

Real-World Study on Upper/Lower Respiratory Tract Microbiome Changes in AECOPD and COPD

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Abstract

Background: Microbiome residing in the respiratory tract has emerged as an important player in the etiology and progression of COPD, but results are conflicting regarding the features of respiratory tract microbiome in COPD and at exacerbations and it is unknown whether these features differ by ethnicity and geography. To address these questions, we enrolled healthy individuals and patients with COPD, including healthy-COPD pairs from same households, from four geographical regions of Yunan province, representative of different ethnicities and/or environmental exposures. Sputum and oropharyngeal swabs were collected from these healthy individuals and from COPD patients at stable state (COPD) or exacerbations (AECOPD) and subjected to 16S amplicon sequencing.

Results: We found that both COPD disease status and region had an impact on alpha-diversity of sputum and oropharyngeal microbiomes, with AECOPD having the lowest microbiome diversity. Shifts in the relative abundance (≥ 1.5 fold, *adj.p* < 0.05) of microbes at healthy, exacerbation and stable COPD. Microbes enriched at exacerbation COPD were primarily *Proteobacteria* and *Firmicutes* phylum in upper respiratory tract. In the lower respiratory tract, population-based study did not find any statistical differential abundance of microbe, however, paired-based study showed phylum of *Proteobacteria*, *Chloroflexi*, *Bacteroidota*, *Acidobacteriota*, *Desulfobacterota*, *Firmicutes* and *Verrucomicrobiota* enriched in exacerbation COPD.

Conclusions: This study was the first attempt to combine the population-based and core-family-based to investigate the microbiome profiling in the upper and lower respiratory tract in COPD patients, and specific microbial flora characteristics may inform future study on the pathogenesis or management of COPD patients.

Background

Chronic obstructive pulmonary disease (COPD) is a heterogeneous complex, progressive respiratory disease featured by persistent symptoms, occasional exacerbation episodes, and chronic airway inflammation, the latter of which may lead to pathological changes in the respiratory tract including parenchymal destruction and airflow limitation. The mechanisms underlying overreactive immunity in the lungs of COPD patients are as yet unclear, which are considered to be an interplay between environmental and exogenous factors. Ethnicity may be a factor associated with the risk and outcome of COPD. Studies have found that the prevalence of COPD differs among the black, white, and Asian populations, and the black group seemed to have more severe exacerbations leading to hospitalization and are at increased risk of mortality[1, 2]. In addition, geographic region may affect individual's predisposition for COPD, evidenced by the geographic disparity in the prevalence of COPD in China.

Microorganisms are increasingly recognised as an important player in the pathogenesis and progression of COPD. Childhood infection in the respiratory tract has been linked to increased risk for COPD in adulthood[3]. A variety of pathogenic bacteria were isolated in the lower respiratory tract of patients with

stable COPD. Even more, pathogen infection was identified to account for 50% of exacerbation events. However, this pathogenic view is being reconsidered as it is now appreciated that even the healthy lower respiratory tract is not sterile but colonized with numerous commensal microbes. Instead of being caused by specific pathogens, it is hypothesized that dysbiosis of respiratory tract microbiome may be involved in COPD development and exacerbation events. Respiratory tract microbiome in COPD has been the subject of several studies, but results were conflicting regarding whether there were marked changes in the respiratory tract microbiome of COPD patients at stable or exacerbation state [4–7]. In this regard and considering the variability of COPD risks by ethnicity and geography, a question that remains to be answered is whether a common respiratory tract microbiome signature featuring COPD exists.

In this study, we aimed to identify the features of respiratory tract microbiome in COPD and reveal the impact of ethnicity and geography on the respiratory tract microbiome. To this aim, we enrolled patients in the core family as the smallest unit in Yunnan province of China. The core families have similar genetic backgrounds and living habits, so as to maximize the control of the influence of these factors on the respiratory tract microbiome. The complex topography in Yunnan province gives rise to varied climates, ranging from frigid, temperate, to tropic zone climate. There is considerable ethnic diversity in this province, where people of one ethnicity usually reside in one region and have similar lifestyle. As such, it is anticipated that respiratory tract microbiomes of individuals from different regions of Yunnan have been shaped by different genetic and/or environmental factors. In addition, we enrolled healthy-COPD pairs from the same households to control non-disease related influences on the microbiome.

Methods

Participant Enrollment

This study was approved by the ethic committees of the 2016YY140; all enrolled participants gave written informed consent. Genetics or lifestyle shapes our microbiome, order to minimize the impact of those factors on upper and lower respiratory tract microbiome, this study enrolled patients in the core family as the smallest unit from four cities in Yunnan province, from 2018 to 2020, including LiJiang, DaLi, Xishuangbanna(BanNa) and KunMing. Total 233 core families (486 samples, dropped 2 samples) were collected, including BanNa (101), DaLi (92), KunMing (204) and LiJiang (87). The healthy family members were defined as healthy control. All COPD patients were doctor-diagnosed, according to their clinical history and pulmonary function tests. The COPD patient was defined as stable if there was no exacerbation for the last month and the patient was not currently have an exacerbation. The AECOPD patient was defined as an acute exacerbation of COPD patient who was suffering a sustained (e.g. 24-48h) increase in cough, sputum production, and/or dyspnea. In this study, we totally collected 280 AECOPD samples, 72 COPD samples and 132 Healthy samples. The detailed study area and sampling location and type have been shown in Fig. 1 and Supplementary Fig. 1. The metadata of the patients were tabulated in Supplementary Table 1.

