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ORIGINAL RESEARCH

Analysis of Respiratory Microbiota Characteristics in Patients with COPD and High-Risk Populations Using 16S rRNA Technology

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Objective: To compare respiratory microbiota across patients with COPD, individuals at high risk for COPD, and healthy controls, and to assess associations with COPD severity.

Methods: From January 2022 to December 2023, participants were enrolled into four groups: previously diagnosed COPD (PVD-COPD, n=16), newly diagnosed COPD (PLD-COPD, n=16), high-risk individuals (HR, n=20), and healthy controls (HP, n=20). Sputum and saliva samples underwent 16S rRNA gene sequencing. Microbial diversity and taxonomic composition were compared among groups. In patients with COPD, correlations between sputum microbiota features and GOLD grade were analyzed.

Results: Seventy-two subjects were included. Alpha diversity (Ace and Chao1) and beta diversity differed significantly among groups (all P<0.05). Dominant phyla were similar across groups (Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria, and Fusobacteria), whereas genus-level profiles differed, with 10 genera showing significant between-group differences (mean abundance >1%). Within the COPD cohort, Ace and Chao1 were positively correlated with GOLD grade (r=0.3659, P=0.0394). A *Streptococcus*-dominant pattern was more frequent in GOLD 1–2, while a *Neisseria*-dominant pattern was more frequent in GOLD 3–4.

Conclusion: Respiratory microbiota composition differs across healthy controls, high-risk individuals, and COPD patients. In COPD, microbiota diversity and dominant genera are associated with disease severity, supporting a link between respiratory microbiota structure and COPD progression.

Keywords: pulmonary disease, chronic obstructive, 16S rRNA, high-risk population, respiratory microbiota

Introduction

Chronic obstructive pulmonary disease (COPD) is a widespread, preventable, and manageable chronic respiratory condition marked by ongoing airflow restriction and related breathing symptoms. Severe comorbidities may affect disease manifestation and mortality.[1] Worldwide, COPD is one of the three most common causes of death, with approximately 90% of deaths occurring in low- and middle-income countries.[2,3] China has an estimated 100 million individuals affected by COPD, representing one of the largest patient populations and highest COPD-associated mortality rates worldwide.[4,5] In China, the rate of pulmonary function testing among patients with COPD is low,[4] and most patients are diagnosed when they already have obvious symptoms or acute exacerbations, leading to delayed treatment. In recent years, early diagnosis and treatment of COPD has become a focus of research attention.

Emerging evidence suggests that the respiratory microbiota may serve as a potential biomarker reflecting early pathophysiological changes in the airway, thereby providing a novel perspective for understanding the clinical manifestations of COPD at its earliest stages. Oral microbiota has complex effects on host disease and health status.[6] Studies have found that healthy individuals also experience microaspiration in daily life,[7] and compared to nasal microbiota, oral

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microbiota composition shows higher similarity with pulmonary microbiota,[8] suggesting a close relationship between oral microorganisms and pulmonary diseases. Liu et al[9] found that oral hygiene was significantly correlated with COPD exacerbations, highlighting the significant impact of oral microbiota on COPD development. Various studies have indicated that oral microbiota might contribute to COPD development via non-specific immune responses, specific immune responses, and the action of proteolytic enzymes.[10–15] Although previous studies have explored the relationship between respiratory microbiota and COPD, the evidence base remains limited. Many existing studies suffer from small sample sizes, lack of appropriate control groups, and insufficient adjustment for potential confounding factors such as smoking history, antibiotic use, and comorbidities. Furthermore, few studies have simultaneously compared microbiota composition across healthy controls, high-risk individuals, and patients with COPD at different disease severity stages. This study aimed to explore the differences in respiratory microbiota composition among healthy controls, high-risk individuals, and patients with COPD, and to investigate the association between microbiota diversity and disease severity, providing scientific basis for early identification and intervention of oral microbiota in high-risk populations for COPD.

Methods

Study Population

This was a cross-sectional observational study conducted at Shanghai Fifth People's Hospital, Gumei Community Health Service Center, and Jiangchuan Community Health Service Center, between January 2022 and December 2023.Twenty patients with COPD in stable condition were selected as the previously diagnosed COPD (Previous diagnosis COPD, PVD-COPD) group. Twenty newly diagnosed patients with COPD were selected as the preliminarily diagnosed COPD (Preliminary diagnosis COPD, PLD-COPD) group. Sixteen high-risk individuals for COPD were selected as the high-risk (High-risk, HR) group, and sixteen healthy individuals were selected as the healthy control (Healthy population, HP) group.

Inclusion criteria:①PVD-COPD group: Documented history of COPD with one or more prior pulmonary function tests demonstrating post-bronchodilator FEV1/FVC < 0.70, consistent with the Global Initiative for Chronic Obstructive Lung Disease (GOLD) 2022 diagnostic criteria,[16] and clinically stable at the time of enrolment (defined as no acute exacerbation within the preceding four weeks).②PLD-COPD group: No prior history of COPD; first-time diagnosis of COPD at enrolment, with post-bronchodilator FEV1/FVC < 0.70 confirmed by pulmonary function testing, consistent with GOLD 2022 diagnostic criteria[H].③HR group: High-risk individuals were identified using the COPD Self-Screening Questionnaire (COPD-SQ). Those with a total score ≥ 16 were considered at high risk, as previously validated.[17] Subsequently, participants with a post-bronchodilator FEV1/FVC ratio ≥ 0.70 on pulmonary function testing were included, thereby excluding those who met the COPD diagnostic criteria of airflow obstruction.④HP group: COPD-SQ total score < 16 points and no history of respiratory disease.⑤Voluntary participation and signed informed consent.

