



Metagenomic Analysis to Uncover the Subgingival and Atherosclerotic Plaque Microbiota in Patients with Coronary Artery Disease

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Abstract The role of periodontal pathogens in the initiation and progression of atherosclerosis has been extensively researched, yet a precise causal mechanism has not been established. The subgingival microbiota may be a source of dissemination and may contribute to the development of atherosclerosis; hence this study attempted to characterize and compare the subgingival and atherosclerotic plaques. Plaque samples were subjected to 16S rRNA-based metagenomics to study microbiota associated with subgingival and atherosclerotic plaques collected from patients with coronary artery disease. The PCoA analysis showed that the microbiomes of subgingival plaques were highly scattered and showed a diverse microbial composition, unlike the atherosclerotic plaques that did not show evident variability in the microbial composition and formed a close distinct group. The abundance of various genera in the subgingival plaques revealed *Fusobacterium* (11%), *Acinetobacter*

(13%), *Veillonella* (9%), and *Prevotella* (11%) among the top ten genera. The atherosclerotic plaques contained *Acinetobacter* (39%), *Chryseobacterium* (9%), *Rhizobium* (5%), and *Staphylococcus* (4%). All the patients examined in this study had either generalized or localized periodontitis with varying degrees of severity. The community microbiota analysis revealed that 22 bacterial genera were shared between two different plaques, with *Acinetobacter* being dominant. Based on the Human Oral Microbiome Database, 55% of the shared microbiota in this study have been listed as periodontal microbiota, with some of them found in increased proportions in patients with periodontitis suggesting the translocation of bacteria from the periodontal pockets into the circulation. This study provides valuable insights into the possible relationship between periodontal pathogens and atherosclerotic cardiovascular disease.

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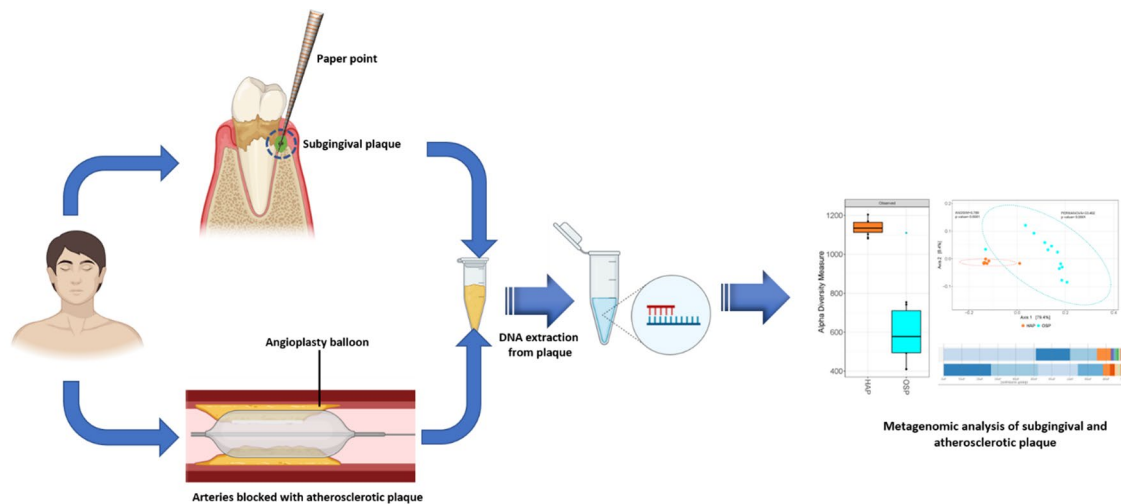
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Graphical Abstract



Keywords Coronary artery disease · Atherosclerosis · Periodontitis · 16S rRNA metagenomics

Introduction

The importance of oral biofilms as multispecies communities is highlighted by current knowledge of periodontal disease. The focus has shifted away from single pathogens to the complexities and interactions of bacterial communities that cause and maintain chronic infection within the biofilms. In the healthy oral cavity, there is a distinct bacterial flora that differs from that of oral disease [1]. This difference is due to the changes observed in the abundance of individual or overall bacterial communities present in the subgingival plaque [2, 3]. The composition of subgingival microbial communities varies among individuals, which is influenced by demography, environmental exposure, lifestyle, and immune response [2, 4, 5].