Study Design

In this study, we took nasopharyngeal swabs or sputum samples from enrolled samples or took both sample types from the same enrolled sample; Sampling was carried out in the same way under the condition of COPD; during the entire enrollment and sampling process, we faithfully carried out enrollment based on the normal clinical procedural. We divided all samples into groups according to their physical condition and sampling type. Each sample type is divided into three groups for pairwise comparison; at the same time, according to the core family, for the same sample type from the same family members, form a paired sample, then performed pairwise comparison. Among the sputum samples, there were only 6 core families including one patient with two COPD stages and corresponding healthy family members. Among the swab samples, such core families were only 10. Therefore, this study did not conduct a three-group pairwise comparison analysis. We incorporated these samples into specific pairwise comparisons based on their characteristics. The detailed study design and sample enrollment have been shown in Table 1.

DNA extraction and microbiome sequencing

Samples were pre-treated, depending on the clinical sample type. Total DNA was extracted from swab and digested sputum samples by use of QIAamp DNA blood kit (Qiagen, Germany) according to the manufacturer's instructions. DNA was eluted from the QIAamp column with 50 µl sterile distilled water, quantified by Nanodrop spectrophotometer (Thermo Fisher Scientific, USA), and qualified by agarose gel electrophoresis.

Sequencing data analysis

The V3-4 hypervariable regions of the 16S rDNA amplicon sequencing was undertaken at KingMed Diagnostics (Guangzhou, China), based on a published standard protocol[8]. The V3-4 region the 16S rDNA was amplified with the universal primer pair 338F/806R. The pooled amplicon was applied to a MiSeq Genome Sequencer (Illumina, USA).

The raw fastq files were trimmed adapter before starting the subsequent analysis. The operational taxonomic units (OTUs) classification analysis was done using published 16S microbial analysis with mothur pipeline[9, 10]. The raw sequences data were merged into contigs, followed by filtering and trimming of reads based on quality score and several other metrics. The filtered sequences data were aligned to the reference Silva v138[11]. The chimera sequences were removed using the vsearch algorithm[12]. Finally, the cleaned sequences data were assigned to taxonomy database for classification, here we used Bayesian classifier and a mothur pre-formatted Silva v138 taxonomy database as reference taxonomy[11]. The corresponding OTU and species annotations in this study can be found in the supplementary table 2.

Statistical Analyses

Statistical analyses were undertaken using R (v4.0.0). *P* value less than 0.05 was considered statistically significant. Core microbiota was defined as those found in more than 10 samples and the relative abundance was more than 0.05%. The final outputs from mothur were exported as count table and

related taxonomy table, then import these files and clinical meta table into Phyloseq (v1.32.0) package to generate S4 object for further analysis[13].

Alpha diversity was calculated on the basis of the taxonomy profile for each sample based on Shannon. Alpha diversity estimates were computed using Microbiome (v2.1.26, <http://microbiome.github.com/microbiome>) package's alpha function. Pair-wised mean comparison was performed.

Principal Coordinates Analysis (PCoA) was used to assess the beta diversity and overall upper and lower respiratory tract microbiomes composition. PCoA plots were generated using the first two principal coordinates according to physical status (Healthy vs AECOPD vs COPD), as well as according to location (BanNa vs DaLi vs KunMing vs LiJiang). The Phyloseq (v1.32.0) package's ordinate function[13] was used to calculate beta diversity and plotted by plot-ordination function. PCoA analysis based on weighted UniFrac distance matrices, PERMANOVA was tested on the same UniFrac distance matrices by Vegan packages' Adonis[14] function with default parameters.

Core microbiota of each group as counts table was input into SPIEC-EASI (v0.1.4, Sparse Inverse Covariance estimation for Ecological Association and Statistical Inference)[14] for the microbial ecological association networks analysis with meinshausen-buhlmann's neighborhood selection method, the networks were plotted using ggnet2 (v0.1.0) with setting the minimum degree (nodes) value to 1.

The Phyloseq object data was converted to the relevant DESeqDataSet object with phyloseq_to_deseq2 function. The pair wised OTU differential abundance analysis was done using the DESeq2 (v1.28.1) [15] negative binomial Wald test.

Results

Overview of sputum and oropharyngeal microbiome

A total of 249 sputum samples and 235 oropharyngeal swab samples were collected. For group comparisons, 58 vs 40, 58 vs 151, and 40 vs 151 sputum samples, and 74 vs 32, 74 vs 129, and 32 vs 129 oropharyngeal swabs were included for comparing healthy controls vs COPD, healthy control vs AECOPD, and COPD vs AECOPD, respectively. For within-household pairwise comparisons, paired groups included numbers of samples ranging from 12 to 48 per group (Table 1). Enrolled individuals were long-term residents in Xishuangbanna, Dali, Kunming, and Lijiang (see Supplementary Fig. 1 for sample distribution and Supplementary Table 1 for individual demographic and clinical characteristics). Medications for COPD maintenance and exacerbations were prescribed as per routine clinical practice.

Using 16S rRNA amplicon sequencing, 1137 and 970 OTUs were identified across all sputum and oropharyngeal swab samples. *Prevotellaceae*, *Streptococcaceae*, *Neisseriaceae*, *Veilonellaceae*, *Fusobacteriaceae*, *Lachnospiraceae*, *Leptotrichiaceae*, and *Pasteurellaceae* were the predominant

bacterial families in both sputum and oropharyngeal microbiomes, whereas *Porphyromonadaceae* and *Micrococcaceae* were also common in sputum and oropharyngeal microbiomes, respectively (Fig. 1).

Alteration of sputum and oropharyngeal microbiome by COPD and resident regions

Although sputum microbiome vs oropharyngeal swab microbiome were not compared within individuals, between-group comparison showed that sputum samples had slightly higher alpha-diversity than oropharyngeal swabs (Fig. 2a, c). The alpha diversity of oropharyngeal swab microbiome was lowest in AECOPD (AECOPD vs COPD, $p < 0.0001$; AECOPD vs healthy control, $p < 0.01$; COPD vs healthy control, $p < 0.01$), and a similar trend was observed in sputum microbiome (healthy control vs COPD, $p < 0.05$; AECOPD vs healthy control, $p < 0.001$). Compared with healthy controls, alpha diversity was increased in oropharyngeal swab microbiome of COPD patients but decreased in their sputum microbiome. Differences in alpha-diversity of microbiome were observed across different geographical locations, with individuals from Lijiang consistently had the least diverse microbiome in both sputum and oropharyngeal swabs (Fig. 2b, d).