Exclusion criteria:① Severe cardiac, hepatic, or renal insufficiency② Patients with oral diseases③ Use of antibiotics or probiotics within 4 weeks before sample collection④ Mental disorders or other conditions preventing cooperation with pulmonary function tests and information collection.

Sample Collection

Saliva Sample Collection

Saliva samples were collected using a commercially available saliva collection device (Simgen, model 3521001, China) using the passive drool method. Participants refrained from eating, drinking, or smoking for at least 30 minutes prior to collection. All collections were performed between 8:00–10:00 AM to control for circadian variation. Samples were immediately placed on ice, centrifuged at 4°C for 10 minutes, and stored at −80°C until analysis. This protocol is consistent with internationally recommended procedures for saliva collection and handling.[18–20]

Sputum Sample Collection

Sputum samples were collected using the deep cough method following established guidelines.[21,22] Participants were instructed to rinse their mouths with water upon awakening, take a deep breath, and cough forcefully to expectorate sputum from the lower respiratory tract. Specimens were collected in sterile, screw-capped containers and transported to the laboratory at room temperature within 2 hours.

High-Throughput Sequencing of Samples

① DNA Extraction, PCR Amplification, and Sequencing

Induced sputum and saliva samples underwent DNA extraction via the CTAB method.[23] DNA extraction quality was verified by agarose gel electrophoresis, and DNA quantification was performed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Specific primers targeting the V3–V4 hypervariable region of the bacterial 16S rRNA gene were synthesized (341F: 5′-CCTACGGGNGGCWGCAG-3′; 805R: 5′-GACTACHVGGGTATCTAATCC-3′) for PCR amplification. Each 25 µL PCR reaction contained 12.5 µL of Phusion Hot Start Flex 2× Master Mix (New England Biolabs, Ipswich, MA, USA), 2.5 µL of each primer, and 50 ng of template DNA. Thermal cycling conditions were as follows: initial denaturation at 98°C for 30s; 35 cycles of 98°C for 10s, 54°C for 30s, and 72°C for 45s; and a final extension at 72°C for 10 min. PCR products were confirmed by 2% agarose gel electrophoresis. PCR products were subsequently purified using AMPure XP beads (Beckman Coulter Genomics, Danvers, MA, USA) and quantified using a Qubit Fluorometer (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). The purified libraries were evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and the Illumina Library Quantification Kit (Kapa Biosciences, Woburn, MA, USA); libraries with a concentration exceeding 2 nM were considered qualified. Qualified sequencing libraries, each bearing unique index sequences, were subjected to gradient dilution, pooled in appropriate proportions based on the required sequencing output, and denatured into single strands using NaOH prior to loading. Paired-end sequencing (2×250 bp) was performed on the Illumina NovaSeq 6000 platform (Illumina, San Diego, CA, USA) using the NovaSeq 6000 SP Reagent Kit (500 cycles).

② Data Processing and Quality Filtering

Raw paired-end sequencing reads were demultiplexed according to barcode information, and adapter as well as barcode sequences were removed. Primer sequences and balance base sequences were subsequently trimmed using cutadapt (v1.9; parameters: -g R1 -G R2 -n 1 -O 17 -m 100). Each pair of paired-end reads was then merged into a longer tag based on overlapping regions using FLASH (v1.2.8; parameters: -m 10 -M 100 -x 0.25 -t 1 -z). Quality filtering was performed using fqtrim (v0.94; parameters: -P 33 -w 100 -q 20 -l 100 -m 5 -p 1 -V -o trim.fastq.gz) via a sliding window approach (window size: 100 bp); reads were truncated from the window start to the 3′ end when the average quality score within the window fell below Q20. Reads shorter than 100 bp after trimming and reads with an ambiguous base (N) content exceeding 5% were discarded. Finally, chimeric sequences were identified and removed using Vsearch (v2.3.4; default parameters).

③ DADA2 Denoising

Denoising was performed using DADA2 (called via qiime dada2 denoise-paired in QIIME 2 v2019.7) for length filtering and error correction, yielding ASV representative sequences and an ASV abundance table Singleton ASVs (ie., ASVs with a total read count of 1 across all samples) were removed as a default filtering step.

④ Diversity Analysis

Alpha and beta diversity analyses were performed based on the ASV representative sequences and abundance table Alpha diversity was assessed using six indices: observed species, Shannon, Simpson, Chao1, Good's coverage, and Pielou's evenness. Beta diversity was evaluated using four distance metrics (weighted UniFrac, unweighted UniFrac, Jaccard, and Bray–Curtis), and group differences were visualized using PCoA, PCA, and NMDS ordination (R v3.4.4; packages: vegan v2.5.4, ade4 v1.7.13, ggplot2 v3.2.0).