The development of periodontitis is accompanied by increased microbial diversity which is different from conditions like dental caries and gastrointestinal inflammatory conditions such as inflammatory bowel disease where decreased microbial diversity is observed [6, 7]. Researchers over the last few decades have hypothesized and supported the role of periodontal pathogens present in the subgingival plaque as an independent risk factor for atherosclerotic cardiovascular disease however, evidence is still lacking [8, 9].

The anatomical proximity of the oral biofilm to the periodontal vasculature facilitates the systemic spread of oral bacteria [10]. In conditions like gingivitis and periodontitis, the periodontal vasculature dilates, increasing in surface area, further facilitating bacteremia [11]. Periodontal pathogens can be carried from the subgingival area through circulation or circulating cells like monocytes to distant sites which can then initiate pathogenic processes. *In-vitro* and *in-vivo* studies

have shown that keystone periodontal pathogens like *Porphyromonas gingivalis* (*P. gingivalis*) have the ability to attach and invade human vascular cells [12–14]. *P. gingivalis* infected human endothelial cells have shown decreased cell surface expression of platelet endothelial cell adhesion molecule and vascular endothelial cadherin with disruption of cellular junctions resulting in endothelial cell permeability [14]. The invasion of periodontal bacteria through compromised endothelial integrity causes mononuclear cell adhesion, increased expression of adhesion molecules, increased procoagulant properties, and the release of proinflammatory cytokines (such as IL-6, IL-8, and MCP-1) leading to atherosclerosis [15, 16]. Hence, inflammatory mechanisms triggered locally or systemically by bacteria associated with periodontitis may have an impact on the initiation or propagation of atherosclerotic lesions [17].

The subgingival plaque microbiota has been stratified into groups or complexes that appear to occur together on the basis of analysis of the microbial profiles of plaque samples. The red complex, which appears later in biofilm development, comprises of species *P. gingivalis*, *Treponema denticola*, and *Tannerella forsythia* and these periodontal pathogens have been found to be associated with advanced and active periodontitis [18]. In our preliminary investigation, periodontal pathogens belonging to the red complex were found in the atherosclerotic plaques of patients with coronary artery disease (CAD) [19]. While some studies have focused on the role of few periodontal pathogens in the events leading to atherosclerosis; however, the role of numerous other non-culturable and unidentified bacteria cannot be undermined. This study thus attempts to fill the lacunae by characterizing the microbiota obtained from subgingival and atheromatous plaques of patients with CAD disease by 16S rRNA-based

metagenomics. This has a major advantage by providing actual representation of the bacterial pool as 16S rDNA sequence is specific for each bacterial genus and the process of sequencing after DNA extraction is similar irrespective of the nature of the pool. We hypothesize that the microbiota of the inflamed subgingival area may have a role to play in the sequence of events leading to atherosclerosis.

Materials and Methods

Experimental Design and Recruitment of Subjects

Twelve patients who reported to K.S. Hegde Charitable Hospital, Mangalore, diagnosed with CAD based on the classic symptoms at presentation and confirmed by electrocardiogram, echocardiography, cardiac biomarkers, and coronary angiograms were selected. Patients who were scheduled for either angioplasty or coronary artery bypass graft (CABG) and had more than 12 teeth were included. Patients who had received periodontal treatment in the previous six months and those who were terminally ill were excluded from the study. The methods and experimental protocols were reviewed and approved by the University's Central Ethics Committee (NU/CEC/2017-2018/0121, NU/CEC/2017-2019/0213). The purpose of the study and the procedure involved was explained to the patients before recruitment, and only those consenting to participate were enrolled.

General biological variables, including gender, age, and biochemical phenotypes relevant to atherosclerosis, were collected from the study participants. The complexity of coronary artery lesions was graded with the Syntax score [20]. The patients with syntax score less than 16 were graded as low, between 16–22 as intermediate and scores above 22 graded as severe condition.

Examination of the Oral Cavity

Subgingival and atherosclerotic plaques were collected from patients diagnosed with CAD and scheduled for angioplasty or CABG. The comprehensive examination of the oral cavity was done where probing depth (PD) and clinical attachment loss (CAL) were recorded by a periodontist using a mouth mirror and graduated periodontal probe. Gingival index (Loe and Sillness) was recorded to evaluate the degree of gingival inflammation. Chronic periodontitis (CP) was further classified into localized or generalized. Based on the definition of Page and Eke (2007), the periodontal condition (PD and/or CAL) was classified as mild, moderate, and severe [21]. The patient's smoking status was also recorded, and they were categorized as smokers (current/ former) and non-smokers. One of the authors, a cardiologist, performed the Syntax scoring to grade the cardiac severity.

