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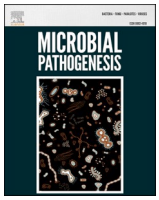

Distinct microbiome variation in children and adults following RSV infection and its association with host response

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ABSTRACT

Respiratory syncytial virus (RSV) hospitalization rates are higher in children than in adults, which may be related to differences in respiratory microbiota composition. The relationship between differences in the pharyngeal microbiome and the host immune response in adults and children infected with RSV remains unclear. This study aims to investigate changes in the microbiota of RSV-infected adult and pediatric patients receiving inpatient and outpatient care, and to explore their relationship with the host immune response. A total of 223 participants were enrolled in the study, including 30 adult RSV patients, 92 pediatric RSV patients, 51 community-acquired pneumonia (CAP) patients, and 50 healthy controls. Throat swabs were collected for 16S rRNA gene sequencing and transcriptome analysis. We found that the abundance of oral anaerobes (*Prevotella* and *Veillonella*) was higher in pediatric inpatients compared to pediatric outpatients. Differences in pharyngeal microbiome composition were observed between pediatric inpatients and outpatients, while not in adult patients. More differentially expressed genes were observed between pediatric inpatients and outpatients than in adults, primarily related to neutrophil chemotaxis and migration pathways. Furthermore, *Alphaproteobacteria* and *Actinobacteria* were positively correlated with the expression of CXCL10 and CXCL11 in pediatric inpatients, suggesting a potential link with neutrophil recruitment and inflammatory responses in these patients. Taken together, these findings improve our understanding of the associations between the host transcriptome and microbiome in the context of RSV infection, which may provide insights into factors related to the increased pathogenicity observed in children.

1. Introduction

Respiratory syncytial virus (RSV) is the leading cause of acute lower respiratory tract infections in both adults and children [1–3]. RSV infections commonly present as a mild cold in adults; however, children can experience severe bronchiolitis, contributing to higher hospitalization rates [4–6]. The factors contributing to different clinical symptoms to RSV include an immature immune system and interactions between RSV and the microbiota [7].

Following RSV infection of airway epithelial cells (AECs), pattern

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recognition receptors are activated, triggering downstream signaling pathways that drive the release of pro-inflammatory cytokines and chemokines, ultimately leading to neutrophil recruitment to the site of infection [8–11]. Notably, while neutrophil infiltration directly contributes to viral clearance, its overactivation can also cause tissue damage, correlating with clinical severity, such as hypoxemia and increased rates of pediatric intensive care unit admission [12–15]. The composition and function of the respiratory microbiome may regulate this process through immune signaling pathways. Previous studies have linked dysbiosis of the respiratory microbiome to disease severity during RSV infection, and have elucidated the role of Toll-like receptor (TLR) signaling and neutrophil/macrophage-mediated inflammation in this process [16,17]. However, it remains unclear whether microbiome-host interactions differ between adults and children following RSV infection.

The aim of this study was to investigate the characteristics of the microbiome and transcriptome in RSV patients, and to explore the relationship and potential pathways of interaction between them. We used 16S rRNA gene sequencing to characterize the composition and function of the pharyngeal microbiota, while transcriptome sequencing data were used to identify differentially expressed genes (DEGs), hub genes, functional enrichment pathways, and immune infiltration patterns. Ultimately, we found that RSV significantly altered both pharyngeal microbiota structure and transcriptional profiles in children, primarily associated with the involvement of chemokines and granulocytes.

