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Upper Airway Microbiome in Adults with Subclinical Atherosclerosis

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Abstract

Cardiovascular disease (CVD), the leading cause of global mortality, is intrinsically linked to atherosclerosis. Recent research suggests that respiratory function may be associated with the development of atherosclerosis. Alterations in the composition of the respiratory microbiome can negatively affect pulmonary function, which in turn may impact cardiovascular health through mechanisms that remain incompletely understood. To analyze the upper airway microbiome abundance and diversity in adults with subclinical atherosclerosis. A case–control study was conducted for atherosclerosis. Oropharyngeal swab samples were collected from participants in the CaRes cohort, for whom carotid Doppler, spirometry, blood chemistry, and clinical history data were available. Sequencing of the 16S ribosomal RNA gene was performed, followed by comparative analyses between subjects with and without atherosclerosis. A total of 100 subjects were analyzed (50 cases and 50 controls). The phylum Bacteroidetes was the most prevalent in both groups, followed by Firmicutes and Proteobacteria. Family Enterobacteriaceae was more abundant among case group, which included species such as Serratia, Klebsiella spp., and Campylobacter. Evaluation of alpha diversity indices revealed lower levels for cases group, with a Shannon diversity index of 2.61 compared to 4.07 in controls ($p = 0.006$). Differences in microbiota composition were observed between cases and controls. Specifically, the family Enterobacteriaceae was associated with the presence of atherosclerosis, suggesting its potential involvement in the progression of cardiovascular disease.

Keywords: upper airway; microbiome; atherosclerosis

Introduction

Cardiovascular disease (CVD) is the leading cause of mortality worldwide [1]. According to the Global Burden of Disease (GBD) report in 2020, it accounted for more than 7.7 million deaths globally, representing 16.8% of all adult mortality [2]. This burden is reflected in the loss of disability-adjusted life years (DALYs) and premature deaths, which in that same year reached 40.8 million DALYs and 36.4 million years of life lost due to premature mortality [3].

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There is a substantial overlap of risk factors common to both CVD and chronic pulmonary disease, such as smoking, unbalanced diet, and others [4], which may partly explain the link between cardiovascular and respiratory disorders. According to recent findings, microbiome may also be one of these factors. It is well established that changes in the composition of the respiratory tract microbiome are associated with pronounced decline in pulmonary function [5,6], making it plausible that through this pathway, cardiovascular deterioration may also be promoted.

With respect to the association between the respiratory tract microbiome and atherosclerosis, few studies have been published to date. Most investigations have focused on the relationship between atherosclerosis and the gut or oral microbiota [7–9]. Some of the most notable analyses have suggested that microbiomes enriched in genera such as *Lachnoclostridium* and *Clostridium* [10], or species such as *Chlamydia pneumoniae* and *Mycoplasma pneumoniae*, are associated with higher prevalence and severity of atherosclerosis [11]. Other authors have found that patients with greater bacterial diversity in the oral cavity exhibited lower presence of carotid atherosclerotic plaques, suggesting that oral health may be linked to cardiovascular health [9].

In Colombia, DALYs attributed to CVD have been estimated in 2800 per 100,000 habitants, with an age-standardized incidence of 502.5 per 100,000 habitants [12]. Respiratory disease are also highly prevalent, accounting for 8.9% of adults with remarkable subnational variations [13–15]. Since cardiovascular and respiratory diseases are concomitant issues in this population, synergic interaction is a plausible scenario, as seen in other regions [16]. Considering that microbiota dysbiosis might be a linking factor between respiratory and cardiovascular health, we hypothesized that airway microbiome abundance and diversity index could be associated with atherosclerosis prevalence in a sample of Colombian adults. To evaluate this hypothesis, we analyzed a sample of adults enrolled in the Cartagena Cohort Study -CaReS-, conducting a nested case–control study for atherosclerosis. Hence, this analysis was aimed to analyze the upper airway microbiome abundance and diversity in adults with subclinical atherosclerosis.