The distinction in overall microbiome composition was evaluated by principal coordinate analysis (PCoA) based on weighted UniFrac distance metrics (Fig. 3). The first and second coordinates captured about 50.3% and 48.3% of variations in the composition of oropharyngeal microbiome and sputum microbiome, respectively. However, both oropharyngeal microbiome and sputum microbiome could not be discriminated by disease group or region. Nevertheless, PERMANOVA showed that both COPD disease status and resident region had an impact on microbiome compositions, suggesting the existence of differences in relative abundances of certain microbial taxa (Supplementary Table 3).

Respiratory Tract Microbiome Taxa Associated With Copd

In order to identify which bacterial clades in the oropharyngeal and sputum microbiome were different in relative abundances between healthy vs COPD stable state vs exacerbations, we performed negative binomial Wald test using DESeq2. A total of 35 OTUs were significantly different in relative abundances with a fold change $\geq .1.5$ and an adjusted p-value ≤ 0.05 in either group comparisons or within-household pair-wise comparisons (Table 2, Supplementary Table 4). We considered differences revealed by pair-wise comparisons more likely to reflect disease effects on microbiome. Compared with healthy controls, oropharyngeal microbiome under COPD stable state had relative higher relative abundances of *Streptococcus*, *Actinomyces*, *Actinobacteria spp.*, *Rothia*, and *Veillonella*. During COPD exacerbations compared with healthy controls, oropharyngeal microbiome consisted of higher relative abundances of *Raoultella*, *Actinobacillus*, *Enterobacteriaceae spp.*, and *Haemophilus*, and sputum microbiome comprised higher relative abundances of *Anaerolineaceae spp.*, *Desulfobacterota spp.*, *Anaerolineae spp.*, *Pedosphaeraceae spp.*, *Rhodobacteraceae spp.*, *gammaproteobacterial spp.*, and *alphaproteobacteria spp.*. No taxa showed significant differences in relative abundances between COPD stable state and exacerbations in pair-wise comparisons using above mentioned cutoffs.

Discussion

Based on respiratory tract microbiome profiles of a cohort of healthy individuals and COPD patients from different geographical regions, representative of diverse ethnicities and environmental exposures, we found that both COPD disease status and geography had an impact on the respiratory tract microbiome. The alterations in respiratory tract microbiome are potentially related to the pathological state or pathogenesis of COPD.

The predominant microbial families in sputum and oropharyngeal swabs are largely the same, indicating a continuum of microbiome composition along the respiratory tract. Previous studies showed that in healthy individuals Bacteroidetes and Firmicutes are two predominant phyla, whereas at the genus level, *Prevotella*, *Veillonella*, and *Streptococcus* are highly abundant in sputum microbiome[16–18]. The sputum microbiome is more diverse than the oropharyngeal swab, consistent with the fact that sputum microbiome is a mixture of oral and upper/lower respiratory tract microbiomes.

Reduced diversity of sputum microbiome under COPD stable state compared with healthy state has been reported in previous studies[6]. Moreover, the diversity of sputum microbiome has been found to be further decreased at exacerbations[6]. We found a consistent decreasing pattern in sputum microbiome; however, the oropharyngeal swab microbiome showed an increase in COPD compared with healthy control, which needs to be confirmed in further studies with balanced sample distribution between groups.

Pair-wise comparison of patients and healthy controls who shared the same household help reduce confounding factors, such as diet and genetic factors, and may reveal true microbiome alterations associated with diseases. Identified enriched or depleted taxa within COPD respiratory microbiome may underlie COPD pathogenesis or be a reflection of the pathological status. Some of the taxa found to be increased under COPD stable state have been reported to be associated with COPD before, such as *Actinomyces*, *Veillonella*, and *Rothia*[19]. In addition, *Actinobacillus* was also markedly enriched under COPD stable state, which was discovered as an aggressive pathogen related to periodontitis. Previous studies have shown that the COPD respiratory tract microbiome became more abundant in Proteobacteria bacteria such as *Pseudomonas spp.* and *Haemophilus spp.*[20–22]. In our study, many bacterial clades under Proteobacteria were observed to be increased in abundance in COPD patients during exacerbations compared with healthy individuals, including *Enterobacteriaceae spp.*, *Gammaproteobacteria spp.*, *Comamonadaceae spp.*, *Alphaproteobacteria spp.*, *Rhodobacteraceae spp.*, *Raoultella*, and *Haemophilus*. *Raoultella* is considered as opportunistic pathogens that may result in pneumonia in individuals with abnormal immunity[23]. *Haemophilus* contains the well-known opportunistic pathogen *Haemophilus influenzae*, and respiratory infection by this bacterium causes airway inflammation and is linked to respiratory diseases including COPD[24]. Though still a conjecture, these clades may contribute to episodic exacerbations in COPD patients.

This study was initially based on the results of the population study, compared with the results generated from the core-family study, and the follow-up microbe's biological function verification study can be

focused on the identical microbes. However, there are still many limitations. First, sample sizes across different groups were imbalanced, due to difficulty in collecting respiratory microbiome samples. Second, the sample sizes for intra-household pairwise comparisons of healthy control vs COPD and healthy control vs AECOPD were quite limited, which still help eliminate confounding factors related to microbiome compositions. Third, few individuals had both sputum and oropharyngeal swab samples available, not allowing for comparing upper and lower microbiome within individuals. Fourth, due to small sample size at one geographical location, whether patients of different ethnics/locations in healthy or COPD respiratory tract microbiome between cannot be investigated.