Plaque Sample Collection

Subgingival plaque samples were collected using sterile paper points inserted into the gingival sulcus and maintained in situ for 10 s from the site with the deepest PD in each quadrant. Subsequently, the paper points were placed in individual sterile microcentrifuge tubes with 1 X Tris–EDTA buffer and stored at -20°C for downstream analysis.

In patients undergoing angioplasty, plaque samples were retrieved from the distal part of the balloon catheters. Atherosclerotic plaques were also collected from patients undergoing CABG procedure from the coronary arteries and transferred in sterile micro-centrifuge tubes containing 1 X Tris–EDTA buffer and stored at -20°C for subsequent analysis.

Genomic DNA Extraction and 16S rRNA Amplicon Sequencing

Samples from CABG procedure were homogenized prior to genomic DNA extraction. The QIAamp DNA Mini Kit (Qiagen, Germany) was used to extract DNA from plaque samples. The quantitative and qualitative estimation of the extracted DNA was performed using a spectrophotometer (Eppendorf, Germany) followed by an agarose gel electrophoresis. PCR was carried out using 40 ng of the purified DNA targeting the V3 and V4 regions of the 16S rDNA gene. Ampure beads were used to remove unused primers from the amplicon of each sample. To prepare the sequencing libraries, an additional eight cycles of PCR were performed using Illumina barcoded adapters. Ampure beads were used to purify libraries, and Illumina MiSeq with the 2X300PE v3 sequencing kit (Biokart India Pvt Ltd, Bangalore, India) was used for sequencing.

Taxonomic Assignment and Metagenome Prediction

Data analysis was performed as described in Lokesh et al. [22]. The FastQ files were used as input to the UPARSE pipeline (usearch version 11.0.667) [23]. First, the forward and reverse reads were merged with a maximum mismatch of 10 and a minimum id fraction of 80%. Primer binding sites were removed from the merged reads and reads were truncated at 400 bp. A quality filter was then applied to the resulting sequences, with the maximum expected error rate set to 1. Dereplication was then performed and reads containing < 10 sequences were discarded. OTUs (operational taxonomic units) were clustered at a threshold of 97% similarity and chimeric sequences were discarded. After clustering, the raw reads were assigned to OTUs by searching the reads as queries against the representative OTU sequences. Taxonomic ranks were assigned to OTUs with the SINTAX algorithm using the Ribosomal Database



Fig. 1 Number of reads obtained for the top taxa identified in subgingival and atherosclerotic plaques at the phylum and genus level. The taxa present in lower abundance (decimals) were rounded off.

Project database (rdp_16s sequences, version v18). Taxonomic assignments with a score of <0.5 were not considered in the downstream analyses. A total of 1,986,897 bacterial sequences with an average 82,787 reads per sample without chimeras were generated from twelve samples. This allowed for rarefaction and uniform sampling of 6000 sequences per sample. Alpha and beta diversity indices were computed and plotted using the R package phyloseq [24]. One-way anova was used to compute the statistical difference in the alpha diversity measures between the two groups. Beta diversity was computed using the Bray–Curtis dissimilarity index and compared using PERMANOVA. Shannon, and Simpson indices were calculated based on the OTU and abundance. The homogeneity of dispersion was computed using PERMDISP as implemented in the R package vegan.

Results and Discussion

This research was carried out to explore, characterize, and compare the bacterial community of subgingival and atherosclerotic plaques of patients from coastal Karnataka with CAD, through metagenomic analysis. We employed Illumina

OSP subgingival plaque samples; HAP atherosclerotic plaque samples; GP Generalised Periodontitis; LP Localized Periodontitis

MiSeq with a 2×300 PE sequencing chemistry to sequence the variable region of the 16S rRNA gene to study microbiota associated with subgingival and atherosclerotic plaques collected from the twelve patients (eight males and four females) with a mean age of 62.7 years (range 40–77 years). Based on the Gingival Index scoring, ten patients exhibited a moderate form, and two had a mild form of gingivitis. On periodontal examination, seven patients had Generalized, and five had Localized CP with varying degrees of severity. Further, the periodontal status revealed that eight patients had moderate, three mild, and one showed severe periodontitis. Based on the syntax scores, seven patients had low, three intermediate, and two patients had high cardiac severity (values greater than 22). It was interesting to note that one patient (P-15) with severe periodontitis also had the highest syntax score (Fig. 1).