Materials and Methods

Subjects were selected from the CaReS cohort, for whom carotid doppler, spirometry, blood chemistry, and clinical history data were available. Prior to sample collection, participants were instructed to perform dental brushing two hours before sampling, to refrain from food intake for at least three hours and from liquids for at least one hour before the examination, and to avoid exposure to antibiotics for at least one month prior to testing.

For microbiome sampling, a sterile Dacron or polypropylene swab was introduced through the palatal arches, avoiding contact with the uvula, cheeks, teeth, and tongue, until reaching the posterior wall of the oropharynx. The swab was rotated 4 to 6 times, then placed into a 1.5 mL microtube, immediately frozen after collection, and stored at −80 °C until DNA extraction. Informed consent was obtained from all participants, and the procedures described were approved by the Ethics Committee of the University of Cartagena.

DNA Extraction and Microbiome Sequencing

DNA was extracted from oropharyngeal samples using the Saliva DNA Isolation Kit (Norgen Biotek, Thorold, Ontario, Canada), following the manufacturer's instructions. The quality and quantity of the extracted DNA were assessed spectrophotometrically using a NanoDrop One instrument (Thermo Scientific). The obtained DNA was amplified by polymerase chain reaction (PCR) employing primers targeting the hypervariable V3–V4 regions of the 16S ribosomal RNA (rRNA) gene (Amplicon PCR Forward Primer 5′ TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG 3′; Amplicon PCR Reverse Primer 5′ GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC 3′), thereby enabling the discrimination of bacterial species present in the microbiome. Amplification of the 16S rRNA gene was performed using a StepOne thermocycler (Thermo Fisher Scientific).

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Libraries were prepared according to the 16S Metagenomic Sequencing Library Preparation protocol (Illumina, San Diego, CA, USA). Sequencing was conducted using the ISeq100 reagent kit (Illumina). Raw sequencing data underwent quality control to remove chimeric sequences, human genomic sequences, food-derived genomic sequences, and low-quality reads. Processed sequences were clustered into operational taxonomic units (OTUs) defined at a 97% similarity threshold. Representative sequences for each OTU were assigned by comparison against the integrated reference database. Taxonomic classification and relative abundance values at all taxonomic levels, as well as alpha and beta diversity indices and intergroup comparisons, were obtained using *EzBioCloud* software with default settings.

Statistical Analysis

Statistical analyses were performed using R software (version 4.3.1). Initially, the Shapiro–Wilk test was applied to assess the normality of the distribution of quantitative variables. Subsequently, the Mann–Whitney U test was used to compare medians between two independent groups. Categorical variables were analyzed using the chi-square test. This non-parametric test is particularly suitable for evaluating differences in microbiome sequence distributions across groups, providing a robust approach when data do not follow a normal distribution.

Results

A total of 100 subjects were included in the study. The sample size comprised 50 individuals in the control group and 50 individuals in the case group. The mean age was 59.7±13.4 years in the case group and 44.9 ± 16.2 years in the control group. The median income was USD $250 for cases and USD $325 for controls. Sociodemographic data is presented in Table 1.

Regarding serological variables, differences were noted in triglyceride and glucose levels, with subjects diagnosed with atherosclerosis exhibiting higher values, even exceeding clinical reference ranges, although these differences were not statistically significant. Cardiovascular variables also differed between groups, particularly in carotid intima-media thickness and cardiovascular risk. Additionally, differences were evident in pulmonary function parameters between cases and controls.

Table 1. Demographic, socioeconomic and clinical characteristics for cases and controls.

