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Characteristics of upper and lower respiratory tract microbiota after lung transplantation

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Abstract

Background The composition and characteristics of airway microbiota offer critical insights for clinical decision-making. Current research on chronic lung diseases shows differences in the composition and characteristics of upper and lower respiratory tract microbiota compared with healthy individuals. However, the temporal changes of these microbial communities in lung transplant recipients remain poorly characterized.

Methods This is a longitudinal prospective study. Respiratory specimens were collected regularly from lung transplant recipients for testing and analysis. A total of 150 bronchoalveolar lavage fluid (BALF) samples, 150 throat swab samples, 51 sputum samples, and 36 lung tissue samples were collected from the recipients, at 7 days, 14 days, 1 month, 2 months, 3 months, and 6 months post-transplant for 16S rRNA gene sequencing and analysis.

Results Our study showed that there were significant differences in α-diversity and β-diversity among lung tissue, throat swab, and sputum samples, although α-diversity did not show a significant difference between lung tissue and BALF. Most amplicon sequence variants (ASVs) belonged to the families *Enterobacteriaceae*, *Pseudomonadaceae*, and *Stenotrophomonas* in BALF, while most ASVs belonged to the genera *Streptococcus*, *Pseudomonadaceae*, and *Stenotrophomonas* in sputum samples. Regarding dynamic changes, *Corynebacterium* and *Staphylococcus* were more prevalent in the early post-operative period but gradually decreased by 7 days post-operatively, while the common microbiota found in healthy populations based on literature became the most abundant ASVs at 6 months post-operatively in our study participants. *Pseudomonadaceae* and *Stenotrophomonas* contributed to the similarity in the composition of upper and lower respiratory microbiota.

Conclusions This study demonstrates that lung transplant recipients exhibit unique characteristics in their upper and lower respiratory tract microbiota, which are distinct ecological profiles, and both undergo significant changes within

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6 months post-operatively. The similarity between upper and lower respiratory tract microbiota is associated with microbial diversity and taxonomic dominance.

Clinical trial The clinical trial was registered at Chinese Clinical Trial Registry (ChiCTR2200056908) in February 2022.

Keywords 16S rRNA, Upper versus lower airway Microbiome, Lung transplant

Introduction

Microbiota occupy almost all surfaces of the human body, including the respiratory tract [1, 2]. In recent decades, with the development of sequencing technology, the microbiota composition in respiratory samples has been assessed, and potential pathogens and symbiotic environments have been identified [3, 4]. The lungs possess diverse innate and adaptive immune defense systems, and the unique symbiotic relationship between microbiota and the host constitutes a delicate balance [5].

Microbial dysbiosis is often associated with adverse clinical manifestations [6]. Recent studies have found that lung infections, as well as acute and chronic rejection in lung transplant recipients may be related to lung microbiota dysbiosis [7–9]. These microbiota and their interactions with the host play crucial roles in immune system regulation, disease occurrence, and prevention [10–12]. Therefore, a more detailed understanding of the respiratory microbial environment in lung transplant recipients is essential for understanding the pathogenesis of post-operative complications and developing new treatment strategies.

Current research has observed that in healthy populations, the upper and lower respiratory tracts differ in microbial composition and load [13–15]. The upper respiratory tract, including the nasal cavity, paranasal sinuses, pharynx, and supraglottic larynx, harbors a large accumulation of microbiota, while the lower respiratory tract, including the subglottic region, trachea, and lungs, exhibits a relatively low microbial load [13, 16].

Lung transplant recipients are prone to pulmonary complications, including both infectious and non-infectious complications [17]. In the clinical diagnosis of severe post-operative complications in lung transplantation, lung tissue obtained through lung biopsy and bronchoalveolar lavage fluid (BALF) are considered relatively ideal specimen sources [18, 19]. However, obtaining these specimens requires invasive procedures, and transplant recipients may not tolerate bronchoscopy. Throat swab and sputum are more easily obtained and therefore widely used as specimen sources for detecting potential respiratory pathogens [20, 21]. However, studies have shown that in other chronic lung diseases, compared to BALF culture as the gold standard, throat swab and BALF cultures show different sensitivities and specificities [22, 23].

Therefore, little is currently known about the characteristics and trends of upper and lower respiratory tract microbiota in lung transplant recipients. It is also unclear to what extent the oropharyngeal microbiota collected by throat swab in lung transplant recipients resembles the microbiota in the lower respiratory tract and lungs. This study aims to compare the microbiota of the upper and lower respiratory tracts by collecting throat swab, sputum, and BALF samples.

Materials and methods

Study population

This study included 51 recipients who underwent allogeneic lung transplantation at the First Affiliated Hospital of Guangzhou Medical University from October 2020 to June 2021 (Table S1). BALF, throat swab, and sputum samples were routinely obtained pre-operatively and post-operatively according to the follow-up schedule, and recipient autologous lung tissue samples were obtained intraoperatively. Samples were collected at the following time points: pre-operative (D0), 7 days post-operative (D7), 14 days post-operative (D14), 1 month post-operative (M1), 2 months post-operative (M2), 3 months post-operative (M3), and 6 months post-operative (M6). A total of 150 BALF samples, 150 throat swab samples, 51 sputum samples, and 36 lung tissue samples were collected. Clinical information collected from lung transplant recipients included demographics, primary disease type, surgical method, and laboratory examinations. Laboratory data, including tumor necrosis factor-α (TNF-α) and complement C3, were provided by the clinical laboratory of the First Affiliated Hospital of Guangzhou Medical University. All recipients received glucocorticoid induction therapy. The post-operative maintenance immunosuppression regimen was routinely consisted of a combination of calcineurin inhibitors, glucocorticoids, and mycophenolate mofetil. All recipients used tacrolimus as the calcineurin inhibitor in the early post-operative period, with a target trough concentration maintained at 10–15 ng/mL. All participants in the study and their surgical procedures involved signed informed consent forms. This study was approved by the Ethics Review Committee of the First Affiliated Hospital of Guangzhou Medical University (No. 128 of 2020).

Sample collection

Lung tissue was obtained from all recipients intraoperatively. Before bronchoscopy, lung transplant recipients were instructed to rinse their mouths repeatedly and cough up sputum from deep within the trachea, which was collected in a sterile sputum cup to obtain sputum samples. Throat swabs were collected by rubbing the swab from beyond the root of the tongue to the pharyngeal isthmus.The swab was then placed in a cryopreservation tube containing saline and vortexed to obtain throat swab samples. BALF collection was performed by experienced bronchoscopists according to a standardized protocol. After adequate sedation, the fiberoptic bronchoscope was passed through the nostril into the right or left nasopharynx, advancing to the oropharynx to examine the glottic structure. Then, 1% lidocaine was applied to the vocal cords before passing through to examine the entire tracheobronchial tree. BALF was obtained by lavage of the transplanted lung. The method for obtaining BALF involved injecting equal amounts of saline (1 mL/kg) into the lobar or segmental bronchi, respectively, and recovering 40-60% of the lavage fluid into sterile containers. All samples were immediately frozen at -80 °C for subsequent microbiota analysis.