Conclusions

This study was the first attempt to combine the population-based and core-family-based to investigate the microbiome changes in the upper and lower respiratory tract in COPD patients. COPD is associated with specific changes in upper and lower respiratory tract microbiomes, whereas exacerbation events confer additional alterations in respiratory tract microbiome compositions. These specific microbiome changes may inform future study on the pathogenesis or management of COPD patients.

Abbreviations

COPD

Chronic Obstructive Pulmonary Disease

AECOPD

Exacerbations Chronic Obstructive Pulmonary Disease

Xishuangbanna

BanNa

OTU

Operational Taxonomic Units

PCoA

Principal Coordinates Analysis

PERMANOVA

Permutational multivariate analysis of variance

SPIEC-EASI

Sparse InversE Covariance estimation for Ecological Association and Statistical Inference

Declarations

Ethics approval and consent to participate

The studies involving human participants were reviewed and approved by The human research ethical board from institutional review board of the First People's Hospital of Yunnan Province approved the

study (2016YY140). The patients/participants provided their written informed consent to participate in this study.

Consent for publication

Not applicable

Availability of data and material

The dataset used for this study can be download in NCBI Sequence Read Archive (SRA), accession PRJNA687932 (<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA687932/>).

Competing interests

The authors declare that they have no conflict of interest.

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Author contributions:

Bing Yuan & Yunhui Zhang designed the study, Yanyan Xu & Nailiang Liu interpreted the data and wrote the paper. MeiYan He, Linfeng Shi, Zhiqin Yang, Mengjie Ma, Rufen Dai, Xianli Li, Zhongyuan Zhang, Liping Chen, Lina Wang, Dailing Yan, Kun Tian, Xingqiu Duan, Xiaohui Yang, Zhenming Huang, Bo Cai, Jianyuan Su and Quan Pan gathered clinical data. Shiyi Zhou, Jingxin Zhang, Jian Qin and Gang Yang conducted data analysis.

Acknowledgements:

Not applicable

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Tables

Due to technical limitations, table 1 and 2 is only available as a download in the Supplemental Files section.

Figures

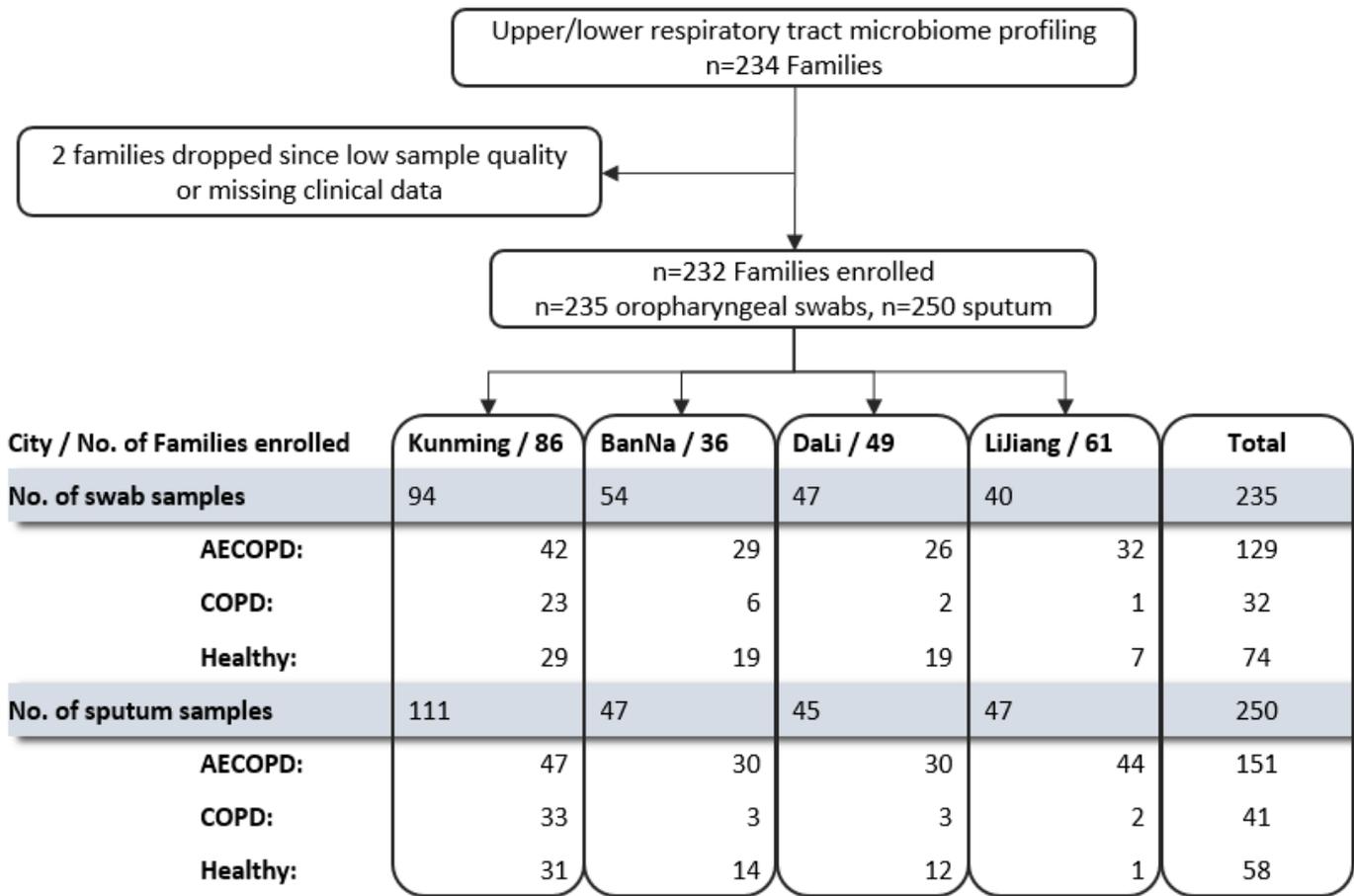


Figure 1

Flow chart of the study sample enrollment. Detailed comparisons between groups are shown in the Table 1.