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Statistical Analysis

All statistical analyses were performed using SPSS (version 26.0, IBM Corp., Armonk, NY, USA). Continuous variables conforming to a normal distribution are expressed as mean ± standard deviation (SD), while non-normally distributed continuous variables are presented as median with interquartile range (IQR). Categorical variables are expressed as frequencies and percentages (n, %). All continuous variables were first assessed for normality using the Shapiro–Wilk test. Variables conforming to a normal distribution: including age, BMI, pulmonary function indices, and alpha-diversity indices, were compared across the four groups using one-way ANOVA, with post-hoc pairwise comparisons performed using Tukey's Honestly Significant Difference (HSD) test to control the family-wise error rate. For microbiome compositional data at the phylum and genus levels, which did not satisfy normality assumptions, the Kruskal–Wallis test was applied, followed by Dunn's test with Bonferroni correction for multiple comparisons. Categorical variables: including sex, smoking history, biomass fuel exposure, respiratory symptoms, mMRC dyspnea score, and GOLD stage distribution, were compared using the chi-square test, with Fisher's exact test applied where any expected cell count fell below five. Shannon index, which measures species richness and evenness; the Simpson index, which reflects the probability that two randomly selected individuals belong to different species; and the ACE and Chao1 indices, which are non-parametric estimators of species richness based on the abundance of rare taxa. Principal Coordinates Analysis (PCoA) based on Unweighted UniFrac distances was performed to visualize and evaluate β-diversity differences in microbial community composition across groups. UniFrac distance incorporates phylogenetic information to quantify the degree of dissimilarity between microbial communities. Spearman correlation analysis was used to assess the relationship between α-diversity indices and clinical parameters, including GOLD grading. Results are reported as correlation coefficients (r) and p-values All statistical analyses and visualizations were performed using GraphPad Prism (version 9.3.1; GraphPad Software, San Diego, CA, USA). A two-tailed p-value of < 0.05 was considered statistically significant.

Results

Baseline Characteristics

A total of 72 subjects were enrolled, including 16 in the PVD-COPD group, 16 in the PLD-COPD group, 20 in the HR group, and 20 in the HP group (Table 1).

Respiratory Microbiota Diversity

Comparison of α-Diversity

Analysis showed that the Shannon indices of sputum samples in the PVD-COPD, PLD-COPD, HR, and HP groups were 6.28±0.75, 5.89±0.73, 6.26±0.82, and 6.61±0.58, respectively. The Simpson indices were 0.95±0.03, 0.95±0.03, 0.94 ±0.04, and 0.96±0.02, respectively. The Ace indices were 539.19±120.93, 402.60±99.40, 540.68±94.34, and 505.02 ±78.61, respectively. The Chao1 indices were 537.29±120.77, 401.83±99.07, 539.48±94.33, and 504.15±78.26, respectively (Supplementary table 1). The PLD-COPD and HP groups exhibited statistically significant differences in the Shannon index, and statistically significant differences in Ace and Chao1 indices between PLD-COPD and the other three groups (Figure 1A).

Analysis showed that the Shannon indices of saliva samples in the PVD-COPD, PLD-COPD, HR, and HP groups were 5.80±1.00, 5.70±1.11, 5.78±0.91, and 6.29±0.64, respectively. The Simpson indices were 0.93±0.07, 0.92±0.13, 0.92±0.06, and 0.95±0.03, respectively. The Ace indices were 371.15±117.86, 56.71±96.00, 374.79±122.2, and 461.15 ±60.83, respectively. The Chao1 indices were 370.91±117.57, 356.35±96.67, 374.29±122.13, and 460.41±61.21, respectively (Supplementary table 2). There were statistically significant differences in Ace and Chao1 indices between PLD-COPD and HP groups (Figure 1B).

Comparison of β-Diversity

Using Unweighted Unifrac distance, Principal Coordinates Analysis (PCoA) revealed the similarity levels among microbial communities in respiratory samples. For sputum samples, PCoA1=19.84%, PCoA2=10.25% (F=7.09, P=0.001, Figure 1C); for saliva samples, PCoA1=17.32%, PCoA2=10.98% (F=6.76, P=0.001, Figure 1D). The results

Table 1 Comparison of Baseline Characteristics Among the Four Study Groups

	HP (n=20)	HR (n=20)	PLD-COPD (n=16)	PVD-COPD (n=16)	P value
Male [n (%)]	4 (20%)	10 (50%)	13 (81.3%)	14 (87.5%)	**<0.001**
Age (year, x±s)	61.25±8.55	67.65±3.38	69.63±2.85	69.63±6.94	**0.002**
Smoking history [n (%)]	4 (20%)	10 (50%)	11 (68.8%)	12 (75%)	**0.004**
History of biomass fuel exposure [n (%)]	6 (30%)	2 (10%)	1 (6.3%)	1 (6.3%)	0.173
BMI, kg/m^2	23.96±2.77	23.17±2.24	22.87±2.47	22.85±3.53	0.692
Clinical symptoms					
Cough [n (%)]	7 (35%)	7 (35%)	6 (37.5%)	15 (93.8%)	**<0.001**
Dyspnea [n (%)]	0 (0%)	13 (65%)	10 (62.5%)	11 (68.8%)	**<0.001**
Wheezing [n (%)]	(0%)	0 (0%)	4 (25%)	6 (37.5%)	**<0.001**
mMRC					**<0.001**
0	14 (70%)	8 (40%)	3 (18.8%)	0 (0%)	
1	6 (30%)	12 (60%)	8 (50%)	6 (37.5%)	
2	0 (0%)	0 (0%)	3 (18.8%)	5 (31.3%)	
3	0 (0%)	0 (0%)	2 (12.5%)	5 (31.3%)	
Lung function					
FVC, L	2.77±0.87	3.14±0.76	2.76±0.60	2.09±0.62	**0.002**
FEV1, L	2.19±0.67	2.37±0.56	1.63±0.45	1.20±0.42	**<0.001**
FEV1pred, %	89.19±15.87	90.67±9.67	62.03±13.94	46.06±13.90	**<0.001**
FEV1/FVC	0.79±0.05	0.75±0.04	0.58±0.06	0.57±0.08	**<0.001**
PEF, L	5.72±1.51	6.41±1.66	4.98±1.80	2.51±1.06	**<0.001**
PEF25-75, L	2.12±0.86	1.90±0.59	0.76±0.26	0.69±0.25	**<0.001**
PEF50, L	2.64±1.15	2.67±1.01	0.94±0.41	0.82±0.36	**<0.001**
PEF75, L	0.80±0.35	0.64±0.20	0.43±0.58	0.36±0.10	**<0.001**
GOLD grade					**<0.001**
1	17(85%)	17(85%)	1(6.3%)	0(0%)	
2	3(15%)	3(15%)	12(75%)	5(31.3%)	
3	0(0%)	0(0%)	3(18.8%)	8(50%)	
4	0(0%)	0(0%)	0(0%)	3(18.8%)	