16 S rRNA gene sequencing and microbiota analysis

Bacterial genomic DNA was extracted from samples using the Qiagen DNA Mini kit (Qiagen, USA) as per the manufacturer's protocol. The extracted DNA yield and purity were evaluated using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). The V4 hypervariable region of the 16S rRNA gene was then PCR-amplified with appropriate reagent controls to avoid contamination introduced into the experiment. Amplified DNA fragments were sequenced using the Illumina NovaSeq 6000 platform. Sequencing reads were processed using QIIME pipeline V.2.0 [24]. Sequences were denoised and the amplicon sequence variants (ASVs) were identified using the dada2 algorithm [25]. Taxonomy was assigned to ASVs using the q2-feature-classifier classify-sklearn Naïve Bayes taxonomy classifier against Greengenes_13_8 database. All samples were rarefied at a depth of 47,560 reads.

For the blank BALF samples, two blank bronchoscope flush samples were generated by flushing the bronchoscope suction channel with sterile saline. For blank throat swab samples, two sterile swabs were used and subjected to the same DNA extraction and sequencing procedures as the actual samples. In addition, two reagent control samples were included in the DNA extraction procedure. These samples were processed and sequenced along with the test samples. The taxonomic profiles for these control samples are shown in Supplementary Table S2.

Statistical analysis

All statistical analyses and visualization were performed using R software (version 3.6). Alpha diversity was assessed using the Shannon index. Beta diversity was generated based on the Bray-Curtis dissimilarity of ASV profiles in each sample and visualized using principal coordinate analysis (PCoA) plots. The association between sample types and microbiota profiles was assessed using permutational multivariate analysis of variance (PERMANOVA, or Adonis) with 999 permutations, implemented in the adonis2 function from the R vegan package [26]. Kruskal-Wallis test was used to test differences of individual taxa in different months. Differential microbiota analysis was conducted using Linear discriminant analysis Effect Size (LEfSe). Clustering analysis and visualization of the temporal patterns of microbiota data were performed using fuzzy c-means algorithm with timeclust function of TCseq package. Procrustes analysis was carried out using vegan package in R [26].

To identify balanced or predominant microbiota subgroups, we performed a hierarchical clustering on the microbiota profiles of all samples (based on Ward's method), and based on the clustered profiles, we defined a sample as "balanced" or "predominated by a given taxon". For the leave-one-out method, we regenerated the microbiota profiles by iteratively excluding each of the 59 genus-level taxa with relative abundance > 0.001 one at a time, and recalculated the Bray-Curtis dissimilarity for the paired sputum-BALF and sputum-throat swab samples. This procedure was conducted using a custom script and pipeline.

Results

Subjects and samples analyzed

The demographic data and clinical characteristics of all subjects are shown in Supplementary Table S1. This study included 51 subjects from 2020 to 2021, and 387 samples were collected during the 6-month follow-up period post-transplantation, including 150 BALF samples, 150 lung tissue samples, 51 sputum samples, and 36 lung tissue samples. The number of samples per subject ranged from 2 to 5. After rarefaction, a total of 45,797 ASVs were identified.

Diversity and community structure of respiratory tract microbiota from different sites

First, we compared the microbial diversity among respiratory samples from different sites. Principal coordinates analysis based on Bray-Curtis dissimilarity matrix showed significant β-diversity differences in the microbial profiles of respiratory samples from different sites (Fig. 1A, $R^2 = 0.07$, $P = 0.001$). We further analyzed the β-diversity of different sampling sites at each time point

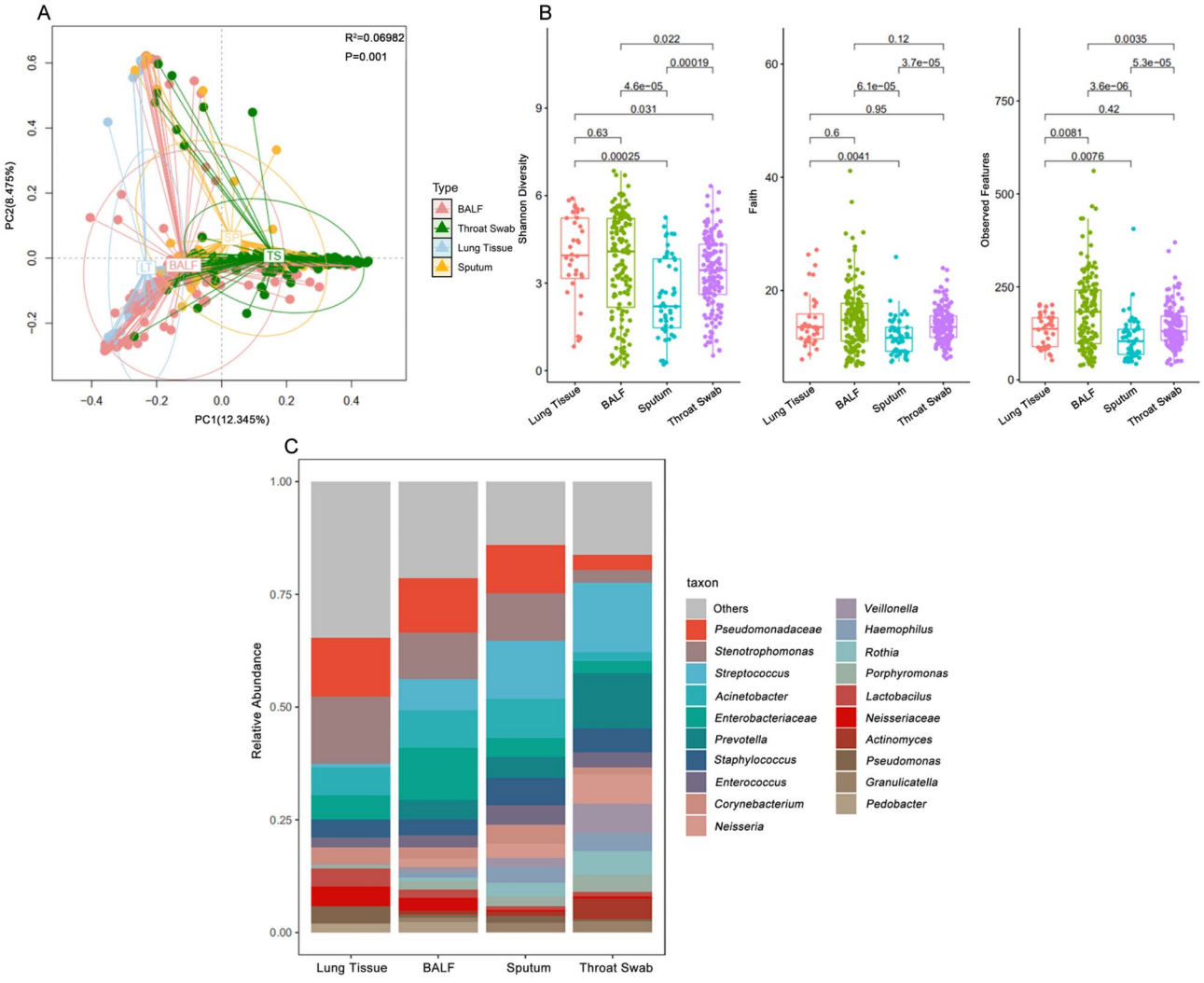

Fig. 1 Community structure and diversity of respiratory microbiota from different sites in lung transplant recipients. BALF: bronchoalveolar lavage fluid. (**A**) Principal coordinates analysis of respiratory samples from different sites based on Bray-Curtis dissimilarity matrix. PERMANOVA was performed to assess the association between the microbiota and sample types. (**B**) α-diversity of microbial communities in respiratory specimens from different sites. Two-sided Wilcoxon rank-sum test was used to compare the Shannon diversity between different groups. **: $P \le 0.01$; *: $0.01 < P \le 0.05$; ns: $P > 0.05$. (**C**) Composition of respiratory microbiota from different sites