2. Materials and methods

2.1. Study population and sample collection

Patients and healthy controls (HC) were recruited from the China-Japan Friendship Hospital and Zibo Municipal Hospital between November 2019 and June 2023. Community-acquired pneumonia (CAP) was diagnosed according to the guidelines of the Infectious Diseases Society of America and the American Thoracic Society [18]. A total of 223 throat swabs were collected, including samples from 30 adult RSV patients, 92 pediatric RSV patients, 51 CAP patients, and 50 HC. Two children were excluded from sequencing due to insufficient sample volume. All samples underwent 16S rRNA gene sequencing. Transcriptome sequencing was performed on samples from 76 children (62 inpatients and 14 outpatients) and 26 adults (16 inpatients and 10 outpatients) with sufficient RNA. RSV infection was diagnosed using the SureX 13 Respiratory Pathogen Multiplex Detection Kit (Cat. No. 1060099, Ningbo Health Gene Technology), the Xpert® Xpress Flu/RSV tests (Cepheid), and rapid antigen tests for RSV (Hangzhou Genesis Biodetection & Biocontrol Co., Ltd). The remaining throat swabs were stored in viral transport medium, aliquoted, and kept at −80 °C until further analysis. All electronic medical records for the study were collected from the participants. The baseline characteristics are summarized in Supplementary Table 1-3. This study was conducted in accordance with the principles of the Declaration of Helsinki and was approved by the Medical Ethics Committees of both the China-Japan Friendship Hospital (Beijing, China) and Zibo Municipal Hospital (Zibo, China). Ethical approval was obtained on May 30, 2022, under reference number 2022-KY-052.

2.2. Microbiome and transcriptome sequencing

DNA was extracted from all throat swabs using the MP Biomedicals MagBeads FastDNA™ Kit for Soil (MP Biomedicals). The quantity and quality were then assessed using a NanoDrop NC2000 spectrophotometer (Thermo Fisher) and agarose gel electrophoresis, respectively. PCR amplification of the bacterial 16S rRNA genes V3–V4 region was performed using the forward primer 338F (5′-ACTCCTACGGGAGGCAGCA-3′) and the reverse primer 806R (5′-GGACTACHVGGGTWTCTAAT-3′) and barcodes were incorporated into the primers for multiplex sequencing. The libraries were sequenced on an Illumina NovaSeq 6000 platform with pair-end length of 250bp. Thermal cycling consisted of initial denaturation at 98 °C for 3 min, followed by 25 cycles consisting of denaturation at 98 °C for 30 s, annealing at 52 °C for 30 s, and extension at 72 °C for 45 s, with a final extension of 5 min at 72 °C. PCR amplicons were purified using Vazyme VAHTSTM DNA Clean Beads (Vazyme, Nanjing, China) and quantified with the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA). Amplicons were then pooled in equimolar amounts, and pair-end 2 × 250 bp sequencing was performed on an Illumina NovaSeq 6000 platform. The median number of raw reads per sample was 76,346.5 (IQR: 68,493–101,972). Samples with fewer than 50,000 reads were excluded from downstream analyses.

RNA was extracted from all throat swabs using the TRIzol Reagent (Invitrogen, Life Technologies). mRNA library was sequenced on Illumina NovaSeq 6000 system (Illumina) of 150 bp paired-end reads.

2.3. Microbiome analysis

Statistical analysis and visualization were performed using R software (v.4.4.1). Raw amplicon sequencing was processed with the EasyAmplicon pipeline (v.1.21) [19]. Low-quality reads were filtered out using VSEARCH (v.2.15.2) [20], and chimeras were removed using USEARCH (v.10.0.240) for non-redundant sequences [21]. Amplicon sequence variants (ASVs) with a total abundance <10 were excluded, and UNOISE denoising incorporated two additional filtering steps in which ASVs with abundance <10 were removed. This conservative strategy helps minimize rare spurious variants that may result from contamination or sequencing errors. The Sintax algorithm was applied for annotation classification, utilizing the RDP database (rdp_16s_v18) [22].