Notes: Bold p-values indicate statistically significant differences among the four groups (p < 0.05).
Abbreviations: HP, Healthy control group; HR, High-risk population; PLD-COPD, Newly diagnosed COPD; PVD-COPD, Previously diagnosed COPD; BMI, body mass index; mMRC, modified Medical Research Council dyspnoea scale; FVC, forced vital capacity; FEV1, forced expiratory volume in 1 second; FEV1%pred, forced expiratory volume in 1 second as a percentage of predicted value; FEV1/FVC, ratio of forced expiratory volume in 1 second to forced vital capacity; PEF, peak expiratory flow; PEF25–75%, forced expiratory flow between 25% and 75% of vital capacity (also referred to as FEF25–75%); PEF50%, forced expiratory flow at 50% of vital capacity (also referred to as FEF50%); PEF75%, forced expiratory flow at 75% of vital capacity (also referred to as FEF75%); GOLD, Global Initiative for Chronic Obstructive Lung Disease.

indicated that there were statistically significant differences in Beta diversity reflecting microbiota structural similarity among the four groups (P<0.05).

Comparison of Bacterial Phyla and Genera

Analysis of Dominant Phyla

Phylum-level analysis showed that the four groups had the same dominant phyla, with the top five dominant phyla in all groups being Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria, and Fusobacteria. However, there were statistical differences in the top five dominant phyla among the four groups (P<0.05) (Figure 2A–C).

Analysis of Dominant Genera

Genus-level analysis showed that the dominant genera differed among the four groups (Figure 2B–D). The top 15 dominant genera were selected for analysis, and their proportions were calculated. In the PVD-COPD and PLD-COPD groups, the top three dominant genera were *Streptococcus*, *Neisseria*, and Rothia; in the HR group, the top three dominant genera were *Neisseria*, *Streptococcus*, and Rothia; in the HP group, the top three dominant genera were

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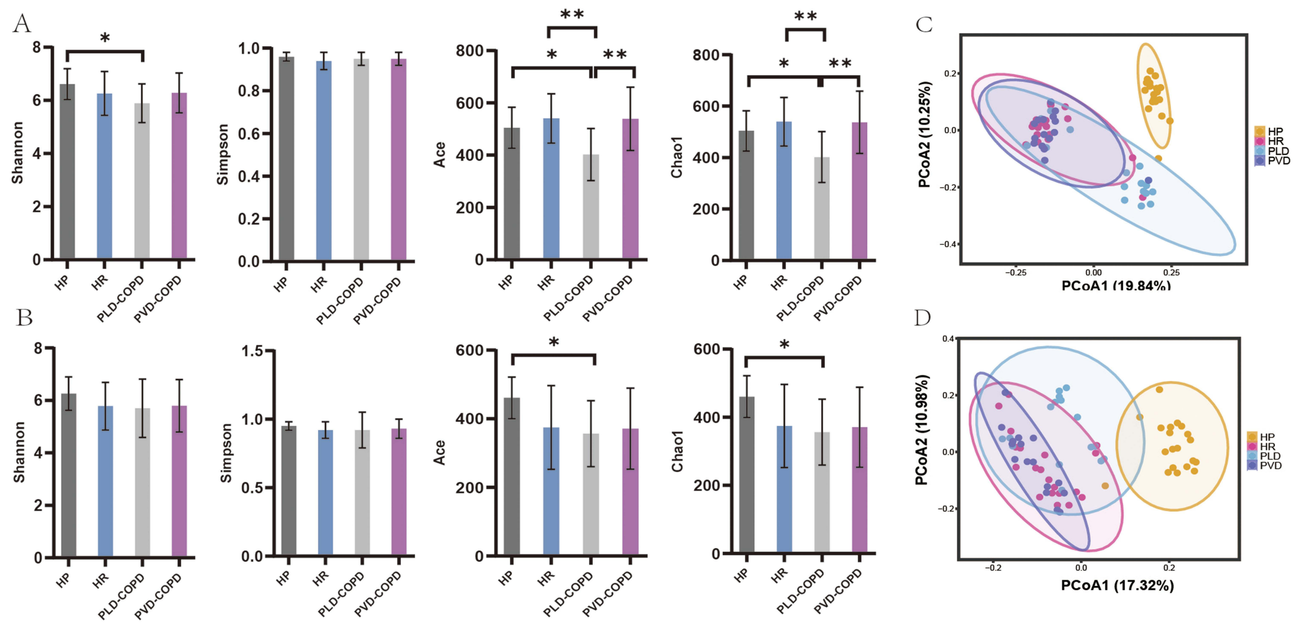

Figure 1 Alpha and Beta Diversity Comparison of Sputum and Saliva Microbiota Among Four Groups. (**A**) Comparison of Shannon index, Simpson index, Ace, and Chao1 in sputum samples among the four study groups. (**B**) Comparison of Shannon index, Simpson index, Ace, and Chao1 in saliva samples among the four study groups. (**C**) The figure shows PCoA plots based on Unweighted Unifrac distance demonstrating the similarity levels between microbial communities in sputum. (**D**) The figure shows PCoA plots based on Unweighted Unifrac distance demonstrating the similarity levels between microbial communities in saliva. *p<0.05. **p<0.01.