Species Richness and Diversity of the Subgingival and Atherosclerotic Plaque Associated Microbiota

A total of 1,986,897 reads with an average 82,787 reads per sample without chimeras were generated from twelve samples and were assigned to 1854 OTUs (1424 OTUs in atherosclerotic plaque and 910 OTUs in subgingival plaques) belonging to different phyla. The rarefaction curves

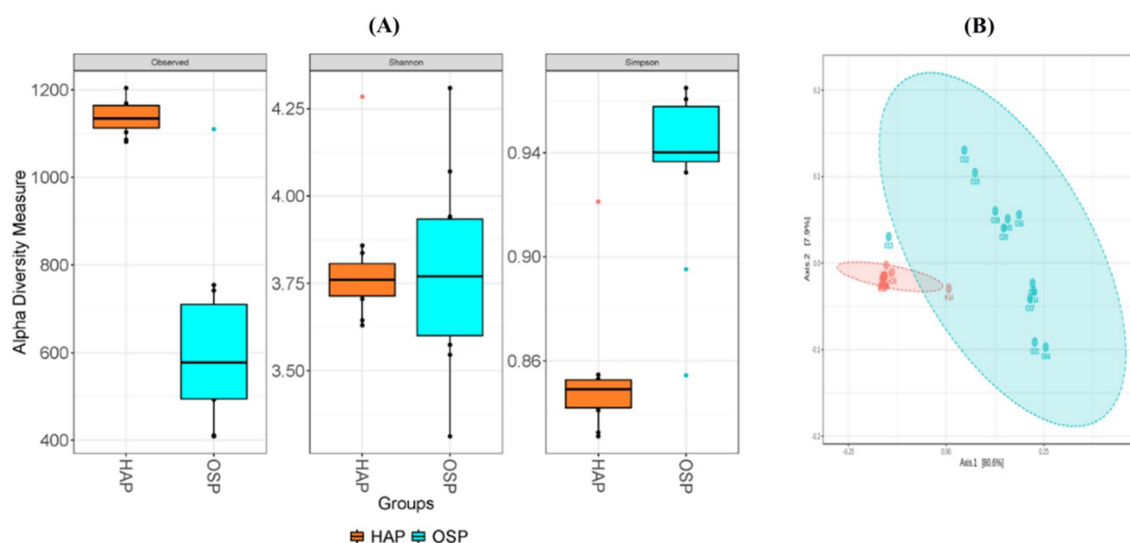


Fig. 2 **A** Alpha diversity indices boxplot of subgingival and atherosclerotic plaque samples; **B** PCoA plots of the bacterial communities' distribution in subgingival (OSP) and atherosclerotic plaque samples (HAP)

showed that an average of 20,000 sequences per sample was adequate to describe the communities' alpha diversity (Fig. 2). Alpha diversity was estimated to understand the species diversity and richness in the microbial communities of subgingival plaque and atherosclerotic plaque. Shannon and Simpson indices capture community diversity, such as species richness and evenness. Greater species diversity in the sample is indicated by a higher Shannon index and smaller Simpson index. The present study revealed a lower Simpson index for the atherosclerotic plaques compared to subgingival plaques. The majority of the arteriosclerotic plaques showed higher species richness than subgingival plaques, whereas subgingival plaques exhibited high sample variation exhibiting a range of species. Though

atherosclerotic plaques recorded the highest number of species, they were not equally abundant as seen by the smaller Simpson index ($p=3.3731e-07$) and a much closer Shannon index ($p=0.049034$) compared to subgingival plaque (Fig. 2A).