and plotted the results separately (Supplementary Figure S1). The microbiota differences among BALF, sputum, and throat swab were significant at most sampling time points, as indicated by the PCoA plots and the plot for the first principal coordinate axis (PCoA1). We observed that at baseline (D0), the microbiota in sputum showed greater similarity to that in throat swab than to BALF. However, post-transplantation, the sputum microbiota gradually became more similar to the microbiota in BALF. Secondly, there were significant differences in α-diversity among lung tissue, throat swab, and sputum samples (all $P < 0.05$), and among BALF, throat swab, and sputum samples (all $P < 0.05$). However, α-diversity did not show significant differences between lung tissue and BALF ($P > 0.05$), indicating that lung tissue and BALF were more similar in species diversity. Sputum samples had the lowest α-diversity, while BALF and lung tissue had the highest α-diversity (Fig. 1B), indicating that BALF and lung tissue samples had more diverse microbial communities.

Figure 1C shows the relative abundance distribution of the top 20 microbial genera in each type of respiratory sample. In this study, *Stenotrophomonas*, *Pseudomonadaceae*, *Acinetobacter*, *Enterobacteriaceae*, and *Lactobacillus* were the most common genera in lung tissue. In BALF, most ASVs belonged to *Enterobacteriaceae*, *Pseudomonadaceae*, *Stenotrophomonas*, *Acinetobacter*, and *Prevotella*. In sputum, most ASVs belonged to *Streptococcus*, *Pseudomonadaceae*, *Stenotrophomonas*, *Acinetobacter*, and *Staphylococcus*. In throat swab, *Streptococcus*,

Prevotella, *Corynebacterium*, *Neisseria*, and *Staphylococcus* were the most abundant. LEfSe analysis highlighted significantly enriched microbial genera in each sample type (FDR < 0.05, LDA > 4.0, Supplementary Figure S2 and Table S3). Specifically, *Enterobacteriaceae* and *Pedobacter* were enriched in BALF; *Stenotrophomonas*, *Pseudomonadaceae*, and *Neisseriaceae* were enriched in lung tissue; *Acinetobacter* and *Corynebacterium* were enriched in sputum, while *Streptococcus*, *Prevotella*, and *Veillonella* were most strongly enriched in throat swab.

Temporal dynamics of upper and lower respiratory tract microbiota composition in lung transplant recipients

To further elucidate the main trends of microbial changes over time, we conducted longitudinal differential analysis and clustering analysis on the throat swab and BALF datasets separately. Finally, 9 clusters were produced for each sample type, with the number of ASVs in each cluster ranging from 91 to 306. Each cluster showed different temporal change patterns. In throat swab, a few clusters were more prevalent in the early post-operative period (Fig. 2A). For example, clusters 1 and 9 were more prevalent at 7 days post-operative and then gradually decreased, mainly including *Granulicatella*, *Staphylococcus*, *Corynebacterium*, *Mycoplasma*, *Oribacterium*, *Streptococcus*, *Rothia*, *Haemophilus*, *Acinetobacter*, and *Lactobacillus*. Additionally, a few clusters were more prevalent in the stable post-operative period. For example, cluster 3 was more prevalent at 3 months post-operative, with *Actinomyces* and *Fusobacterium* being the most abundant ASVs; cluster 7 (including *Gemellaceae* and *Parvimonas*, etc.) showed higher abundance at 6 months post-operative. In contrast, more microbial communities showed a transient increase in abundance only from 14 days to 2 months post-operative. For example, *Pseudomonadaceae*, *Veillonella*, *Enterococcus*, *Neisseria*, *Porphyromonas*, *Enterobacteriaceae*, *Ralstonia*, etc.

In BALF, a few clusters were more prevalent in the early post-operative period (Fig. 2B). For example, clusters 1 and 9 were more prevalent at 7 days post-operative and then gradually decreased, including *Corynebacterium*, *Staphylococcus*, *Pseudomonas*, *Enhydrobacter*. Additionally, a few clusters were more prevalent in the stable post-operative period. For example, cluster 2 (represented by *Streptococcus*) was more prevalent at 3 months post-operative; cluster 5 (represented by *Prevotella*, *Fusobacterium*, *Haemophilus*, *Actinomyces*, and *Neisseria*) showed higher abundance at 6 months post-operative. In contrast, more microbial communities showed a transient increase in abundance from 14 days to 2 months post-operative. For example, *Enterobacteriaceae*, *Enterococcus*, *Acinetobacter*, *Ralstonia*, *Veillonella*, *Porphyromonas*, *Rothia*, etc.

To assess whether the temporal dynamics of the microbiota could be attributed to the taxa with low prevalence, we filtered out the genera with prevalence less than 10% and 50% and repeated the time-based clustering analysis for the microbiota of both BALF and throat swab (Supplementary Figure S3). In general, we observed similar patterns of temporal dynamics as those from the full dataset. For instance, in BALF, we continued to observe clusters with peaks at D14, M1, M2, and M4, and in throat swab, clusters with peaks at D14, M1, and M2 remained present. This indicates that the dynamic trends of microbiota are not likely a result of the low-prevalent microbial taxa.

Furthermore, to explore potential shared temporal patterns of microbiota between throat swab and BALF samples, we conducted a temporal analysis of the genera commonly identified in both sample types, combining paired BALF and throat swab samples (Supplementary Figure S4 and Table S4). This approach enables a more intuitive comparison of trends in these genera across the two sample types. Indeed, we observed consistent temporal patterns for multiple genus-level taxa in throat swab and BALF samples, suggesting that certain microbial taxa changed in synchrony in the upper and lower airways over time. These observations indicate that upper and lower respiratory tract microbiota underwent dramatic fluctuations and changes in the early post-operative period.

Temporal dynamics of microbiota similarity between upper and lower respiratory tracts in lung transplant recipients

To further elucidate the similarity between upper and lower respiratory tract microbiota, we performed Bray-Curtis dissimilarity analysis on 150 paired throat swab-BALF samples grouped by different time points. We compared the Bray-Curtis dissimilarity between throat swab and BALF at different time points. The results showed that the Bray-Curtis dissimilarity between BALF and throat swab was highest pre-operatively, rapidly decreased within 7 days post-operatively, then stabilized, reaching the lowest value at 6 months post-operative (Fig. 3). This indicates that the upper and lower respiratory tract microbiota differed significantly pre-operatively but gradually became more similar post-operatively. There was a heterogeneous pattern of temporal trajectory of BALF-throat swab microbiota similarity at the individual level (Supplementary Figure S5A).