Microbial diversity in the samples was assessed using alpha diversity, with the Chao1 and Shannon indices reflecting species richness and evenness. Beta diversity, which captures differences in microbial composition and distribution between samples, was evaluated through principal coordinate analysis (PCoA) based on Bray-Curtis distance to identify sample clustering. Adonis analysis with 999 permutations was used to identify statistically significant differences. Homogeneity of multivariate dispersion was tested using the betadisper function in the R package " vegan " (v. 2.6–8), with significance assessed by permutest. Microbial composition was normalized based on relative abundance and visualized using stacked bar charts. Linear discriminant analysis effect size (LEfSe) was employed to identify biomarkers and genomic signatures in high-dimensional data, revealing microbial taxa that differ significantly between groups. Linear discriminant analysis (LDA) effect sizes were used to identify ASVs with significant differences between groups. Differential abundant bacteria were the cover results across both LEfSe and STAMP analyses. Microbial differences between groups were also tested using MaAsLin2 (v. 1.16.0), adjusting for onset season, antibiotic exposure, and steroid use in pediatric RSV patients. For adult RSV patients, models were adjusted for antibiotic exposure. PICRUSt2 is designed to make functional predictions about microbial communities through an open-source platform (https://bioincloud.tech/) [23]. Differential analysis and visualization of the results from functional annotation of bacterial genes, based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, were performed using STAMP software to compare species differences [24].

2.4. Co-occurrence analysis

Co-occurrence networks for the microbiomes of pediatric inpatients and outpatients were constructed based on SparCC. Genera present in at least 80 % of samples were retained [25]. Network edges were estimated with SparCC with permutation tests. Significance was determined using p-values with Benjamini–Hochberg false discovery rate (FDR) correction, retaining only correlations with $|r| \geq 0.5$ and FDR ≤ 0.05. Network topology was reconstructed in igraph and visualized using Gephi

(v.0.10.1), where we calculated density, average degree, and modularity software [19,26,27].

2.5. Transcriptome analysis

Raw RNA-sequencing reads were quality filtered by Fastp (v. 0.21.0). These clean data were then mapped to the human genome (GRCh38) using Hisat2 (v. 2.2.1). Gene expression counts were generated using FeatureCounts (v. 1.6.3). The sequencing depth per sample was 6 Gb. The median number of raw counts per sample was 643,195.5 (IQR: 197,116–1,239,569.25). These raw counts were used as input for differential expression analysis in the R package "edgeR," (v. 4.4.0) within a generalized linear model (GLM) framework, with normalization performed by the TMM method. DEGs were identified using a fold change (FC) ≥ 2 and a FDR < 0.1. Gene Ontology (GO) and KEGG pathway analyses were performed using the R package "clusterProfiler" (v. 4.14.3) [28]. Screening criteria included a Benjamini-Hochberg method-adjusted p-value <0.05 and a q-value <0.1. Information on the protein interactions and predictions of DEGs was obtained using the Search Tool for the Retrieval of Interacting Genes (STRING) database (http://string-db.org/, v.12.0). Network visualization and identification of hub genes were performed using Cytoscape software (v. 3.7.2) along with the cytoHubba plugin (v. 0.1). The maximum clique centrality (MCC) algorithm was applied to evaluate and select hub genes. Correlation analyses between hub genes and differential bacteria, as identified through STAMP software and LEfSe analyses, were conducted using the R package "corrplot" (v. 0.95). CIBERSORT, a deconvolution algorithm based on gene expression data, was used to analyze 22 infiltrating immune cell types. The LM22 gene signature matrix, which contains data on infiltrating immune cells, was obtained from a previous publication [29]. The xCell algorithm was used to validate the immune infiltration analysis [30]. The Wilcoxon test was used to assess differences between groups.

2.6. Statistical analysis

Statistical analyses were conducted using IBM SPSS Statistics 25 software. Continuous data were analyzed using the Mann-Whitney *U* test, while categorical data were compared using the Chi-square test or Fisher's exact test. A two-sided p-value <0.05 was considered statistically significant. P-values were adjusted by the Benjamini-Hochberg method.