Beta Diversity of the Subgingival and Atherosclerotic Plaques

Beta diversity is a measure for comparing microbial composition between different samples, habitats, or ecosystems (between-sample diversity). An obvious separation was observed between the two groups viz atherosclerotic and subgingival plaques. Microbiomes of subgingival plaques

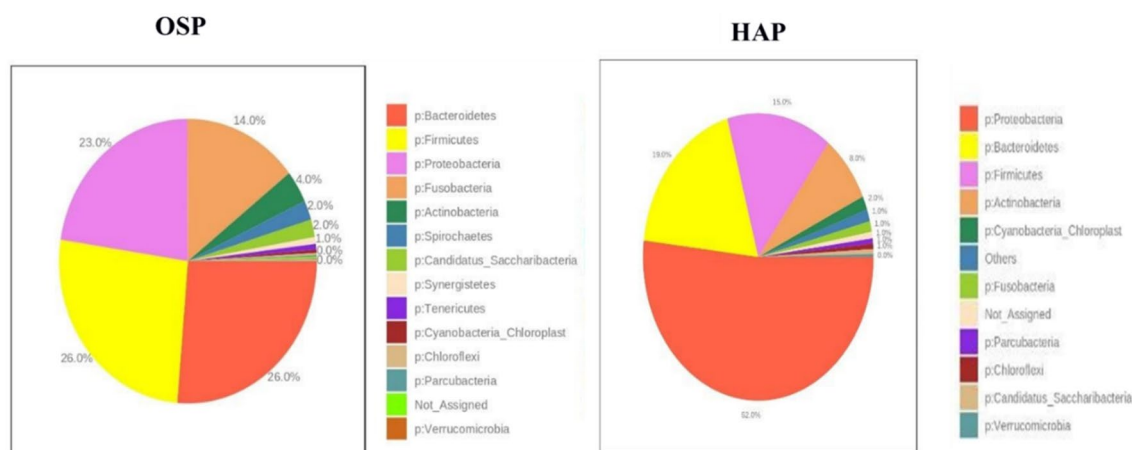


Fig. 3 Relative abundance of predominant bacterial phyla in subgingival plaque (OSP) and atherosclerotic plaques (HAP). Comparison between groups was calculated using parametric *t* test for paired samples

were highly scattered and showed a diverse microbial composition, unlike the atherosclerotic plaques. Atherosclerotic plaques did not show evident variability in the microbial composition and formed a close distinct group. Based on the membership, bacterial communities of the atherosclerotic plaques clustered together and separated from those of subgingival plaques, which explains the variation (80.6%), seen in PCoA axis 1 (Fig. 2B). The diverse communities present in subgingival areas have an abundance of microflora due to increased nutritional resources available as a result of periodontal inflammation [2]. The differences in bacterial community composition were assessed using the nonparametric test, analysis of similarity (ANOSIM), which demonstrated the remarkable differences in the bacterial communities of subgingival and atherosclerotic plaques (ANOSIM = 0.815; p -value < 0.001). Likewise, the permutational multivariate analysis of variance (PERMANOVA) between the two different groups further revealed differences in the bacterial community between subgingival and atherosclerotic plaques (PERMANOVA = 41.153; p -value < 0.001). Interestingly, the subgingival plaque of the patient (P-15) with a clinical

finding of severe periodontitis showed deviation from the rest and clustered with the atherosclerotic plaques.

Relative Abundance of the Prominent Taxa in the Subgingival and Atherosclerotic Plaques

The microbial taxonomic composition of subgingival and atherosclerotic plaques differed according to the relative abundance of significant taxa (Fig. 3). Bacteroidetes (26%), Firmicutes (26%), Proteobacteria (23%), Fusobacteria (14%) and Actinobacteria (4%) are the top five taxa of the subgingival plaques whereas in atherosclerotic plaques, Proteobacteria (52%), Bacteroidetes (19%), Firmicutes (15%), Actinobacteria (8%) and Cyanobacteria (2%) constituted the top microbiota. From the analysis, it is evident that subgingival plaques of CAD patients possessed evenly distributed bacterial communities belonging to different phyla. On the other hand, it was interesting to observe that atherosclerotic plaque samples were dominated by members of Proteobacteria representing skewed bacterial communities which was also indicated by a lower Simpson index. Similar findings