Variable	Controls (n=50)	Cases (n=50)
Age, y.o. mean ± SD	44.9 ± 16.2	59.7 ± 13.4
Sex, % (n)		
Women	34.2 (17)	46.4 (23)
Men	65.8 (33)	53.6 (27)
Economic activity, % (n)		
Housewives	20.8 (10)	31.0 (16)
Retired	10.8 (5)	8.3 (4)
Unemployed	13.1 (7)	11.9 (6)
Employee	51.9 (26)	47.0 (24)
Unspecified	3.4 (2)	1.8 (1)
Monthly income, USD median [IQR]	325 [150; 600]	250 [125; 381.3]
Smoking status, % (n)		
Never	81.2 (41)	71.4 (36)
Former	14.5 (7)	22.0 (11)
Current	4.3 (2)	6.5 (3)
Pack-years median [IQR]	4.35 [2.3; 10.6]	8.6 [2.7; 17.9]
Pulmonary function (Pre-bronchodilator), mean ± SD		
FVC (L)	3.22 ± 0.67	2.85 ± 0.65

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FEV_1 (L)	2.51 ± 0.54	2.05 ± 0.48
FEV_1/FVC	78.36 ± 6.65	72.7 ± 7.89
Pulmonary function (Post-bronchodilator), mean ± SD		
CVF (L)	3.18 ± 0.63	2.74 ± 0.58
VEF_1 (L)	2.59 ± 0.54	2.11 ± 0.49
FEV_1/FVC	81.82 ± 6.22	76.85 ± 6.20
Carotid Intima-Media Thickness, mean ± SD	0.52 ± 0.19	0.91 ± 0.33
Framingham 10-year risk, mean ± SD	18.78 ± 20.22	42.37 ± 22.05

Upper Airway Microbiome

From the sequences obtained for the control group, 94.2% of valid reads were retained. Among the discarded sequences, 7.81% corresponded to low-quality reads, 0.001% to non-bacterial sequences, and 1.03% to chimeric sequences. The mean read fragment length was 248 bp, with a species-level discrimination rate of 25.61%. For the case group, 94.2% of valid reads were retained. Among the discarded sequences, 4.62% corresponded to low-quality reads, 0.04% to non-bacterial sequences, and 0.36% to chimeric sequences. The mean read fragment length was 250 bp, with a species-level discrimination rate of 20.38%.

When comparing genus, in the case group, the predominant presence of Prevotella was observed, accounting for 36.23%, followed by Veillonella at 8.84%, Pasteurellaceae_uc at 7.17%, and Alloprevotella at 5.42%. Conversely, in the control group, Prevotella was also the predominant genus, representing 35.93%, followed by Fusobacterium at 7.28%. Additionally, Alloprevotella was present at 6.29%, and Haemophilus at 2.37%. Unclassified bacteria were detected in both groups, though at different proportions: 6.54% in cases and 0.19% in controls. The presence of Porphyromonas was notably higher in the control group (4.74%) compared to the case group (1.19%). The remaining microorganisms accounted for less than 2% in each study group (Figure 1).