We observed that for certain patients, there was a high degree of similarity in microbiota between BALF and throat swab samples at D7 and D14 (Supplementary Figure S5B). Analysis of these samples revealed that their microbiota was predominantly composed of genera such as *Pseudomonadaceae*, *Staphylococcus*, and

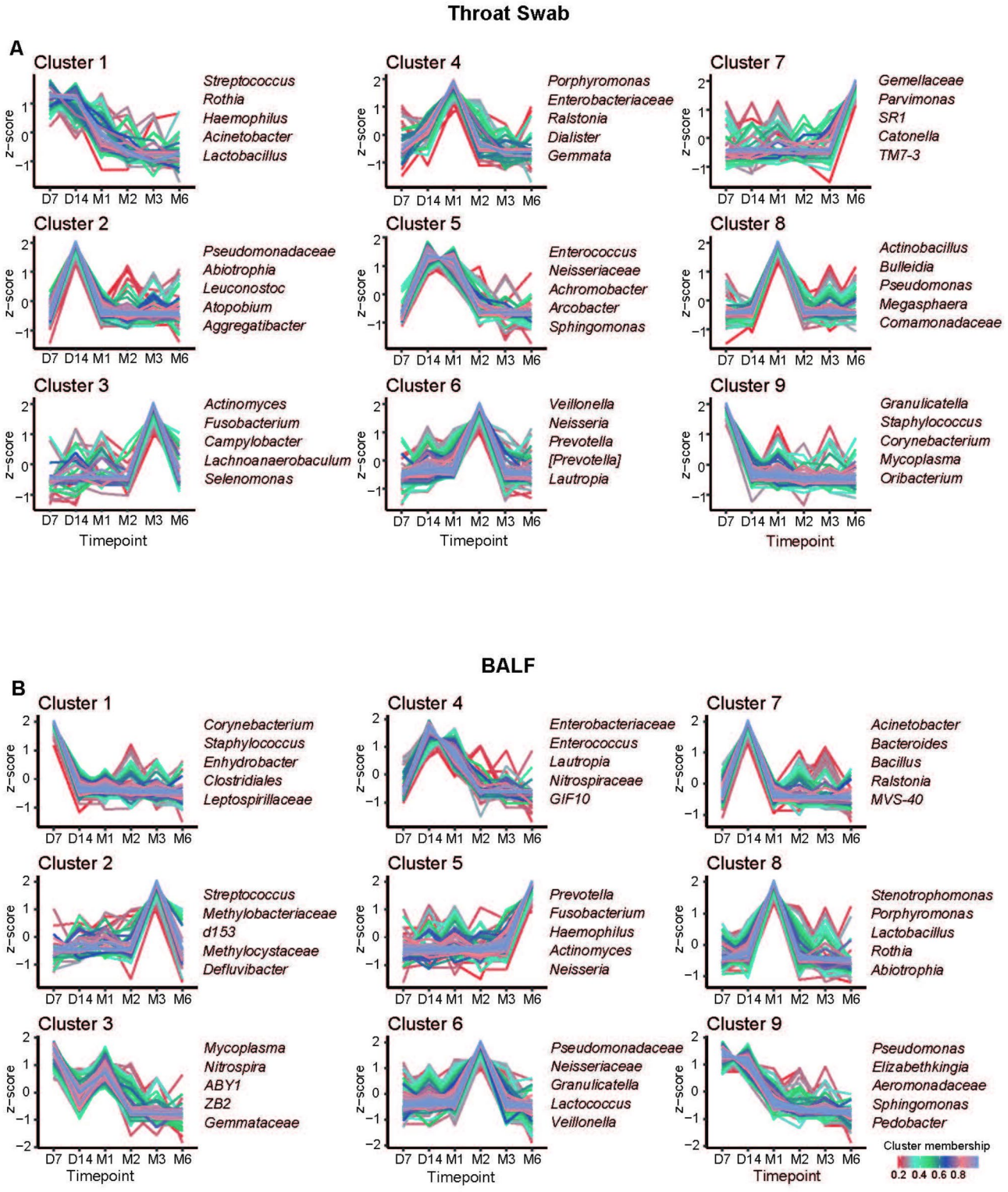

Fig. 2 Differential and clustering analysis of respiratory microbiota over time. Line graphs represent the trends of z-scores of ASVs relative abundance over time. The 5 most abundant ASVs are listed on the right side of each graph. Colors indicate membership values, representing the degree to which data points belong to clusters. D7: 7 days post-operative; D14: 14 days post-operative; M1: 1 month post-operative; M2: 2 months post-operative; M3: 3 months post-operative; M6: 6 months post-operative. (**A**) Differential and cluster analysis of upper respiratory tract microbiota changes over time represented by throat swab. (**B**) Differential and cluster analysis of lower respiratory tract microbiota changes over time represented by BALF

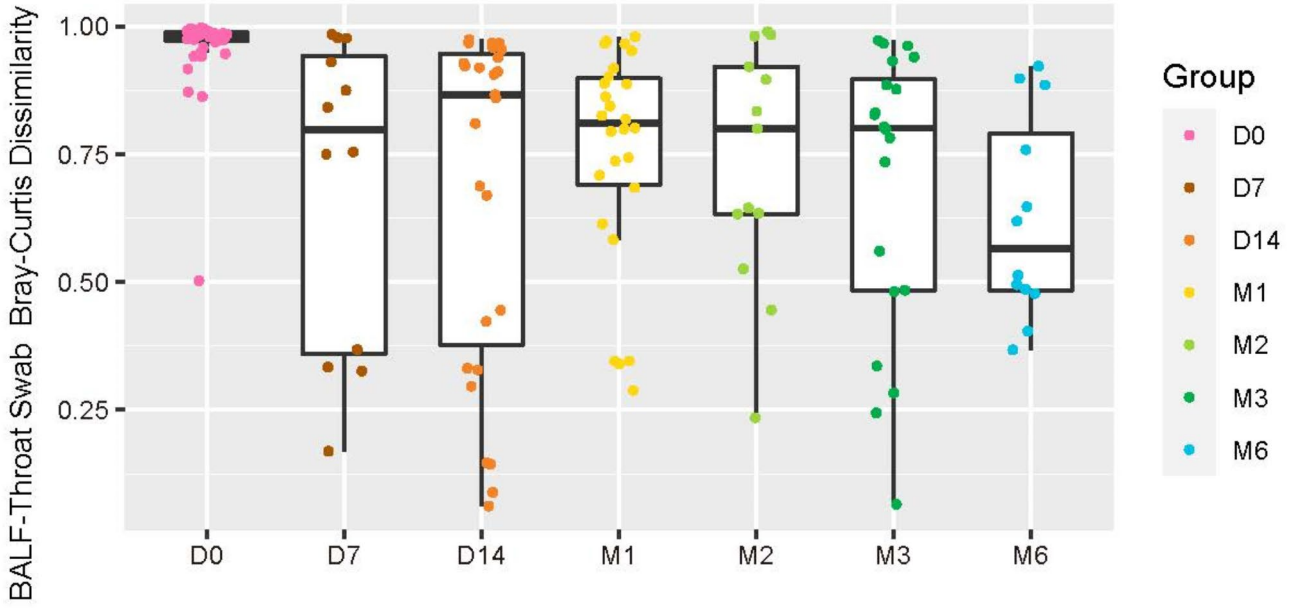

Fig. 3 Bray-Curtis dissimilarity of paired throat swab-BALF samples at different time points. D0: pre-operative or intraoperative; D7: 7 days post-operative; D14: 14 days post-operative; M1: 1 month post-operative; M2: 2 months post-operative; M3: 3 months post-operative; M6: 6 months post-operative

Stenotrophomonas, which are known to include respiratory pathogens(Supplementary Figure S5C). This suggests that when dominated by certain pathogenic taxa, the microbiota of the upper airways may exhibit greater similarity to that of the lower airways.