Abbreviations: HP, Healthy control group; HR, High-risk population; PLD-COPD, Newly diagnosed COPD; PVD-COPD, Previously diagnosed COPD.

Neisseria, *Prevotella*, and *Streptococcus* (Supplementary table 3). There were 10 genera that showed significant differences and had mean abundance >1% across all four groups (Table 2).

Microbiota Characteristics of COPD Group

Further analysis was conducted on sputum samples from the COPD group, with grouping based on dominant genera to analyze clinical characteristics of different dominant genera groups. There were 15 cases in the Neisseria-dominant group, 11 cases in the Streptococcus-dominant group, and 6 cases in the other genera-dominant group (Table 3).

Spearman correlation analysis showed that the α-diversity ace index in the COPD group was directly related to GOLD grade (r=0.366, P=0.039), and chao1 was also positively correlated with GOLD grade (r=0.366, P=0.039) (Figure 3A). However, α-diversity did not significantly differ among the various genus groups.(Supplementary table 4). The *Streptococcus*-dominant group had a higher proportion of GOLD 1–2 [8(73%) vs 3(27%)], while the Neisseria-dominant group had a higher proportion of GOLD 3–4 [7(47%) vs 8(53%)], but the statistical difference was not significant (P = 0.465) (Figure 3B).

Discussion

This study found that the respiratory microbiota of COPD patients and high-risk populations exhibited distinct characteristics compared to healthy controls. Alpha diversity indices differed significantly across the four groups, and beta diversity analysis further revealed significant structural differences in microbiota composition between groups. At the genus-level, analysis identified 10 genera with significant differences in mean abundance across groups. In COPD patients, the alpha diversity Ace and Chao1 indices were positively correlated with GOLD grade, and the dominant genus composition differed between GOLD 1–2 and GOLD 3–4 patients, suggesting a potential link between respiratory microbiota diversity and disease severity.

While sputum samples can directly reflect lower respiratory tract microbiota characteristics, their collection faces significant challenges. Patients with COPD often cannot provide qualified sputum specimens due to dry cough, difficulty in expectoration, or low compliance. Additionally, sputum production can alternate between exacerbation and remission.[24] The advantage of saliva samples lies in their non-invasive nature, repeatable collection, and high patient acceptance, making them particularly suitable for large-scale screening and long-term monitoring of high-risk