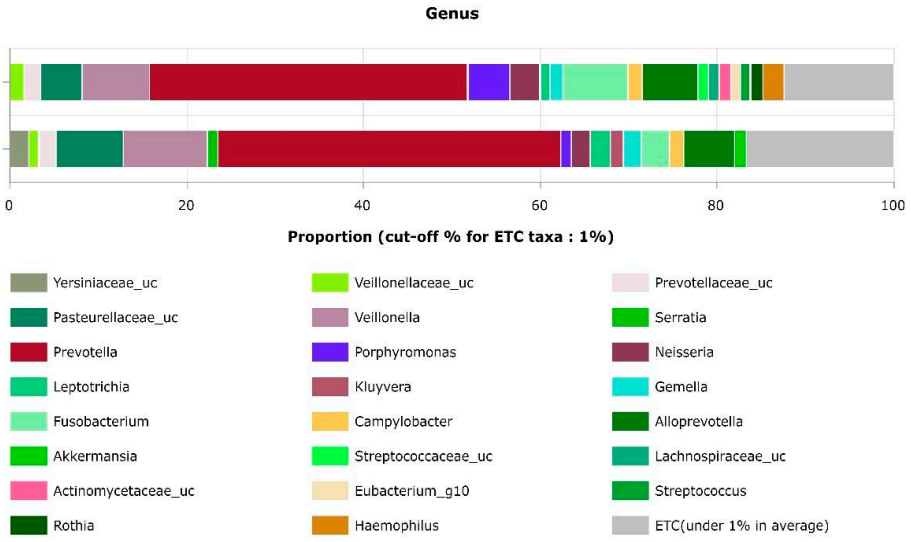

Figure 1. Relative abundance of the main bacterial taxa between cases and controls at the species level. The X-axis represents the case and control groups, while the Y-axis indicates the percentage distribution of the bars for each taxonomic composition. The color of each bar denotes the corresponding bacterial taxon, with grey bars representing taxa unclassified at this level. An abundance threshold of 1.0% was applied; therefore, only taxa representing more than 1% of the total bacterial community are shown. In the case group, Prevotella_uc was predominant, accounting for 28.17% of the total, followed by unclassified bacteria at 22.90%. A significant presence of Veillonella_uc (8.17%) and Alloprevotella_uc (5.10%) was also observed. Conversely, in the control group, Prevotella_uc remained prominent, representing 23.64%, while the proportion of unclassified bacteria was lower, at 14.48%.

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At the species level, in the case group, a dominant presence of Prevotella_uc was observed, representing 28.17%, followed by Veillonella_uc at 8.17% and Alloprevotella_uc at 5.10%. Unclassified bacteria accounted for 22.90% in this group. In the control group, Prevotella_uc remained prominent, representing 23.64%, followed by Fusobacterium_uc at 6.74% and Veillonella_uc at 6.81%. The proportion of unclassified bacteria was lower, at 14.48%. Notable differences in species distribution were observed between groups; for instance, Prevotella_uc was more abundant in the case group compared to controls. Moreover, unique species were identified in each group, such as Serratia_uc in cases and Porphyromonas endodontalis in controls. The presence of Neisseria_uc was higher in the control group (2.65%) compared to the case group (1.00%) (Figure 2).

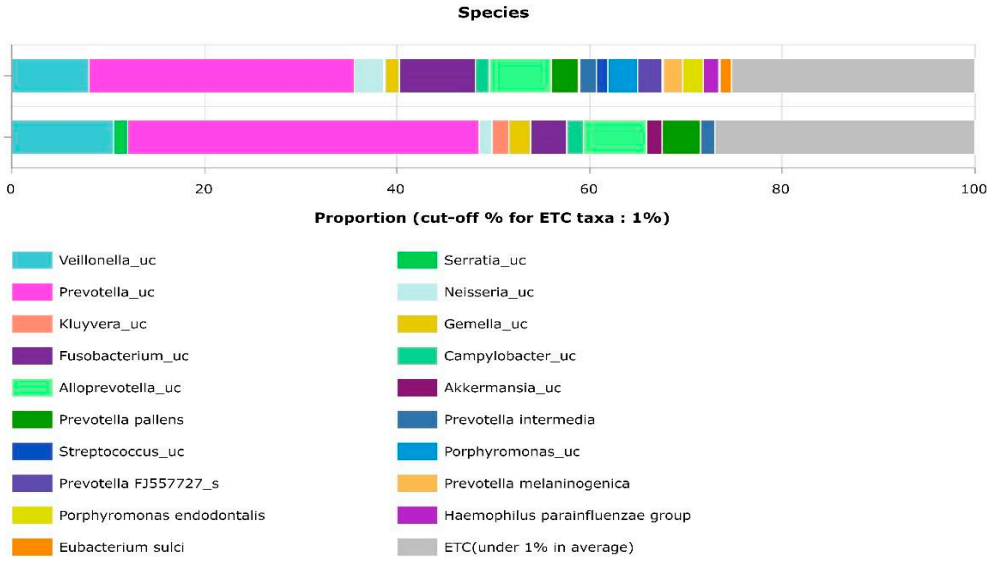

Figure 2. Relative abundance of the main bacterial taxa between cases and controls at the species level. The X-axis represents the case and control groups, while the Y-axis indicates the percentage distribution of the bars for each taxonomic composition. The color of each bar denotes the corresponding bacterial taxon, with grey bars representing taxa unclassified at this level. An abundance threshold of 1.0% was applied; therefore, only taxa representing more than 1% of the total bacterial community are shown. In the case group, Prevotella_uc was predominant, accounting for 28.17% of the total, followed by unclassified bacteria at 22.90%. A significant presence of Veillonella_uc (8.17%) and Alloprevotella_uc (5.10%) was also observed. Conversely, in the control group, Prevotella_uc remained prominent, representing 23.64%, while the proportion of unclassified bacteria was lower, at 14.48%.