Relationship between similarity of upper and lower respiratory tract microbiota and microbial diversity

The density plot for Bray-Curtis dissimilarity between BALF and throat swab samples showed a bimodal distribution with an approximate splitting point of 0.6 (Fig. 4A), while the density plot for weighted UniFrac distance generally followed a normal distribution. Based on the Bray-Curtis dissimilarity between paired throat swab and BALF microbiota, we divided the all paired throat swab and BALF samples into two categories (based on Bray-Curtis dissimilarity cutoff of 0.6, Fig. 4A): microbiota similar group and microbiota dissimilar group, and analyzed the differences in diversity between the two groups. The results showed that in the similar group, compared to the dissimilar group, the Shannon diversity of both BALF and throat swab samples was lower (Fig. 4B). This indicates that lower diversity in the upper and lower respiratory tract microbiota is associated with higher similarity in microbial composition.

We performed a Fisher's exact test to assess the impact of patient identity and time on the categorization of BALF-throat swab microbiota similarity and dissimilarity. The results showed that patient identity had no significant effect ($P = 0.336$), while time had a highly significant effect ($P = 9.00\text{e-}4$). A mixed-effects model yielded similar findings, with time as a fixed effect showing a significant influence on the BALF-throat swab Bray-Curtis dissimilarity ($P = 2.02\text{e-}06$), while patient identity, as a random effect, had no significant impact ($P = 0.064$). Furthermore, the significant impact of time on BALF-throat swab similarity was primarily driven by the D0 time point (Supplementary Figure S6). At D0, all samples were categorized as dissimilar between BALF and throat swab. This is possibly due to the fact that the lung tissue sampled at D0 was the explanted lung, which was highly injured and could have exhibited significant dysbiosis, leading to a high dissimilarity in microbiota compared to the upper airways. Indeed, when excluding D0, the effects of other time points on the BALF-throat swab similarity were not significant (Fisher's exact test $P = 0.389$). Therefore, the split based on time reveals a distinct BALF-throat swab microbiota similarity pattern at D0 but does not highlight a unique subset of patients.

According to the hierarchical clustering of all BALF and throat swab microbiota profiles (Supplementary Figure S7 and S8), we divided all BALF and throat swab samples into a balanced microbiota and a "predominant" microbiota group (where the microbiota was dominated by a single taxon), and further analyzed the Bray-Curtis dissimilarity between throat swab and BALF in these two groups of samples (Fig. 4C). The results showed that the Bray-Curtis dissimilarity between upper and lower respiratory tract microbiota in the "predominant" microbiota group were lower than those in the balanced microbiota group, but the difference was more significant in throat swab, with statistically significant differences ($P < 0.05$).

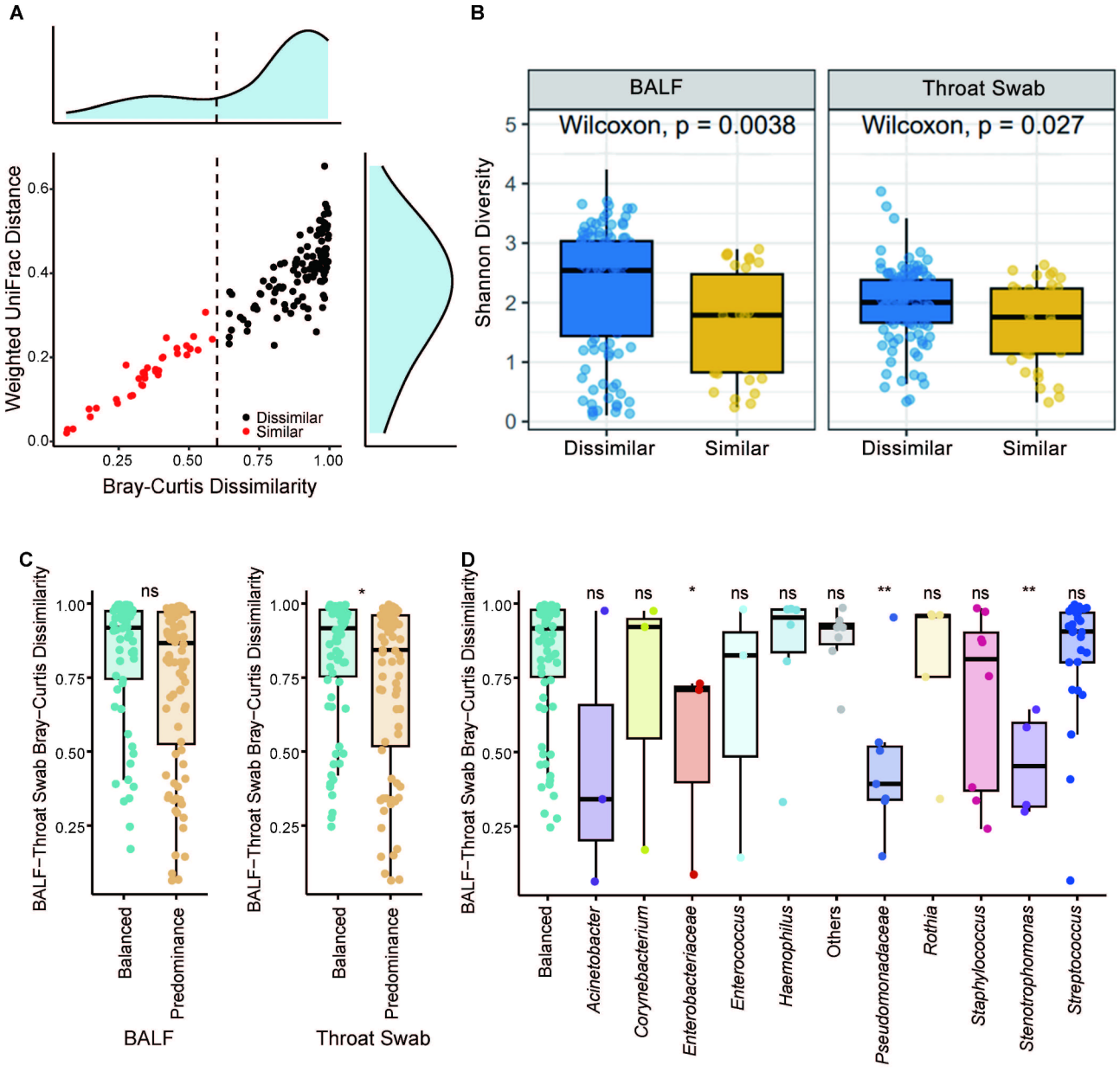

Fig. 4 Throat swab-BALF microbial similarity and its association with microbial diversity and predominance. BALF: bronchoalveolar lavage fluid. (**A**) Bray-Curtis and Weighted UniFrac distance of throat swab-BALF microbiota. Samples were divided into similar and dissimilar groups based on whether the Bray-Curtis dissimilarity is greater than 0.6. (**B**) Shannon diversity of the BALF-throat swab similar group and dissimilar groups. (**C**) BALF-throat swab Bray-Curtis dissimilarity in balanced and predominance microbiota groups. (**D**) Differential analysis of BALF-throat swab dissimilarity was performed for different predominant microbiota groups in comparison to the balanced group. Two-sided Wilcoxon rank-sum test was used to compare the Shannon diversity between different groups. **: $P \le 0.01$; *: $0.01 < P \le 0.05$; ns: $P > 0.05$