3. Results

3.1. Difference on pharyngeal microbiota of adult RSV patients compared with CAP patients and HC

The microbiota profiles of pharyngeal swabs from adults infected with RSV and HC were compared. Compared to HC, RSV patients showed significantly lower Chao1 and Shannon indices (Fig. 1A and B), indicating a downregulation of bacterial richness and homogeneity in RSV patients. For beta diversity, PCoA and Adonis analysis revealed significant differences and distances between the microbiota of the three groups (R2 = 0.237, p-value = 0.001). The result showed a clear separation of microbiota among the groups (Fig. 1C). Microbial composition at both the phylum and genus levels was further analyzed. At the phylum level, the dominant phyla in RSV patients were *Firmicutes* (32.9 % for RSV, 19.5 % for HC) and *Proteobacteria* (30.5 %, 27.1 %), followed by *Bacteroidetes* (13.4 %, 30.6 %) and *Actinobacteria* (18.3 %, 6.9 %) (Fig. 1D). At the genus level, there was a significant decrease in the relative abundance of oral anaerobes (*Prevotella* and *Veillonella*) and a significant increase in *Neobacillus* and *Desulfovibrio* in RSV patients compared to HC (Fig. 1E). LEfSe analysis showed that *Proteobacteria*, *Actinobacteria*, *Bacillales*, *Neobacillus*, *Desulfovibrio*, and *Micrococcales* were significantly enriched in RSV patients (Fig. 1F and G). The results of the MaAsLin2 analyses were consistent with the trends described above (Supplementary Fig. 1A). In summary, RSV patients exhibited a lower abundance of oral anaerobes (*Prevotella* and *Veillonella*) and a higher abundance of other bacteria compared to HC.

Compared to CAP patients, RSV patients had lower Chao1 and Shannon indices (Fig. 1A and B), indicating that the diversity of pharyngeal microorganisms was even lower in RSV patients than in CAP patients. At the genus level, RSV patients exhibited a decrease in the relative abundance of *Prevotella* and *Veillonella* and an increase in the relative abundance of *Neobacillus* and *Desulfovibrio* compared to CAP patients (Supplementary Fig. 1B). Thus, RSV patients exhibited a greater reduction in oral anaerobes (*Prevotella* and *Veillonella*) and a higher increase in other bacteria compared to CAP patients.

3.2. Difference on pharyngeal microbiome between pediatric inpatients and outpatients but not in adult patients with RSV

We observed pharyngeal microbiome dysbiosis in adult RSV patients. To explore the clinical significance of this finding, we investigated the microbiome composition in adult and pediatric RSV patients, categorizing them by inpatient and outpatient status.

No significant differences in alpha diversity and microbial composition were observed between adult inpatients and outpatients, based on PCoA and differential bacterial abundance analyses (LEfSe, STAMP,and MaAsLin2) (Supplementary Fig. 2A-E, I). However, we observed significant differences in the pharyngeal microbiome between pediatric inpatients and outpatients.

Both PCoA and Adonis analyses demonstrated a distinct separation between pediatric inpatients and outpatients (R2 = 0.035, p-value = 0.019) (Fig. 2A). However, the small effect size (low R^2 value) suggests that the overall difference in community composition was limited. This could be explained by the limited sample size. The major phyla in pediatric inpatient were *Firmicutes* (32.2 % for inpatient, 26.7 % for outpatient) and *Proteobacteria* (27.6 %, 30.4 %), followed by *Bacteroidetes* (19.3 %, 10.2 %) and *Actinobacteria* (13.8 %, 20.5 %) (Fig. 2B, Supplementary Fig. 2H). At the genus level, the relative abundance of *Prevotella* and *Veillonella* was higher in pediatric inpatients than in pediatric outpatients. Differential abundance testing using STAMP revealed that pediatric inpatients, compared to outpatients, had a higher relative abundance of *Haemophilus* and *Actinomyces* (genera containing some opportunistic pathogens) and a lower relative abundance of *Sphingomonas* (Fig. 2B). LEfSe analyses revealed that *Veillonella*, and *Prevotella* were significantly enriched in pediatric inpatients (Fig. 2C and D). After multivariable adjustment in the MaAsLin2 analyses, this finding was further corroborated (Supplementary Fig. 2J). In summary, while there was no difference in the pharyngeal microbiota composition between adult inpatients and outpatients, pediatric inpatients showed an increased abundance of oral anaerobes and a decreased abundance of opportunistic pathogens compared to pediatric outpatients.