The richness between groups, assessed using the abundance-based coverage estimator (ACE) method ($p = 0.631$), showed no statistically significant differences. However, when evaluating the alpha diversity index between groups, subjects with atherosclerosis exhibited lower diversity levels, with a Shannon index of 2.61 compared to control subjects, who presented a diversity index of 4.07 ($p = 0.006$) (Figure 3).

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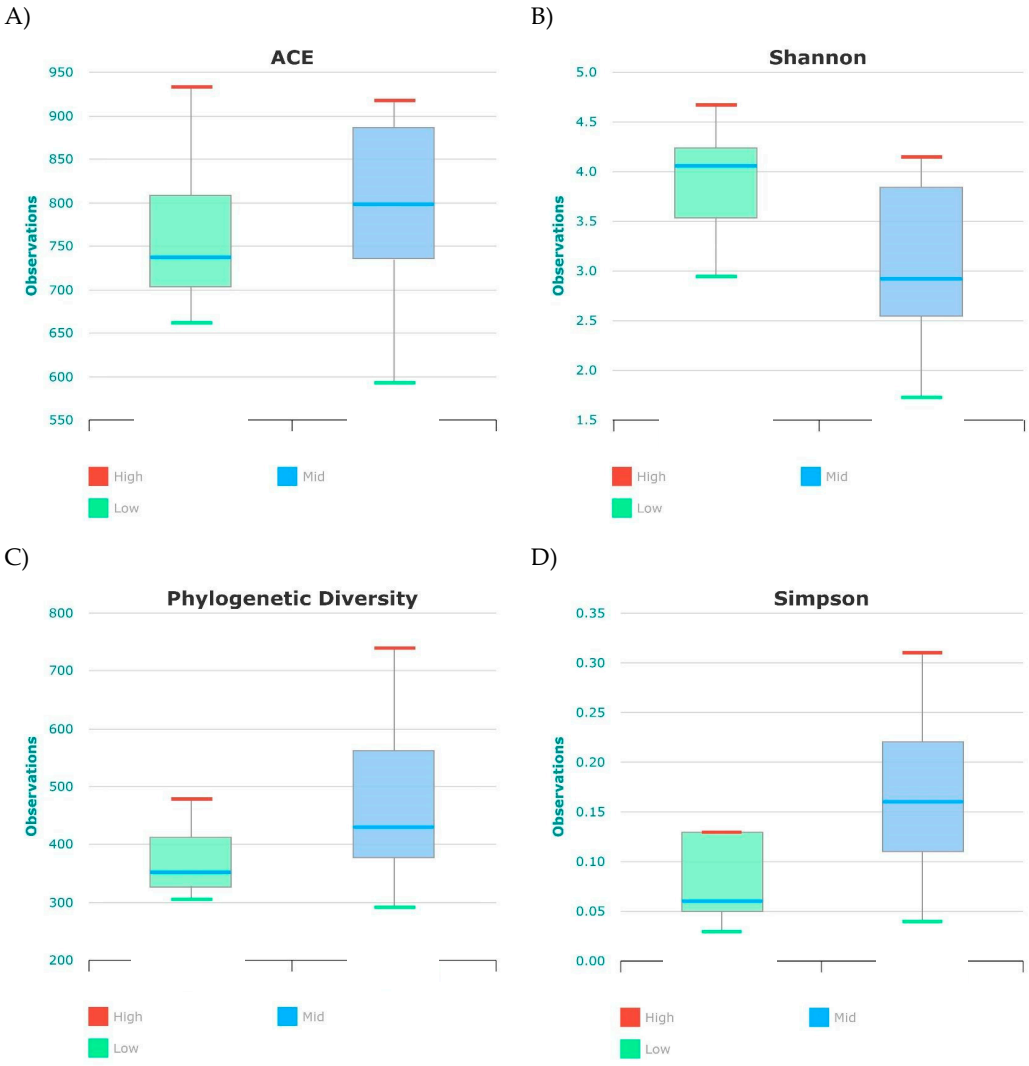