This suggests that when a certain taxon was predominant in the microbiota, the upper and lower respiratory tract microbiota are more similar. Therefore, to further elucidate the relationship between the predominance of specific single taxon and the similarity of upper and lower respiratory tract microbiota, we further analyzed the Bray-Curtis dissimilarity between upper and lower respiratory tracts in samples with different taxon predominance types in throat swab (Fig. 4D). The results showed that among the 7 single taxon predominance types obtained, 3 had significantly lower Bray-Curtis dissimilarity between upper and lower respiratory tracts, with statistically significant differences ($P < 0.05$). This indicates that *Pseudomonadaceae*, *Staphylococcus*, and *Stenotrophomonas* predominance types are significantly associated with a higher similarity between upper and lower respiratory tracts, suggesting that these genera are more likely to enter the lower respiratory tract when

predominant and can better reflect the microbiota of the lower respiratory tract. Moreover, we plotted the Bray-Curtis dissimilarity between BALF and throat swab of different taxon predominance patterns in different time periods (Supplementary Figure S9). We defined time points up to D14 as "early post-transplant" and time points starting from M1 as "late post-transplant" There was a significantly lower BALF-throat swab Bray-Curtis dissimilarity in samples with the predominance of *Pseudomonaceae*, *Rothia*, *Staphylococcus*, *Stenotrophomonas*, and *Streptococcus* during early post-transplant timepoints. However, this trend was non-significant in the late-transplant timepoints. Therefore, these results suggest that the close resemblance of upper and lower airway microbiota, in particular in individuals whose microbiota were predominated by microbial genera that contain respiratory pathogens, was more pronounced during the early post-transplant timepoints.

Association between sputum microbiota similarity to upper and lower respiratory tracts and microbial diversity

To further analyze the similarity of sputum microbiota to upper and lower respiratory tract microbiota, we included 51 paired sputum-throat swab-BALF samples. We analyzed the Bray-Curtis dissimilarity of all paired samples and divided the samples into 4 groups based on the Bray-Curtis dissimilarity between sputum and BALF, and between sputum and throat swab, respectively (Fig. 5A). Samples more similar only to BALF were classified into the BALF group (only BALF-sputum Bray-Curtis dissimilarity < 0.5), those more similar to throat swab were classified into the Throat Swab group (only throat swab-sputum Bray-Curtis dissimilarity < 0.5), those similar to both were classified into the Both group (both throat swab-sputum and BALF-sputum Bray-Curtis dissimilarity < 0.5), and those similar to neither were classified into the Neither group (both throat swab-sputum and BALF-sputum Bray-Curtis dissimilarity > 0.5). We then analyzed the microbial diversity of the 4 groups of samples (Fig. 5B). The results showed that in the Both group, the Shannon diversity of all samples was significantly lower than that in the Throat Swab group and the Neither group, with statistically significant differences (all $P < 0.05$). In the BALF group, the Shannon diversity of BALF samples was significantly lower than that in the Throat Swab group and the Neither group, with statistically significant differences ($P < 0.05$), but there was no significant statistical difference compared to the Both group ($P > 0.05$). These results indicate that when BALF diversity is lower, sputum microbiota is more similar to BALF microbiota.

Likewise, a Fisher's exact test was also conducted to assess the impact of patient identity and time on the group of similarity/dissimilarity of sputum with BALF and throat swab in their microbiota (Supplementary Figure S10A). Again, time showed a significant effect ($P = 0.020$), while patient identity did not ($P = 0.708$). We then established two mixed-effects models, one for the BALF-sputum Bray-Curtis dissimilarity and another for the throat swab-sputum Bray-Curtis dissimilarity (Supplementary Figure S10B and S10C). In both models, patient identity was treated as a random effect, and time was treated as a fixed effect. Both models revealed a significant influence of time ($P = 0.014$ for BALF and $P = 0.033$ for throat swab) and a non-significant influence of patient identity ($P = 0.102$ for BALF and $P = 0.684$ for throat swab) for the group of similarity/dissimilarity. Similar with the results for BALF-throat swab microbiota comparison, the influence of time is also likely associated with D0, at which there is a higher proportion of sputum samples that were dissimilar with both BALF and throat swab in the microbiota than the other timepoints.

In addition, we attempted to identify effective clinical indicators to assist in assessing the similarity of sputum to upper and lower respiratory tract microbiota. In our study, we found that TNF-α was lower in the BALF or Both groups, while complement C3 was lower in the throat swab or both groups (Supplementary Figure S11).

Subsequently, to elucidate the relationship between specific single taxon predominance types and the similarity of sputum-BALF microbiota, we further analyzed the species composition characteristics of all BALF samples. Based on the hierarchical clustering of all BALF and throat swab microbiota profiles (Supplementary Figure S7 and S8), they were divided into a balanced microbiota and a "predominant" microbiota group (where microbiota was predominated by a single taxon, such as *Acinetobacter*, *Enterobacteriaceae*, *Enterococcus*, *Pseudomonadaceae*, *Staphylococcus*, *Stenotrophomonas*, and *Streptococcus*). We then analyzed the differences in Bray-Curtis dissimilarity between sputum and BALF among these predominance types (Fig. 5C). The results showed that the Bray-Curtis dissimilarity of *Pseudomonadaceae* predominance type and *Stenotrophomonas* predominance type were significantly lower than those of the microbiota balanced type, with statistically significant differences ($P < 0.05$). This indicates that they are significantly associated with higher similarity between sputum and BALF, suggesting that when these genera cause lower respiratory tract microbial dysbiosis, sputum samples have greater clinical reference value.

Finally, we used the leave-one-out method to analyze the contribution of different taxa to the similarity between sputum and upper and lower respiratory tract microbiota (Supplementary Figure S12). The results showed that after removing *Stenotrophomonas*, the Bray-Curtis dissimilarity between sputum and BALF, and sputum and throat swab, both decreased significantly,