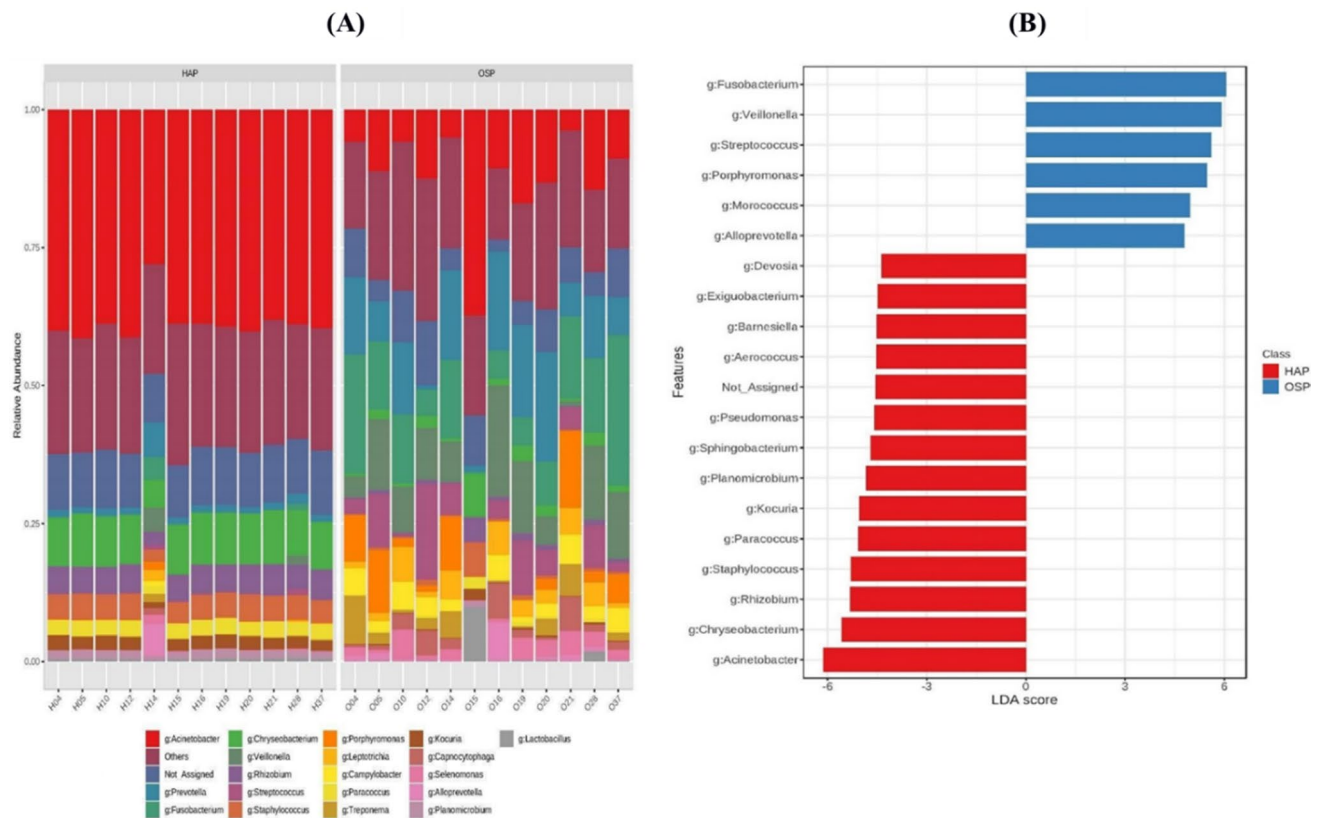


Fig. 4 **A** Characterization of the microbiome communities (genus level showing top 20 taxa, the group “Others” is the sum of taxa which were present in low abundance) of the atherosclerotic plaque and subgingival plaque samples collected from 12 patients scheduled for angioplasty or coronary artery bypass graft. Comparison between

groups was calculated using parametric t test for paired samples. **B** Plot from LEfSe analysis (alpha value ≥ 0.05 , logarithmic LDA score threshold ≥ 3.5) indicating enriched bacterial genera associated with subgingival and atherosclerotic plaque samples

have been reported in other studies where significantly higher levels of Proteobacteria were observed in the atherosclerotic plaques [25–27].

The onset of periodontal condition is accompanied by a shift in the composition of subgingival microbial communities, dominated by members of phylum Firmicutes and Bacteroidetes, which outgrow health-associated taxa [28]. Abundance of Spirochaetes, Fusobacteria, and Bacteroidetes have been reported in patients with periodontitis in few studies while Actinobacteria have been observed with increased abundance in microbial communities of the healthy periodontium [3, 29]. In this study, Firmicutes and Bacteroidetes were the dominant phyla in the subgingival plaques while the health associated Actinobacteria was at just 4%. Sample-wise comparison at the phylum level in this study consistently revealed higher abundance of Fusobacteria in the subgingival plaques as compared to atherosclerotic plaques which had relatively lower abundance (Fig. 1). In comparison to the other subgingival plaques, one of the samples (O-15) that clustered with the atherosclerotic plaques had Proteobacteria in higher abundance and Fusobacteria in lower abundance. Also, the microflora of this patient remained distinct from other subgingival plaque samples of patients who had mild or moderate periodontitis.