3.3. Significant differences of microbial interaction between pediatric inpatients and outpatients

Due to the differences in pharyngeal microbiota between pediatric inpatients and outpatients, we further analyzed the difference in their co-occurrence networks. Microbiome co-occurrence networks were constructed for both pediatric inpatients and outpatients (Fig. 3A and B). Compared with pediatric outpatients, the network of pediatric inpatients exhibited markedly reduced density (0.464 vs. 0.776) and average degree (61.7 vs. 92.4), indicating a loss of overall connectivity among genera. Conversely, modularity increased in pediatric inpatients (0.15 vs. 0.03), suggesting that the community became more segregated into distinct modules. While the network of pediatric outpatients was dominated by positive correlations, reflecting extensive cooperative associations, the network of pediatric inpatients showed a higher proportion of negative correlations, consistent with increased competition

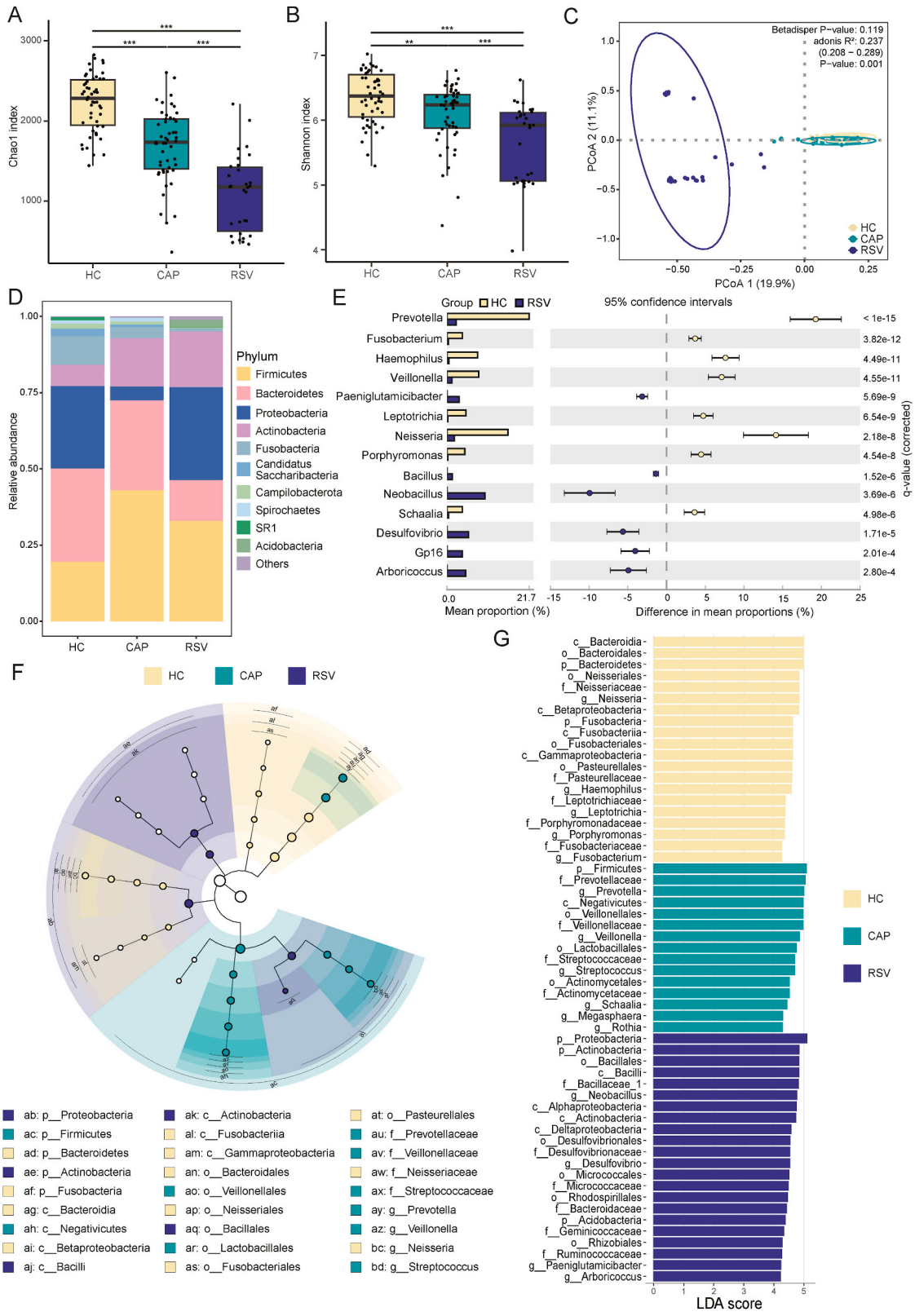

Fig. 1. The pharyngeal microbiota of adult patients. (A) Chao1 index (alpha diversity indices) among RSV patients (n = 30), CAP patients (n = 51), and HC (n = 50). (B) Shannon index (alpha diversity indices) among RSV patients, CAP patients, and HC. (C) PCoA based on the Bray–Curtis distance. (D) Phylum-level relative abundance profiles of RSV patients, CAP patients, and HC. Bar plots show the relative abundance over time of the 10 most abundant phyla in each ecosystem. (E) Differential abundant genera between RSV patients and HC. (F) Lefse analysis of differential taxa among RSV patients, CAP patients, and HC. Yellow nodes indicate enrichment in the HC; green nodes indicate enrichment in the CAP patients; blue nodes indicate enrichment in the RSV patients; white nodes indicate no significant differences. (G) The LDA scores of different taxa. Only taxa with an LDA score >4.2 are shown.