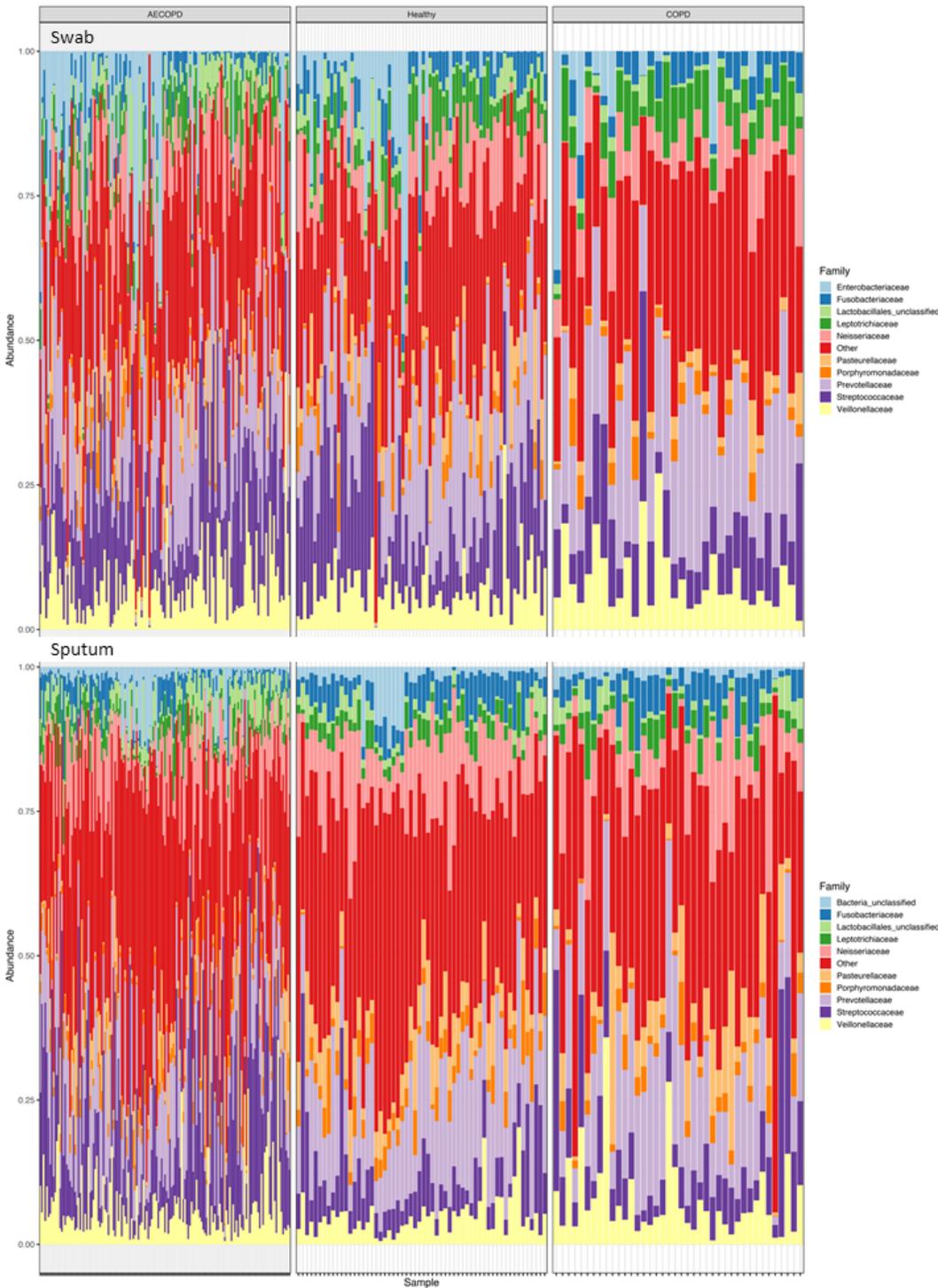


Figure 2

Relative abundance of major family in AECOPD, Healthy and COPD. (Swab, Sputum) shows the relative abundance of the bacterial species at the family levels. Each column of the bar graph represents a sample, and each patch represents a class of microorganisms in this group in proportion. Only top 10 family were plotted, and rest of family were classified as other. OTUs were annotated by default mothur method with default settings and the Silva v138 database.

