundance of *S. mitis* in smokers and Healthy #1 and #3 is noticeable. Healthy 1 and 3 shares unique restriction patterns that cluster them with smokers. The sequence data are in full agreement showing similar richness as smokers of the genera *Neisseria*, *Granulicatella* and *Pyrobaculum* in these individuals indicating deteriorating oral health.

tools employing genetic markers of oral health or of infectious disease and support the expansion of probiotic therapies and intervention.

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