Figure 3. Comparison of species richness and diversity metrics between subjects with atherosclerosis and controls. (A) Species richness estimated by the abundance-based coverage estimator (ACE) (p = 0.335); (B) Shannon diversity index, showing significantly lower values in cases compared to controls (p = 0.006*); (C) Phylogenetic diversity, also reduced in cases (p = 0.029)*; (D) Simpson index, indicating decreased diversity in controls (p = 0.004).

Discussion

The present study conducted a microbiological characterization of the oropharyngeal microbiome in subjects with and without carotid atherosclerosis using next-generation sequencing (NGS). Our preliminary findings suggest differences in microbiota composition between the analyzed groups, implying that microbiome composition may condition the presence of systemic pathologies. According to our results, the most abundant phylum was *Bacteroidetes*, followed by *Proteobacteria* and *Firmicutes*, which have been reported in other studies of the oropharyngeal mucosa [17–19].

Taxonomic distribution differed between groups: *Bacteroidetes* were observed in both cases and controls, *Firmicutes* predominated in controls, whereas *Proteobacteria* were more abundant in cases. These variations are consistent with previous descriptions, suggesting a relationship between microorganisms belonging to these phyla—particularly *Proteobacteria*—and the development of atherosclerosis [20–22]. Schenkein et al. (2016) reviewed associations between bacteria involved in atherosclerotic plaque formation and coronary artery disease, integrating results from 63 independent studies including 1791 patients [22]. This review confirmed the presence of 23 oral commensal bacteria within atherosclerotic plaques in patients undergoing carotid endarterectomy or

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similar procedures. Some were well-known periodontal pathogens (*A. actinomycetemcomitans, P. gingivalis, T. forsythia, P. intermedia, F. nucleatum*), others were oral bacteria associated with dental caries (*S. gordonii, S. mutans, S. oralis*), and additional uncultivable members of *Proteobacteria, Chlamydiae, Fusobacteria, Tenericutes, Bacteroidetes,* and *Firmicutes* [22]. The detection of odontopathogens in coronary artery plaques suggests that these microorganisms invade endothelial cells and induce chronic vascular inflammation [21]. Furthermore, evidence indicates that other oral anaerobes can degrade immunoglobulins, inhibit the complement system, and produce toxic components such as lipopolysaccharides and exotoxins [23].

Current evidence suggests that atheromas harbor a complex microbiome with organisms cohabiting in different anatomical sites. Proteobacteria constitute the largest phylum within the bacterial kingdom. Based on phylogenetic analysis of the 16S rRNA gene, *Proteobacteria* are subdivided into six classes: Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Deltaproteobacteria, Epsilonproteobacteria, and Zetaproteobacteria [24]. In humans, *Proteobacteria* are present in diverse sites such as the skin, oral cavity, tongue, vaginal tract, intestine, and feces [24,25]. Notably, several common human pathogens belong to this phylum: *Brucella* and *Rickettsia* (Alphaproteobacteria); *Bordetella* and *Neisseria* (Betaproteobacteria); and *Escherichia, Shigella, Salmonella,* and *Yersinia* (Gammaproteobacteria). Guan et al. (2018) analyzed the association between *Proteobacteria* community composition and bronchiectasis severity, finding significant differences between patients and controls ($p < 0.001$). The relative abundance of *Haemophilus* spp. ($p = 0.002$), *Pseudomonas* spp. ($p < 0.001$), and *Moraxella* spp. ($p = 0.005$) correlated positively with disease severity [26]. Similarly, Wang et al. (2016) reported increased abundance of *Proteobacteria* in COPD patients during exacerbations, associated with reduced lung function and increased symptom severity [27].