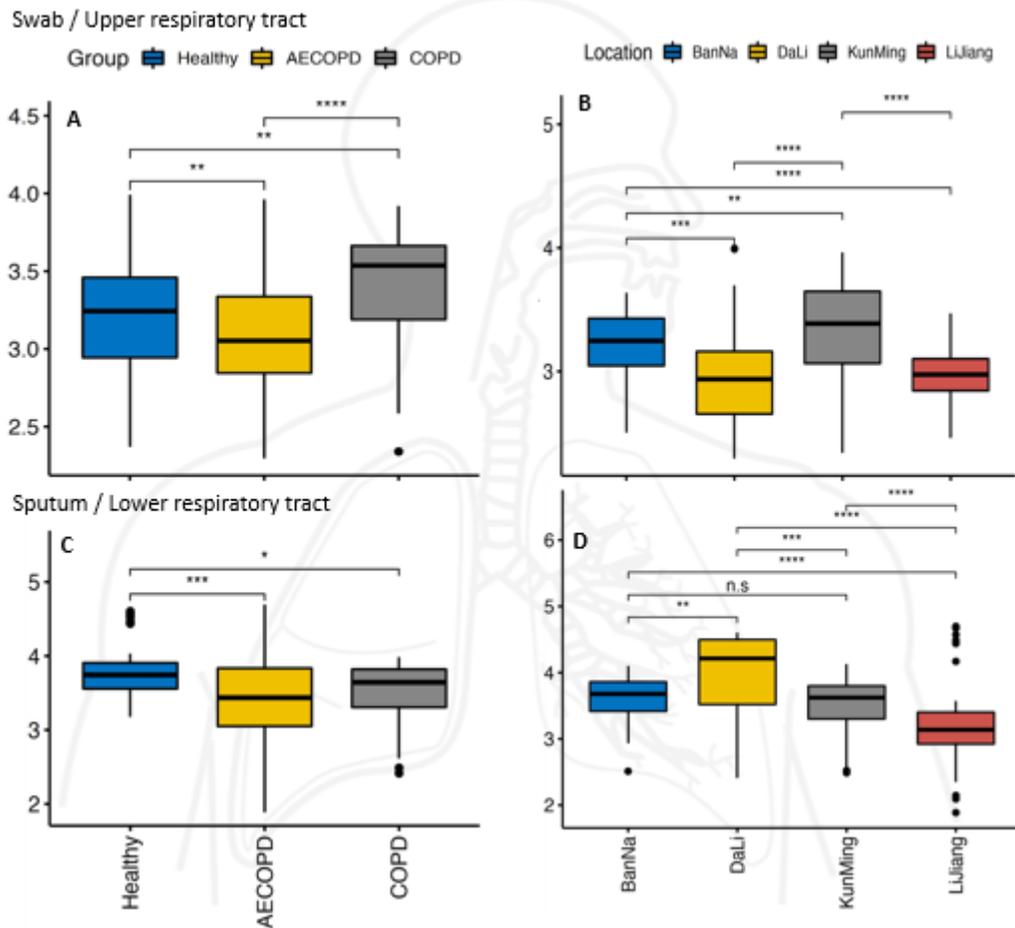


Figure 3

Alpha diversity of bacteria identified in different groups, as well as in different locations. (A) Alpha diversity of swab samples among Healthy, AECOPD and COPD groups; (B) Alpha diversity of swab samples among BanNa, DaLi, KunMing and LiJiang locations; (C) Alpha diversity of sputum samples among Healthy, AECOPD and COPD groups; (D) Alpha diversity of sputum samples among BanNa, DaLi, KunMing and LiJiang locations.

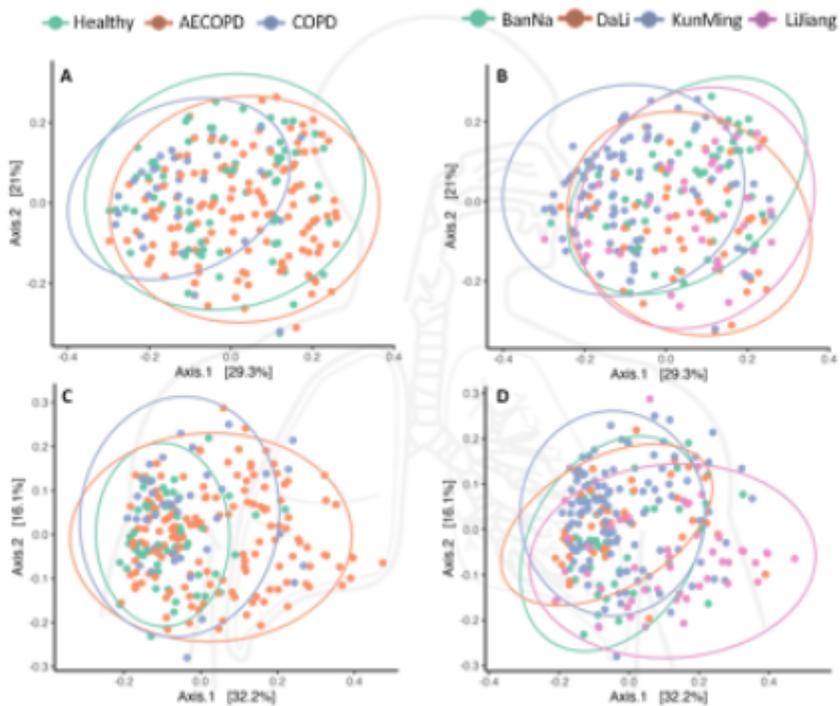


Figure 4

Beta diversity of bacteria identified in different groups, as well as in different locations. (A) Beta diversity of swab samples among Healthy, AECOPD and COPD groups; $p=0.001$, $R^2=0.0302$; (B) Beta diversity of swab samples among BanNa, DaLi, KunMing and LiJiang locations; $p=0.001$, $R^2=0.121$; (C) Beta diversity of sputum samples among Healthy, AECOPD and COPD groups; $p=0.001$, $R^2=0.06704$; (D) Beta diversity of sputum samples among BanNa, DaLi, KunMing and LiJiang locations. $p= 0.001$, $R^2= 0.11176$.