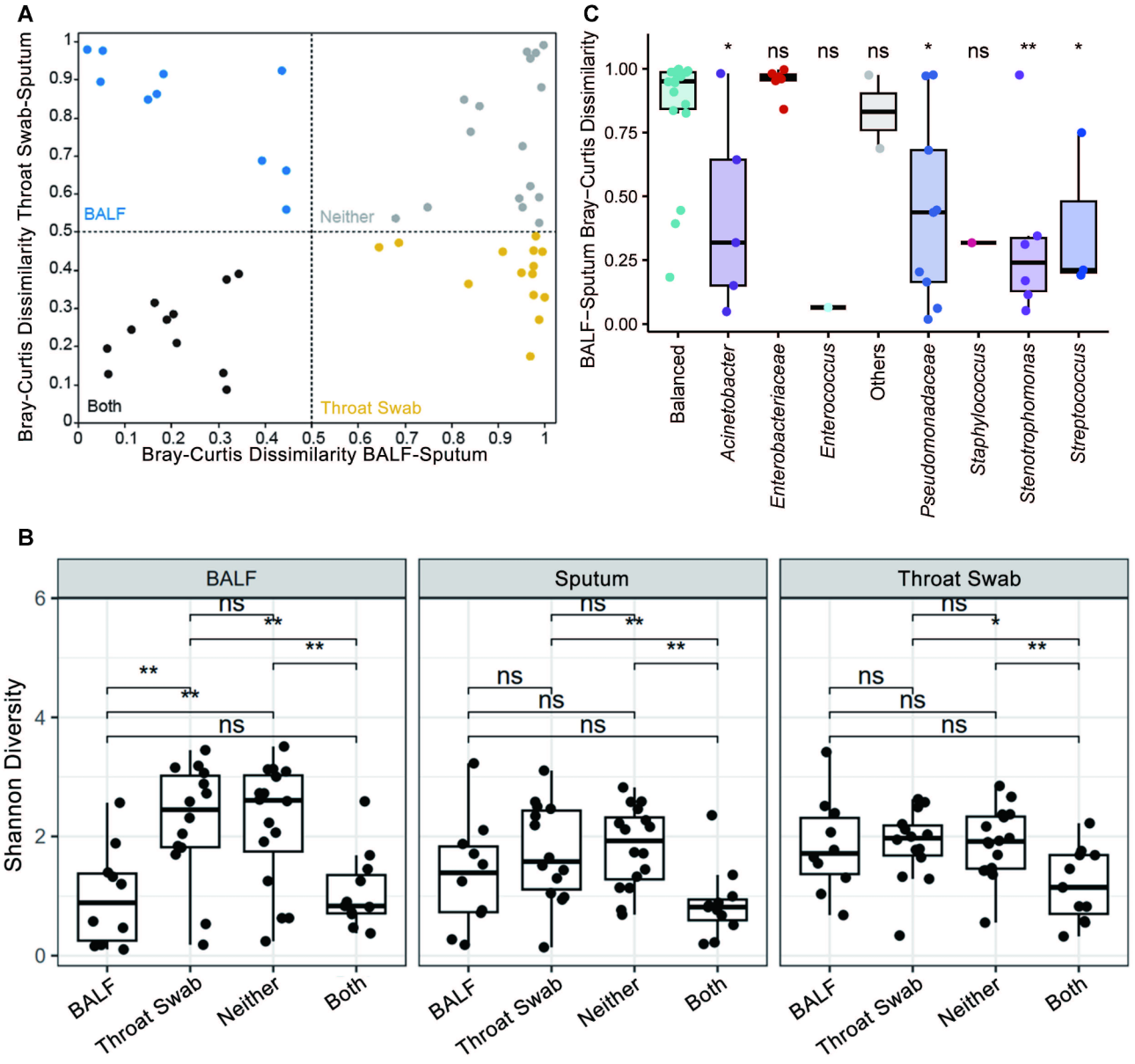

Fig. 5 Characterization of microbial similarity of sputum to throat swab and BALF. (**A**) Classification of sputum microbiota as "BALF", "Throat swab", "Both" and "Neither" based on their Bray-Curtis dissimilarity to throat swab and to BALF. (**B**) Shannon diversity of each group as defined in panel A. (**C**) Comparison of the BALF-sputum Bray-Curtis dissimilarity between predominance microbiota groups and the balanced microbiota group. Two-sided Wilcoxon rank-sum test was used to compare the Shannon diversity between different groups. **: $P \leq 0.01$; *: $0.01 < P \leq 0.05$; ns: $P > 0.05$

suggesting that *Stenotrophomonas* may be the key taxon driving the similarity between sputum and upper and lower respiratory tract microbiota.

Discussion

In this prospective study, we characterized for the first time the dynamic changes in upper and lower respiratory tract microbiota of lung transplant recipients and identified factors associated with their compositional shifts.

We observed no significant differences in microbial diversity between BALF and lung tissue microbiota, with comparable genus composition between the two. However, marked differences emerged when comparing BALF to throat swab and sputum samples, the latter two exhibiting significantly reduced microbial diversity. Both upper and lower respiratory tract microbiota underwent rapid, significant remodeling within 6 months post-transplantation. Although divergent patterns of change were observed between upper and lower tract communities, they shared similar trends, with specific microbial taxa demonstrating parallel shifts. Regarding microbiota convergence, pre-operative samples showed pronounced dissimilarity between upper and lower tract microbiota, but post-operative longitudinal analysis revealed progressive

compositional harmonization. This convergence correlated strongly with reduced microbial diversity and predominance of single taxa. Additionally, sputum microbiota similarity to both upper and lower tract communities was similarly influenced by diversity loss and monopolization by individual species.

Obtaining lung tissue through transbronchial biopsy is the current gold standard for diagnosing pulmonary complications (including infections and allograft lung rejections) [18, 27]. Our findings demonstrated comparable microbial diversity and taxonomic composition between BALF and lung tissue microbiota, suggesting BALF could reliably substitute for lung tissue-based microbiota assessment. Generally, BALF is considered to be an ideal specimen for pathogen detection in the diagnosis of post-transplant complications. However, many recipients are unable or unwilling to undergo the bronchoscopy test as it is an invasive procedure and/or the bad clinical condition is not permit them to tolerate the test. Consequently, clinicians increasingly rely on non-invasive alternatives like throat swabs and sputum samples. Although studies in healthy adults suggest oropharyngeal microbiota seed the lung microbiota [1, 28], our data revealed stark compositional divergences across sample types: BALF was predominated by *Stenotrophomonas*, *Pseudomonadaceae*, *Acinetobacter*, and *Enterobacteriaceae*, whereas throat swab and sputum exhibited preferential enrichment of *Streptococcus* and *Staphylococcus*.

Consistent with the prior researches on lung transplantation, our study showed that throat swab and sputum displayed significantly reduced microbial diversity compared to BALF. Marie et al. [28]found that the microbial diversity of the respiratory part was significantly higher than that of the conductive part in lung transplant recipients. Sharma et al. [29] confirmed reduced oral microbiota diversity relative to BALF via 16 S rRNA profiling. Notably, while healthy populations based on literature typically exhibit attenuated lower respiratory tract microbial abundance compared to upper regions [23, 30, 31]. Our findings highlight distinct microbiota characteristics in transplant recipients, including compartment-specific diversity patterns and taxon enrichment.