Abundance of Various Genera in Subgingival and Atherosclerotic Plaques of CVD Patients

A cursory glance of the top ten genera present in subgingival plaques of CAD patients showed *Acinetobacter* (13%) followed by *Fusobacterium* (11%), *Prevotella* (11%) and, *Veillonella* (9%), which were equally abundant as reflected by the higher Simpson index. On the other hand the atherosclerotic plaques had a skewed microbial community dominated by *Acinetobacter* (39%) followed by other genera such as *Chryseobacterium* (9%), *Rhizobium* (5%), *Staphylococcus* (4%). Though not recorded in the top ten genera, some of the known perio-pathogens were present in relatively lower abundance in atherosclerotic plaques, and their contribution to the onset of plaque cannot be undermined (Fig. 4A). The study also found inter-patient variation in the relative abundance of some of these genera in the subgingival plaque of CAD patients, which could be linked to lifestyle and physiological differences. Inter-subject variability in the composition and taxonomic differences in the specific bacterial taxa outnumbering in health and periodontitis has been reported [2]. The role of *Fusobacterium* as a core member in plaque biofilm has been reaffirmed in the present study as it was observed in all the subgingival plaque samples except one (O-15) [29]. A positive correlation between abundance of *Fusobacterium* in subgingival plaques with LDL and cholesterol levels in patients with atherosclerosis has been shown [25].

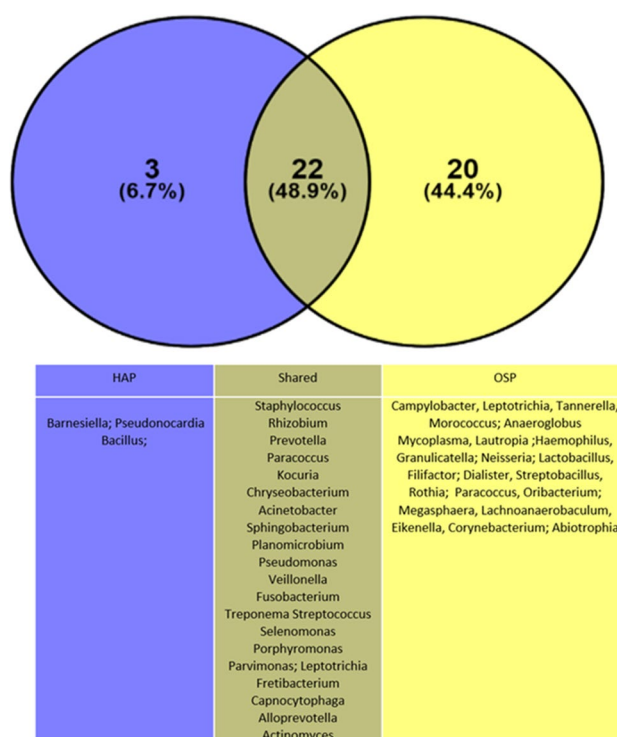


Fig. 5 Venn diagram representing the shared and unique taxa in oral subgingival plaques (OSP) and atherosclerotic plaque samples (HAP)

The lone (O-15) subgingival plaque sample was unique by the fact that it contained *Lactobacillus* (10%) and showed a striking resemblance with its corresponding atherosclerotic plaque in its microbial composition. It was interesting to note that the percentage of *Prevotella* and *Porphyromonas* detected in this sample was also low compared to the other subgingival samples which could be attributed to the presence of *Lactobacilli*. An *in-vitro* study has shown *Lactobacilli* to possess an antimicrobial resistance against periodontal pathogens like *P. gingivalis* and *Pr. intermedia* [30].

Atherosclerotic plaques contained a significantly higher proportion of *Proteobacteria*, with *Acinetobacter* being dominant. Similarly *Acinetobacter*, *Acidovorax*, and *N. polysaccharea* have also been detected in atherosclerotic samples taken from patients with symptomatic atherosclerotic disease [31].