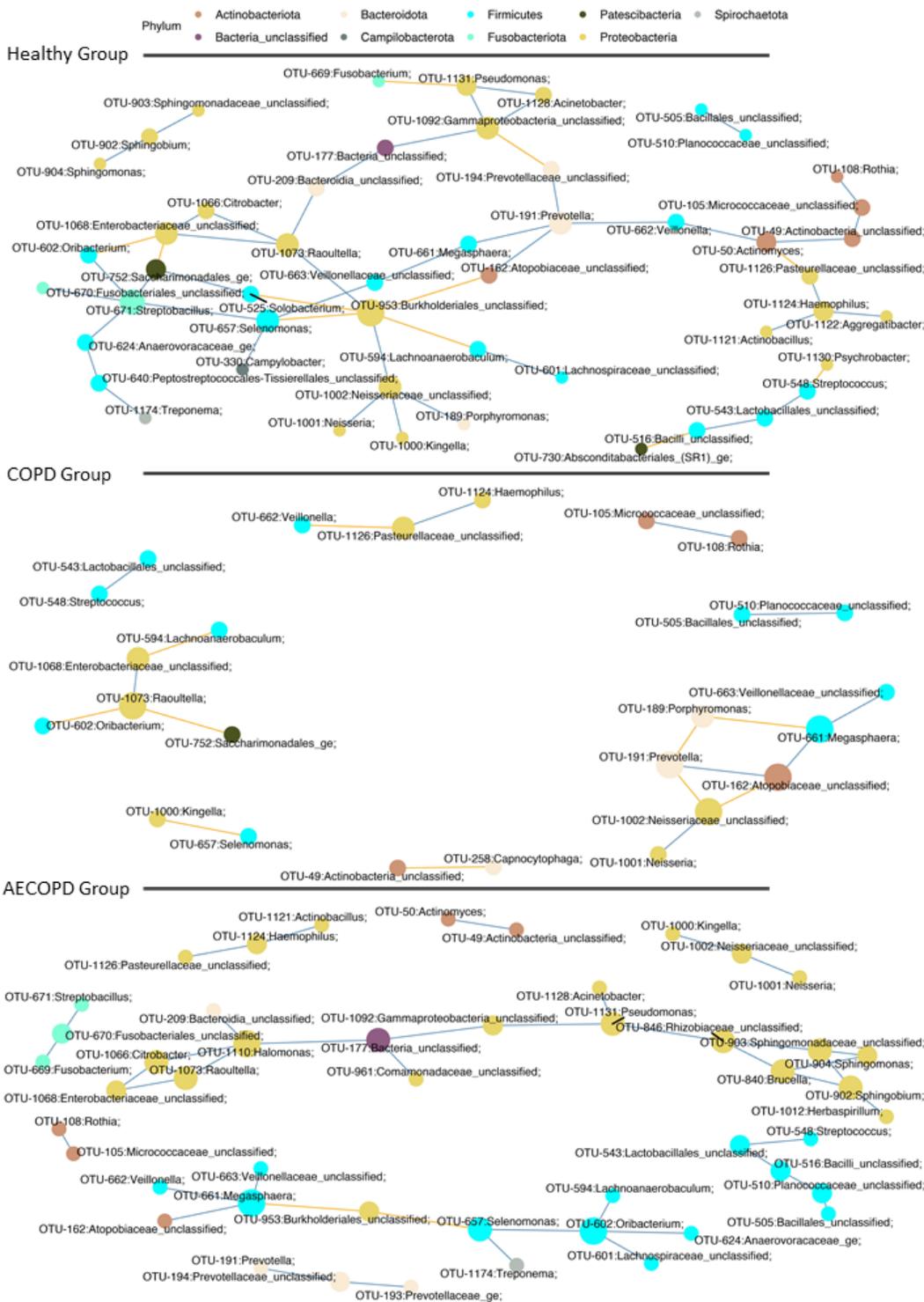


Figure 5

Upper respiratory tract microbial community structure in different groups. Here, we only plotted those microbes with degree > 1 in groups, including Healthy, COPD and AECOPD groups, which were conclusively associated with certain clinical status.