A relevant finding was the identification of the family *Enterobacteriaceae* with higher abundance in subjects with atherosclerosis, encompassing species such as *Escherichia coli, Klebsiella* spp., and *Enterobacter*—pathogens linked to systemic diseases in previous studies [28–30]. This may explain the predisposition of patients with atherosclerosis to cardiovascular disease progression. Jie et al. (2017) reported increased *Enterobacteriaceae* in patients with atherosclerosis compared to healthy controls, suggesting a possible connection between intestinal dysbiosis and atherosclerosis pathogenesis [31]. Jia et al. (2018) similarly identified increased *Streptococcus* and *Enterobacteriaceae* species in heart failure patients, accompanied by reduced abundance of *Faecalibacterium prausnitzii* and *Roseburia intestinalis* [32]. Zhu et al. (2018) reported increased *Enterococcus, Escherichia,* and *Shigella*, along with decreased *Roseburia, Faecalibacterium,* and *Eubacterium rectale*, all known butyrate producers [33].

Another notable result was the association between atherosclerosis and variations in oropharyngeal microbiota richness. Faith's phylogenetic diversity index was high in both groups, suggesting adequate capture of oropharyngeal microbiota diversity. Genera such as *Neisseria* spp., *Rothia* spp., and anaerobes including *Veillonella* spp., *Prevotella* spp., and *Leptotrichia* spp. are described as part of the commensal microbiota of the upper respiratory tract [34,35]. However, median Faith's index was higher in cases (429.00) than in controls (351.00), with statistical significance ($p = 0.029$). This suggests greater phylogenetic diversity in atherosclerosis cases, potentially reflecting dysbiosis [36–38].

Bach et al. (2021) characterized the oropharyngeal microbiome of 18 healthy adults sampled weekly for 40 weeks using NGS, reporting high alpha diversity, uniformity, and temporal stability. Interindividual variation was greater than intraindividual variation [39]. Similarly, Kato et al. (2022) conducted a cross-sectional case-control study comparing oral microbiome composition between subjects with atherosclerotic cardiovascular disease and healthy individuals. Saliva samples analyzed by NGS (V1–V2 regions) revealed lower diversity in patients with atherosclerosis and higher abundance of periodontal-associated bacteria compared to controls [40]. Thus, a higher Faith's index in atherosclerosis cases may reflect a more diverse but potentially less functional microbiome, consistent with dysbiosis. Chen et al. (2021) evaluated intestinal microbiota variations in carotid atherosclerosis (CAS) patients ($n = 82$; 31 cases, 51 controls). Risk factors were significantly higher in CAS patients, with enrichment of 21 species and 142 metabolic pathways in cases, versus 10 species

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and 1 pathway in controls. *Bacteroides eggerthii, Escherichia coli,* and *Klebsiella pneumoniae* were most abundant in CAS, whereas *Parabacteroides* spp., *Prevotella copri, Bacteroides* sp3_1_19, and *Haemophilus parainfluenzae* predominated in controls [41].

The comparative analysis of the number of OTUs identified in each group did not reveal statistical differences. Similarly, when comparing richness using the abundance-based coverage estimator (ACE) method ($p = 0.631$), no significant differences were observed. However, when evaluating alpha diversity indices between groups, subjects with atherosclerosis exhibited lower levels, with a Shannon diversity index of 2.61 compared to 4.07 in controls ($p = 0.006$) [42]. This finding is consistent with results reported by Cuesta-Zuluaga et al. (2018), who investigated the relationship between fecal short-chain fatty acids (SCFAs), intestinal microbiome dysbiosis, and cardiometabolic risk factors. Elevated fecal SCFA concentrations in subjects with metabolic syndrome were inversely associated with microbiota diversity, correlating with increased cardiovascular risk [43]. This relationship between microbiome diversity and disease development has been highlighted in numerous studies [44–46].