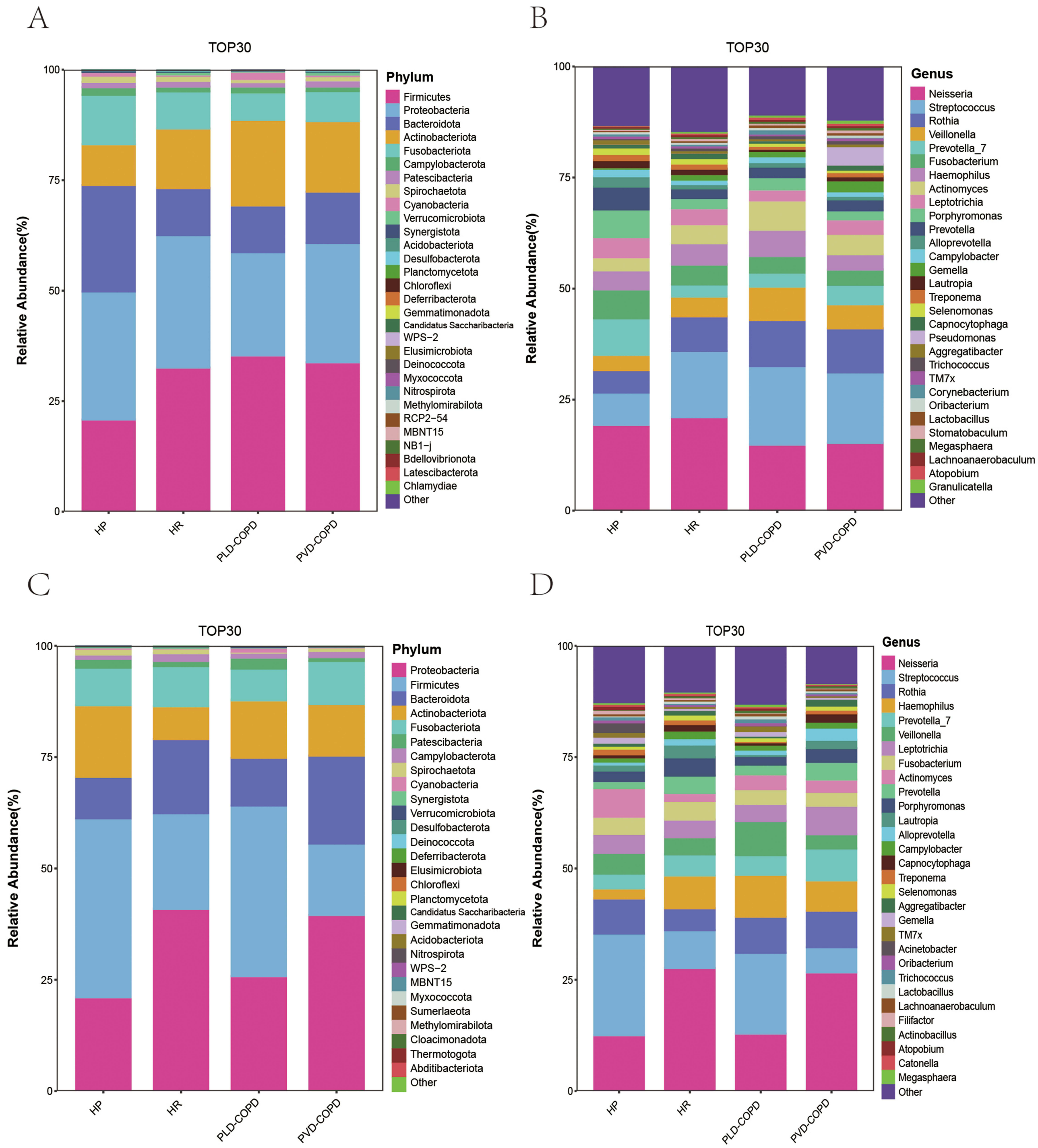

Figure 2 Analysis of dominant phyla and genera among the four groups. (**A**) Phylum-level dominant bacteria in sputum samples of the four groups. (**B**) Genus-level dominant bacteria in sputum samples of the four groups. (**C**) Phylum-level dominant bacteria in saliva samples of the four groups. (**D**) Genus-level dominant bacteria in saliva samples of the four groups.

Abbreviations:HP, Healthy control group; HR, High-risk population; PLD-COPD, Newly diagnosed COPD; PVD-COPD, Previously diagnosed COPD.

populations in the community. Studies have found that lower respiratory tract microbiota shows better consistency with oral microbiota.[8] Notably, research demonstrated that the upper respiratory bronchial tree is richer but less diverse in microorganisms than the lower bronchial tree, and *Streptococcus* was the most common genus identified across both compartments, with Rothia, *Neisseria*, *Prevotella*, and *Veillonella* also commonly detected.[25] Therefore, we collected

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Table 2 Significantly Different Genera Among the Four Groups

Dominant Genus (%)	HP	HR	PLD-COPD	PVD-COPD	P value
Streptococcus	7.28	14.91	17.68	15.84	**<0.001**
Prevotella	13.41	4.85	5.54	6.86	**<0.001**
Veillonella	3.42	4.45	7.50	5.42	**0.008**
Actinomyces	2.94	4.28	5.92	4.58	**0.015**
Pseudomonas	0.04	0.01	0	4.14	**0.002**
Fusobacterium	6.51	4.5	3.73	3.43	**0.025**
Leptotrichia	4.55	3.60	2.49	3.26	**0.021**
Gemella	0.35	1.25	1.24	2.45	**<0.001**
Porphyromonas	6.22	2.30	2.76	2.04	**0.017**
Campylobacter	1.72	1.03	1.31	1.04	**0.009**

Notes: Bold p-values indicate statistically significant differences among the four groups ($p < 0.05$).
Abbreviations: HP, Healthy control group; HR, High-risk population; PLD-COPD, Newly diagnosed COPD; PVD-COPD, Previously diagnosed COPD.

Table 3 Comparison of Baseline Characteristics Among Different Genus Groups

	Neisseria-Dominant Group (n=15)	Streptococcus-Dominant Group (n=11)	Other Genera-Dominant Group (n=6)	P value
Male[n(%)]	14 (93.3%)	10 (90.9%)	3 (50%)	**0.04**
Age (year, x±s)	68.53±5.13	69.91±5.80	71.83±4.26	0.432
Smoking history[n(%)]	13 (86.7%)	7 (63.6%)	3 (50%)	0.191
History of biomass fuel exposure[n(%)]	0 (0%)	0 (0%)	2 (33.3%)	**0.011**
BMI, kg/m^2	22.39±3.49	23.43±1.88	22.94±3.66	0.594
Clinical symptoms				
Cough[n(%)]	10 (66.7%)	7 (63.6%)	4 (66.7%)	0.986
Dyspnea [n(%)]	12 (80%)	6 (54.5%)	3 (50%)	0.281
Wheezing[n(%)]	0 (0%)	2 (18.2%)	2 (33.3%)	0.096
mMRC				0.423
0	1 (6.7%)	1 (9.1%)	1 (16.7%)	
1	6 (40%)	5 (45.5%)	3 (50%)	
2	3 (20%)	3 (27.3%)	2 (33.3%)	
3	5 (33.3%)	2 (18.2%)	0 (0%)	
Lung function				
FVC, L	2.43±0.60	2.62±0.73	2.08±0.82	0.302
FEV1, L	1.34±0.44	1.57±0.48	1.31±0.59	0.360
FEV1pred, %	48.75±13.84	59.39±17.20	57.47±16.76	0.238
FEV1/FVC	54.25±8.10	59.54±4.91	61.95±5.62	0.069
PEF, L	3.27±1.82	4.26±1.58	4.01±2.69	0.305
PEF25-75, L	0.67±0.23	0.80±0.27	0.72±0.28	0.460
PEF50, L	0.79±0.31	0.96±0.41	0.94±0.51	0.597
PEF75, L	0.46±0.60	0.36±0.09	0.31±0.09	0.465
GOLD grade				0.359
1	0 (0%)	1 (9.1%)	0 (0%)	
2	7 (46.7%)	7 (63.6%)	3 (50%)	
3	6 (40%)	2 (18.2%)	3 (50%)	
4	2 (13.3%)	1 (9.1%)	0 (0%)	

Notes: Bold p-values indicate statistically significant differences among the four groups ($p < 0.05$).
Abbreviations: BMI, body mass index; mMRC, modified Medical Research Council dyspnoea scale; FVC, forced vital capacity; FEV1, forced expiratory volume in 1 second; FEV1%pred, forced expiratory volume in 1 second as a percentage of predicted value; FEV1/FVC, ratio of forced expiratory volume in 1 second to forced vital capacity; PEF, peak expiratory flow; PEF25–75%, forced expiratory flow between 25% and 75% of vital capacity (also referred to as FEF25–75%); PEF50%, forced expiratory flow at 50% of vital capacity (also referred to as FEF50%); PEF75%, forced expiratory flow at 75% of vital capacity (also referred to as FEF75%); GOLD, Global Initiative for Chronic Obstructive Lung Disease.