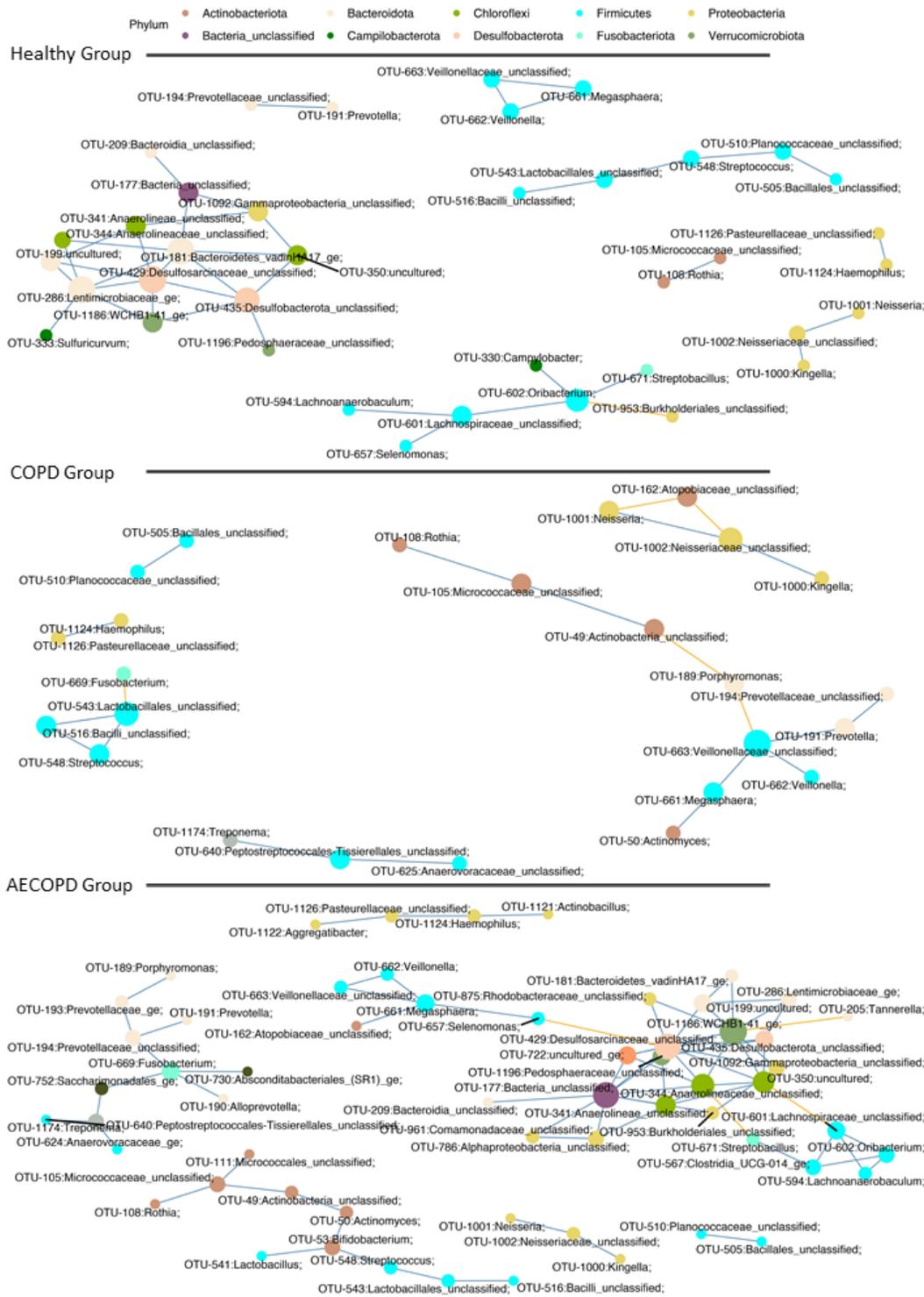


Figure 6

Lower respiratory tract microbial community structure in different groups. Here, we only plotted those microbes with degree > 1 in groups, including Healthy, COPD and AECOPD groups, which were conclusively associated with certain clinical status.

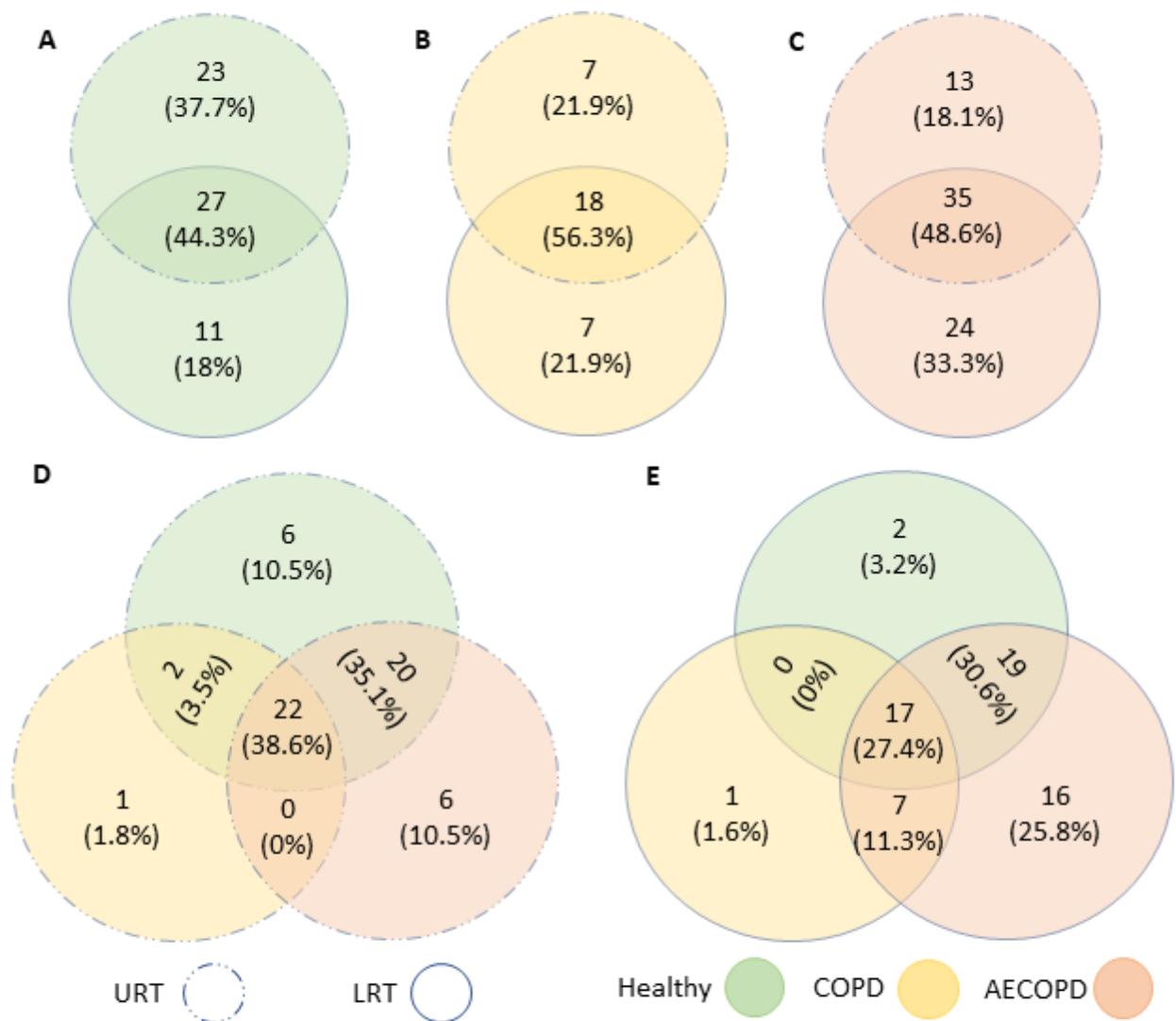


Figure 7

Identical and unique core microbiota in different groups with microbial community structure's degree > 1. Dash line represented core microbiome profile from upper respiratory tract, and solid line for lower respiratory tract; The different color represented different groups, green for Healthy group, yellow for COPD group and pink for AECOPD group.

Supplementary Files

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