To identify specific members of the core microbiome enriched in the plaque samples, linear discriminant analysis effect size (LEfSe) with LDA score cutoff of 3.5 (p 0.05) was performed. LEfSe revealed a distinct microbial distribution between subgingival and atherosclerotic plaques collected from CAD patients. 46 OTUs were significantly enriched between the two groups, with six abundant in subgingival plaques and 40 in atherosclerotic plaques. The significantly abundant taxa with their corresponding LDA scores is shown in (Fig. 4B).

Shared and Distinct Taxa in the Subgingival and Atherosclerotic Plaques of CVD Patients

Shared and distinct taxa between the plaques at the two sites were identified as an approach to explore the possible spread of bacteria from infected periodontal pockets to the coronary arteries. The shared and distinct microbiome was identified using QIIME, and its presence in at least 90% of samples at the relative abundance of 0.01% was chosen as a conservative representation of the core microbiome. The 45 bacterial genera constituted the shared and distinct microbiome of subgingival and atherosclerotic plaques collected from patients with CAD. The whole community of subgingival plaques, comprised of 42 bacterial genera, while the atherosclerotic plaques consisted of 25. 48.9% (22) comprised of the shared bacterial genera; subgingival plaques showed 44.4% (20) unique bacterial genera while 6.7% genera (3) (*Barnesiella*, *Pseudonocardia* Bacillus) were exclusively present in atherosclerotic plaques (Fig. 5). The shared genus included *Staphylococcus*, *Rhizobium*, *Prevotella*, *Paracoccus*, *Kocuria*, *Chryseobacterium*, *Acinetobacter*, *Sphingobacterium*, *Planomicrobium*; *Pseudomonas*, *Veillonella*, *Fusobacterium*, *Treponema*, *Streptococcus*, *Selenomonas*; *Porphyromonas*, *Parvimonas*, *Leptotrichia*, *Fretibacterium*, *Capnocytophaga*, *Alloprevotella*, *Actinomyces*. Based on the Human Oral Microbiome Database 55% of the shared microbiota in this study (*Actinomyces*, *Kocuria*, *Capnocytophaga*, *Porphyromonas*, *Prevotella*, *Selenomonas*, *Staphylococcus*, *Streptococcus*, *Veillonella*, *Fusobacterium* and *Treponema*) have been listed as periodontal microbiota with some of them found in increased proportions in patients with periodontitis [32, 33]. *Prevotella* was found across all the twelve atherosclerotic samples. The shared microbiota could suggest a bacterial translocation from the periodontal pockets to coronary arteries.

This study demonstrated the presence of pathogens belonging to the red complex group in the atherosclerotic plaques. Although, the percentage of *P. gingivalis* (bacteria belonging to the complex) was low, there is evidence that periodontal destruction can be caused by even low levels of colonization by *P. gingivalis* which tend to dysregulate innate immunity pathways, leading to an increase in the overall biomass causing an imbalance between periodontal microbiota and host inflammatory response [34, 35]. Pathogens belonging to the red complex have shown to increase the threat of cardiovascular disease either directly or indirectly [36, 37]. Invasive strains of *P. gingivalis* have been shown to induce their uptake by macrophages and enhance foam cell formation one of the earliest events in atherogenesis [38].

One interesting observation was the combined detection of *Acinetobacter*, *Chryseobacterium*, *Rhizobium*, *Veillonella*, and *Staphylococcus* in the atherosclerotic plaque samples

across patients. *Veillonella* and *Streptococcus* abundance in atherosclerotic plaques has been linked to their abundance in the oral cavity [25].

In this study, *Acinetobacter* was the dominant microflora detected in both subgingival and atherosclerotic plaques; however, subgingival plaques had a relatively lower number than atherosclerotic plaques. This organism has been isolated from oral biofilms and is known to be a frequent cause of nosocomial infections, especially pneumonia [39]. It has also been detected in significantly higher frequencies in subgingival samples of CP patients with bleeding on probing and periodontal destruction [40].

Periodontal diseases and CAD share several systemic inflammatory mechanisms. This study is an attempt to investigate the role of periodontal pathogens in atherosclerosis development by characterizing and comparing the subgingival and atherosclerotic plaques. Although the study demonstrated the presence of periodontal pathogens in the atherosclerotic plaques, many of the bacteria detected in the atherosclerotic plaques were not indigenous to the oral cavity; their source and role needs to be investigated. Further, the application of multi-omics studies could provide insights into the role of microbial communities and their complex interactions. This may help to understand the pathogenesis of CAD and its sequelae due to the interplay between the oralomes at the nexus of the oral cavity and heart.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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