Our longitudinal analysis revealed rapid, marked restructuring of both upper and lower respiratory tract microbiota within 6 months post-transplantation. Consistently, previous studies have suggested that there are complex dynamic changes in respiratory tract microbiota within one year after lung transplantation [32–34]. Early post-operative microbial dynamics appeared driven by several factors such as donor lung microbiota displacement, immunosuppression, and antibiotic exposure, collectively shaping diversity patterns and community composition. While upper and lower tract communities exhibited divergent trajectories, they shared temporal patterns of microbial succession. For example, *Corynebacterium* and *Staphylococcus* were most abundant in the early post-operative period and then gradually decreased. *Enterococcus*, *Ralstonia*, *Porphyromonas* and *Veillonella* transiently increased from 14 days to 2 months post-operatively. In the relatively stable period at 6 months post-operative, *Prevotella*, *Fusobacterium*, *Haemophilus*, *Actinomyces*, and *Neisseria*, which are common taxa in healthy populations, were the most common genera in the lower respiratory tract. The upper respiratory tract microbiota was most abundant in *Gemellaceae*, *Parvimonas*, *SR1*, *Catonella*, and *TM7-3*, which are common oral taxa. Our study results suggest that the detection of taxa common in healthy populations in the lower respiratory tract is a sign that the status of lung transplant recipients is tending towards stability. In addition, we found that some genera peaked later in BALF than in throat swab, such as *Streptococcus*, *Porphyromonas*, and *Stenotrophomonas*. However, *Pseudomonas* and *Ralstonia* peaked earlier in BALF than in throat swab. Previous studies have suggested that the recipient's upper respiratory tract microbiota can rapidly replace the original donor microbiota in the transplanted lung [32–34]. Our findings align with reports of rapid donor microbiota replacement by recipient upper tract communities, suggesting bidirectional microbiota crosstalk between transplanted lungs and native upper airways during immunological adaptation. Furthermore, our study suggests that the emergence of health-associated taxa in stabilized lower respiratory tract microbiota may serve as an ecological indicator of post-transplant recovery.

Previous studies have found spatial and temporal heterogeneity in the respiratory tract microbiota of lung transplant recipients, but few studies have explored the similarity and characteristics of airway microbiota at different sites in the respiratory tract post-operatively [28, 35]. We found that upper and lower respiratory tract microbiota differed significantly pre-operatively and in the early post-operative period, but the difference rapidly decreased thereafter, with the highest similarity at 6 months post-operative in this study. Further research found that lower diversity of upper and lower respiratory tract microbiota was associated with higher similarity. The upper and lower respiratory tract microbiota predominated by *Pseudomonadaceae*, *Staphylococcus*, and *Stenotrophomonas* had higher similarity. This suggests that when these genera are predominant, they are more likely to enter the lower respiratory tract, and at this time, throat swab can better reflect the microbiota of the lower respiratory tract. *Pseudomonas*, *Staphylococcus*, and *Stenotrophomonas* include pathogenic bacteria and are also common multidrug-resistant (MDR) bacteria following lung transplantation. Lung transplant recipients with MDR infections have a six-fold higher

in-hospital mortality rate compared to non-MDR lung transplant control groups [36, 37]. Therefore, early identification and treatment are of great significance for improving recipient prognosis. Our study suggests that when the microbiota of lung transplant recipients was predominated by taxa containing these pathogens such as MDR bacteria, the upper airway microbial information obtained through throat swab can reflect the lower airway microbiota detected by bronchoscopy, which is conducive to rapid non-invasive pathogen detection sampling in clinical practice. The lung microbiota of lung transplant recipients in the early postoperative period is influenced by numerous factors, including (1) the use of various antimicrobial agents, such as antibacterial, antifungal, and antiviral drugs; (2) changes in the recipient's environment after transplantation, such as transitioning from the hospital to the home setting; (3) alterations in immune status due to the administration of immunosuppressive agents; and (4) interactions between the microbiota of the native lung and the transplanted lung in single-lung transplant recipients. These factors often interact with one another, resulting in complex clinical scenarios. Due to the limited sample size at each time point, it is challenging in this study to directly link the observed microbiota changes to specific clinical events. A larger sample size, combined with comprehensive documentation of all clinical characteristics, may enable future time-series statistical modeling to associate temporal microbiota changes with specific clinical events.

The upper and lower respiratory tracts are anatomically continuous, and the sputum collection site is located between the upper and lower respiratory tracts. The microbiota of sputum may have some characteristics of both upper and lower respiratory tract microbiota. At the same time, like throat swab, the effectiveness of sputum for pathogen detection is also controversial [38]. Currently, the postoperative convergence patterns between upper and lower airway communities remain understudied in lung transplant recipients. Our study found that when the lungs are predominated by genera containing pathogens, the diversity of microbiota in BALF is lower, and the microbiota of sputum is more similar to that of BALF compared to throat swab. When pulmonary microbiota is balanced, sputum cannot reflect the microbiota status of the lower respiratory tract. Notably, communities predominated by *Pseudomonadaceae* and *Stenotrophomonas* exhibited heightened cross-compartment similarity, suggesting these taxa may preferentially colonize both niches. Our research results suggest that when BALF is not available for pathogen detection in the clinical practice, sputum is a better substitute for BALF than throat swab.

Limitations

This study provides the first longitudinal characterization of upper and lower respiratory tract microbiota in lung transplant recipients during the critical 6-month post-operative window. Three key limitations merit discussion: (1) As an exploratory single-center study, our cohort size limits generalizability to broader populations. Paired sample acquisition was further challenged by heterogeneous recovery trajectories and clinical complexities. The small sample size limits the statistical power to resolve fine-scale temporal dynamics of airway microbiota, in particular in the subgroup analyses based on predominance profiles of the microbiota. (2) The 6-month observation window precludes analysis of long-term microbiota evolution, particularly beyond the acute post-transplant phase where chronic ecological shifts may emerge. (3) While 16 S rRNA gene amplicon sequencing can identify species-level taxa for certain taxonomic groups, particularly with the use of new algorithms [25], in general, sequencing one or a few hypervariable regions of the 16 S rRNA gene results in a relatively low proportion of sequences that can be confidently assigned to a species-level taxon [39]. Consequently, we adopted genus-level taxonomy to ensure analytical rigor, although future metagenomic sequencing could provide enhanced functional and strain-level insights. In addition, exclusive focus on bacterial communities neglects potential fungal/viral interactions critical to post-transplant ecology.

Conclusion

This study provides the first comparative analysis of diversity and compositional dynamics between upper and lower respiratory tract microbiota in lung transplant recipients. Longitudinal tracking revealed progressive convergence of upper and lower tract microbial communities over the 6-month post-operative period, with initial dissimilarity gradually transitioning toward ecological harmonization These findings establish sputum as a potential ecological proxy for monitoring cross-compartment microbiota dynamics in lung transplant recipients, particularly during phases of low diversity and pathogen predominance.

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

Supplementary Material 6

Supplementary Material 7

Supplementary Material 8

Supplementary Material 9

Supplementary Material 10

Supplementary Material 11

Supplementary Material 12

Supplementary Material 13: **Table S1**. Demographics and clinical characteristics of the study cohort. **Table S2**. Taxonomic profiles of negative control samples. **Table S3**. Significantly differentiated ASVs ccross different sample types identified by LEfSe. **Table S4**. Clustering of common taxonomic groups found in both BALF and throat swab.

Author contributions
YHC, YCF, AC, CRJ, JL, and XZY conceived the study, directed the project, and designed the experiments; JQC and LLW participated in sample collection and performed experiments; YHC and AC interpreted the results, and wrote the manuscript; YCF, ZW, and XZY performed the microbial sequencing analysis; XHW, CRJ and JL assisted in resource acquisition and manuscript improvement. all authors read and approved the final manuscript.

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Data availability
The 16 S rRNA gene sequencing data have been deposited in the NCBI Sequence Read Archive database under accession number PRJNA1202372.

Declarations

Ethics approval and consent to participate
The Ethics Commission of the First Affiliated Hospital of Guangzhou Medical. University approved the study (No. 128 of 2020). All participants in the study signed informed consent forms. The study was conducted in accordance with the ethical standards established by the 1964 Declaration of Helsinki.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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