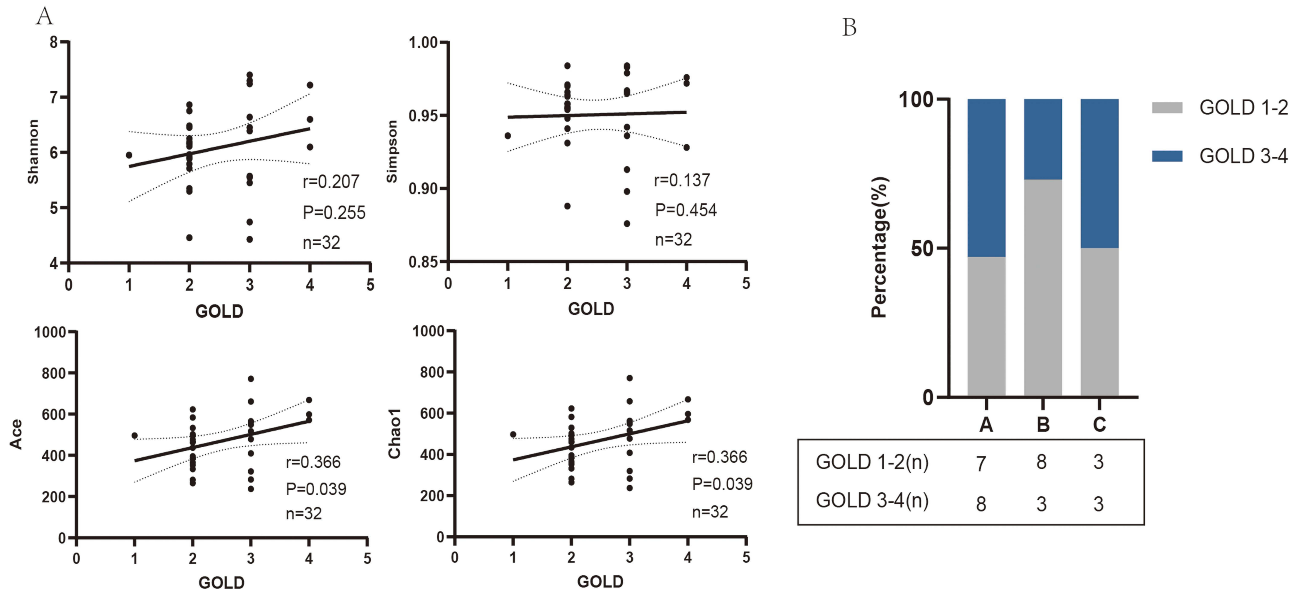

Figure 3 The relationship between different bacterial communities and lung function. (**A**) Relationship between α-diversity and GOLD Grade of Pulmonary Function in COPD Patients. (**B**) Relationship between Different Dominant Genus Groups and Pulmonary Function. (**A**) *Neisseria*-dominant group; (**B**) *Streptococcus*-dominant group; (**C**) Other genera-dominant group.

Abbreviation: GOLD, Global Initiative for Chronic Obstructive Lung Disease.

saliva samples from patients to comparatively study oral microbiota, aiming to provide a more convenient method for respiratory sample collection in patients with COPD and high-risk populations. Our research found that respiratory microbiota detection in sputum and saliva samples showed good consistency, particularly in core microbiota such as *Streptococcus*, *Neisseria*, and *Rothia* genera, which showed significant correlation. Notably, saliva may be interfered with by oral colonizing bacteria, and future studies need to improve its reliability through standardized decontamination procedures or machine learning model correction.

The development of COPD may be significantly correlated with airway microbiota dysbiosis.[26] This study observed high similarity in microbiota characteristics between high-risk populations and patients with COPD, mainly manifested as: 1) markedly reduced alpha diversity in comparison to the healthy control group; 2) an increase in the Rothia genus and a decrease in the *Prevotella* genus were observed in the respiratory tract. This phenomenon suggests that airway microbiota imbalance may occur before the manifestation of clinical symptoms, even preceding the decline in pulmonary function. The study reported that the presence of Rothia, Moraxella, and Granulicatella rose during episodes of COPD exacerbation,[27] potentially modulating the pathogenesis of COPD exacerbations through innate immune mechanisms like Type I interferon and Toll-like receptor signaling pathways. The resulting airway inflammation can be partially objectified by exhaled biomarkers: alveolar nitric oxide concentration (CAlv) has been shown to be significantly elevated in stable COPD patients compared with healthy subjects, and to correlate independently with small airway function indices including the single-breath nitrogen washout slope (dN2) and the diffusing capacity per unit alveolar volume (%DL(CO)/VA), suggesting that excess peripheral-airway nitric oxide generation is a measurable hallmark of the inflammatory microenvironment in which microbial dysbiosis operates.[28] In this regard, Su et al also observed that increased proportions of Rothia and decreased levels of *Prevotella* and *Alloprevotella* were characteristic features of acute exacerbations, and that *Veillonella* abundance showed a positive correlation with lung function.[29] Studies have found that *Prevotella* in the lungs has a protective effect due to its limited inflammatory capacity and its ability to enhance lung tolerance to respiratory pathogens.[8,30] Ramshehd et al[31] found that healthy individuals had significantly higher abundance of *Prevotella*, and its abundance was significantly correlated with improved pulmonary function and reduced symptoms in patients. Consistent with this, multiple reviews have corroborated that decreased *Prevotella* and *Veillonella* are associated with increased COPD disease severity, while increased *Proteobacteria*, *Pseudomonas*, and *Haemophilus* are linked to lung function impairment and more frequent exacerbations.[25,29,32]

Although the respiratory microbiota in high-risk populations already shows a "COPD-like state", the causal relationship with disease progression remains unclear. Future prospective cohort studies combined with metabolomics are needed to determine whether microbiota dysbiosis directly participates in lung tissue damage or is merely a concomitant phenomenon secondary to inflammation.