A noteworthy observation was the comparison of beta diversity indices between cases and controls. In contrast to Shannon's index, Simpson's index revealed lower diversity values in controls ($D = 0.06$) compared to cases ($D = 0.16$). The boxplot for controls showed reduced dispersion and a median close to the first quartile, whereas cases exhibited greater dispersion and a central median. One possible explanation is Simpson's sensitivity to dominant species within the community [47]. When dominant species are present, Simpson's index tends to be lower, indicating reduced diversity. In controls, the lower Simpson's index ($D = 0.06$) may reflect the predominance of one or a few species at disproportionately high abundance, suggesting a less diverse or more uniform community. Conversely, in cases, the higher Simpson's index ($D = 0.16$) indicates reduced dominance and greater heterogeneity [47–49]. This variability may be influenced by sample size limitations or specific factors associated with atherosclerosis, resulting in a more diverse and heterogeneous microbial community [50,51].

Among the strengths of this study, it is important to highlight that the data originated from a standardized NGS protocol implemented de novo in a research and development laboratory. Samples were collected, processed, and analyzed under controlled conditions, and sequence quality was maintained at appropriate standards for interpretation. Limitations include the fact that samples were collected at a single time point. Longitudinal data could provide broader insights into individual microbiome variations influenced by extrinsic factors and shared characteristics across subjects, allowing discrimination of microbiome-driven differences. Despite this temporal limitation, the results offer an intriguing perspective on the complexity and dynamics of the oropharyngeal microbiome, incorporating the cardiovascular variable related to atherosclerosis and underscoring the need for longitudinal studies to capture extrinsic and shared influences over time.

In conclusion, this study addressed the microbiological characterization of the oropharyngeal microbiome in subjects with and without carotid atherosclerosis. Differences in microbiota composition were observed between the two groups, with *Proteobacteria* being more abundant in cases. Specifically, the family *Enterobacteriaceae* showed a significant association with the presence of atherosclerosis, suggesting its possible implication in the progression of cardiovascular diseases. Although no differences in richness were observed between groups, the reduction in alpha diversity in subjects with atherosclerosis points to a potential cardiovascular risk marker. These findings underscore the importance of the microbiome in the pathogenesis of cardiovascular diseases and highlight the need for future longitudinal investigations in this area.

Author Contributions: Conceptualization, Diana Mena-Yi, Josefina Zakzuk, Marlon Munera-Gomez, Gustavo Mora-Garcia, Fernando Manzur-Jattin and Maria Stephany Ruiz-Diaz; Methodology, Diana Mena-Yi, Alejandra Puerto-Lopez, Sara Mestra, Gustavo Mora-Garcia and Maria Stephany Ruiz-Diaz; Formal analysis, Diana Mena-Yi, Gustavo Mora-Garcia and Maria Stephany Ruiz-Diaz; Investigation, Diana Mena-Yi, Alejandra Puerto-Lopez, Sara Mestra and Gustavo Mora-Garcia; Data curation, Diana Mena-Yi, Alejandra Puerto-Lopez, Sara Mestra,

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Gustavo Mora-Garcia and Maria Stephany Ruiz-Diaz; Writing – original draft, Diana Mena-Yi and Gustavo Mora-Garcia; Writing – review & editing, Josefina Zakzuk, Marlon Munera-Gomez, Gustavo Mora-Garcia, Fernando Manzur-Jattin and Maria Stephany Ruiz-Diaz; Supervision, Josefina Zakzuk, Marlon Munera-Gomez, Gustavo Mora-Garcia, Fernando Manzur-Jattin and Maria Stephany Ruiz-Diaz; Project administration, Gustavo Mora-Garcia and Maria Stephany Ruiz-Diaz; Funding acquisition, Josefina Zakzuk, Marlon Munera-Gomez, Gustavo Mora-Garcia, Fernando Manzur-Jattin and Maria Stephany Ruiz-Diaz.

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Institutional Review Board Statement: The procedures were approved by the Ethics Committee of the University of Cartagena (protocol code 156-02-05-22-5-9, date of approval May 2nd, 2022).

Informed Consent Statement: Informed consent was obtained from all participants.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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Conflicts of Interest: The authors declare no conflict of interest.

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