The study revealed that alpha diversity indices exhibited a "decline-then-rise" pattern across healthy controls, high-risk populations, and COPD patients. The alpha diversity indices of respiratory microbiota in COPD patients and high-risk populations were lower than those in healthy individuals. Further analysis indicated that Ace and Chao1 indices in the COPD group showed positive correlations with GOLD classification, meaning that GOLD grade 4 patients had significantly higher diversity compared to GOLD grade 1–2 patients. This finding aligns with the research results of Xue et al.[33] Notably, the increased diversity indices observed in patients with COPD do not signal disease improvement, but rather represent pathological microbial dysbiosis. This is because alpha diversity indices only reflect the quantity and distributional evenness of microbial communities, without revealing their functional significance.[34] Current evidence suggests that the composition of the lung microbiota in mild-to-moderate COPD may be relatively similar to that of healthy subjects, while more pronounced dysbiosis emerges in severe disease and during exacerbations.[32] The significant differences in respiratory microbial composition among the four groups further substantiate this viewpoint. Casadevalld et al[35] discovered a direct association between respiratory microbiota and systemic inflammatory patterns as well as clinical disease severity: on one hand, bacterial abundance correlates with plasma levels of several pro-inflammatory markers, with IL-8 being particularly significant; on the other hand, lung function decline over the past year and the frequency of acute exacerbations show positive correlations with relative microbial abundance. However, the relationship between respiratory microbiota and COPD pathogenesis requires further investigation.

The mechanisms by which respiratory microbiota dysbiosis contributes to COPD progression have been increasingly elucidated. The "vicious cycle" hypothesis proposes that harmful stimuli such as smoke exposure and air pollution damage the airway epithelium and impair mucociliary clearance and macrophage function, thereby increasing the risk of pathogen colonization. Colonized pathogens in turn drive abnormal inflammatory responses through Toll-like receptor (TLR) signaling, inducing cytokines such as IL-6 and IL-13, recruiting neutrophils and macrophages that release reactive oxygen species and matrix metalloproteinases (eg., MMP-12), and disrupting the protease-antiprotease balance, ultimately leading to progressive airflow limitation.[25] Notably, this inflammatory cascade extends to the peripheral airways, where heightened nitrosative stress results in measurably elevated alveolar NO concentrations. CAlv has been found to correlate significantly with small airway dysfunction parameters (dN2 and %DL(CO)/VA) in stable COPD patients, suggesting that alveolar NO may serve as a non-invasive surrogate marker of the chronic low-grade inflammation perpetuated by dysbiotic microbial communities in the distal lung.[28] This dysbiosis-inflammation cycle perpetuates and amplifies itself, and is further exacerbated during acute exacerbations.[32] A complementary model proposed by Xiao et al suggests that COPD dysbiosis is characterized not merely by shifts in community composition, but by a reduction in antagonistic (negative) bacterial interactions, with *Haemophilus* spp. emerging as a dominant pathogen that exerts disproportionately large negative interactions within the lung microbiota network; a decrease in such antagonistic interactions was associated with worse dyspnea scores, lower lung function, exaggerated neutrophilic inflammation, and higher risk of exacerbation.[32] Furthermore, the gut–lung axis may play an additional modulatory role: COPD patients have been shown to exhibit increased intestinal permeability, and gut microbiota-derived metabolites and microbial translocation via the portal circulation or mesenteric lymphatics can influence pulmonary inflammation and dysbiosis.[29,32] Animal models have demonstrated that gut microbiota alterations correlate with increased lung inflammation, emphysema, and airway remodelling, highlighting the bidirectional nature of gut–lung communication.[32] These mechanistic insights underscore the importance of viewing respiratory microbiota dysbiosis not as an epiphenomenon but as an active contributor to COPD pathogenesis, and suggest that microbiota-targeted interventions may represent a promising therapeutic avenue.

This study has certain limitations: first, no follow-up was conducted on patients, and the relationship between respiratory microorganisms and pulmonary function decline in patients with COPD and high-risk populations was not explored; second, the study included a relatively small number of participants, which was determined based on recruitment feasibility and reflects the exploratory nature of this work. And this may limit the statistical power of certain comparisons and the generalisability of our findings. Future studies with larger, well-powered cohorts are warranted to validate and extend these findings; third, the mechanisms by which different respiratory microorganisms affect pulmonary function status were not further investigated.

In conclusion, respiratory microbiota composition and diversity differ significantly across healthy controls, high-risk individuals, and COPD patients. Specifically, alpha diversity indices (ace and chao1) were reduced in patients with COPD and high-risk populations compared to healthy individuals. Furthermore, alpha diversity indices and dominant genera composition were positively correlated with COPD severity as measured by GOLD grade, supporting a link between respiratory microbiota dysbiosis and disease progression.

Ethics Approval and Informed Consent

The study was approved by the Ethics Committee of Shanghai the Fifth Peoples Hospital in accordance with the ethical requirements of the Declaration of Helsinki (2021 Ethics Approval NO.176). We ensured the confidentiality of the personal information of the participants in this study. The patients/participants provided their written informed consent to participate in this study.

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Disclosure

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