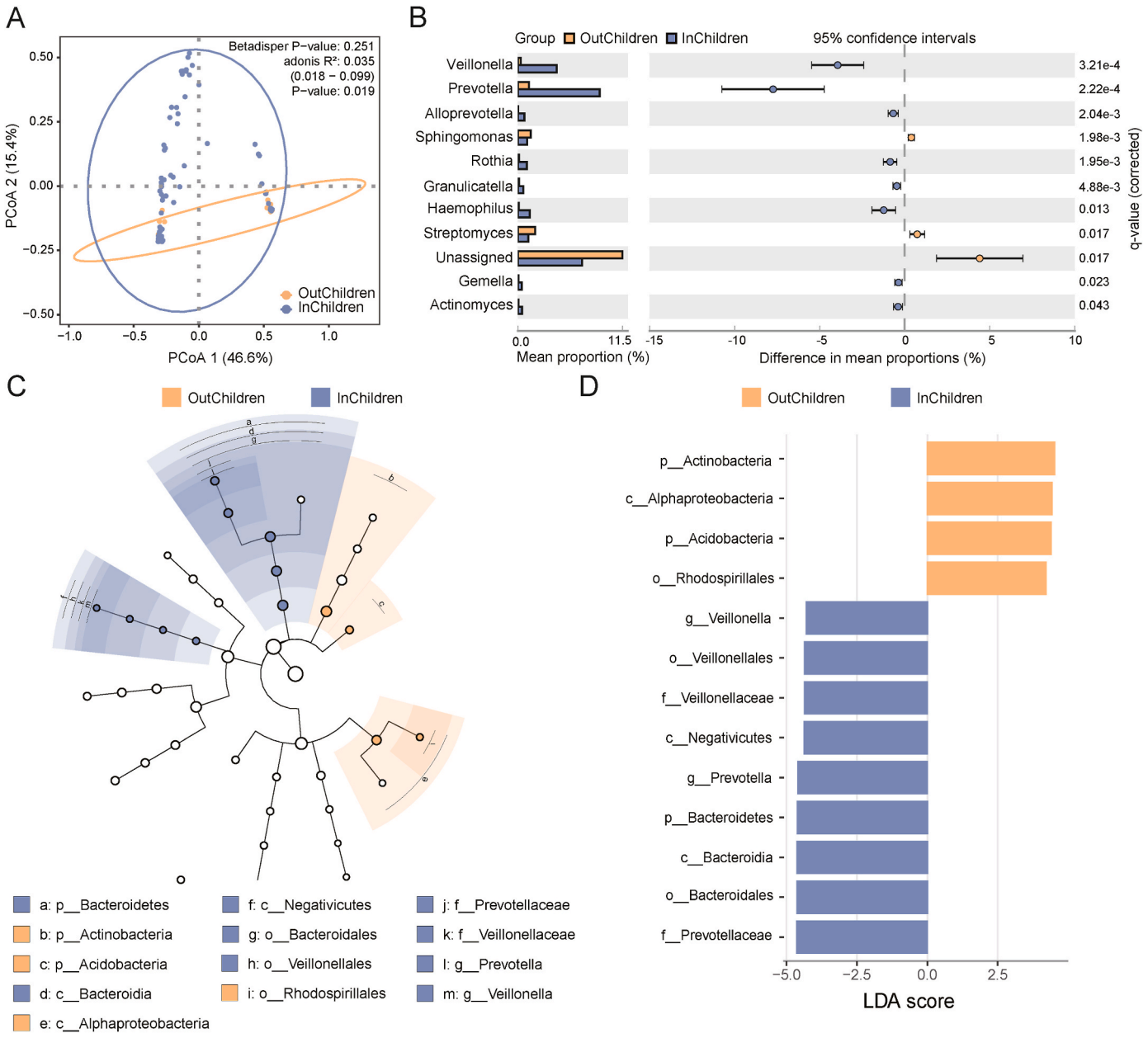

Fig. 2. The pharyngeal microbiota of pediatric patients infected with RSV. (A) PCoA based on the Bray–Curtis distance. (B) Differential abundant genera between pediatric inpatients and outpatients. (C) Lefse analysis of differential abundant taxa between pediatric inpatients and outpatients. Yellow nodes indicate enrichment in the pediatric outpatients; blue nodes indicate enrichment in the pediatric inpatients; white nodes indicate non-significant differences. (D) The LDA scores of differential abundant taxa. Only taxa with an LDA score >4.2 are shown.

or niche exclusion. Together, these changes suggest that the microbial community in pediatric inpatients is less cohesive and more fragmented, potentially reflecting reduced ecological stability under disease pressure.

3.4. *Microbiome function analysis*

Different microbial taxa were associated with various functional genes. Functional predictions based on the KEGG database using PICRUSt2 revealed that the microbiomes of adult patients infected with RSV were significantly less enriched in pathways related to energy metabolism and nutrient metabolism compared to HC, such as metabolism of cofactors and vitamins (Fig. 4A). Additionally, the microbiomes of adult patients were significantly less enriched in pathways related to antimicrobial resistance, but more enriched in pathways related to lipid metabolism, amino acid metabolism, and carbohydrate metabolism (Fig. 4A). These findings suggest differences in predicted metabolic functions of the microbiome associated with RSV infection.

Microbiome function analysis was also conducted between pediatric inpatients and outpatients, but not in adult patient subgroups due to the lack of differential abundant bacteria. The microbiomes of pediatric inpatients were significantly more enriched in pathways related to replication and repair and antimicrobial resistance compared to pediatric outpatients (Fig. 4B). Pediatric inpatients, who likely received more antibiotic treatments, showed more functional replication and repair pathways (Fig. 4B).

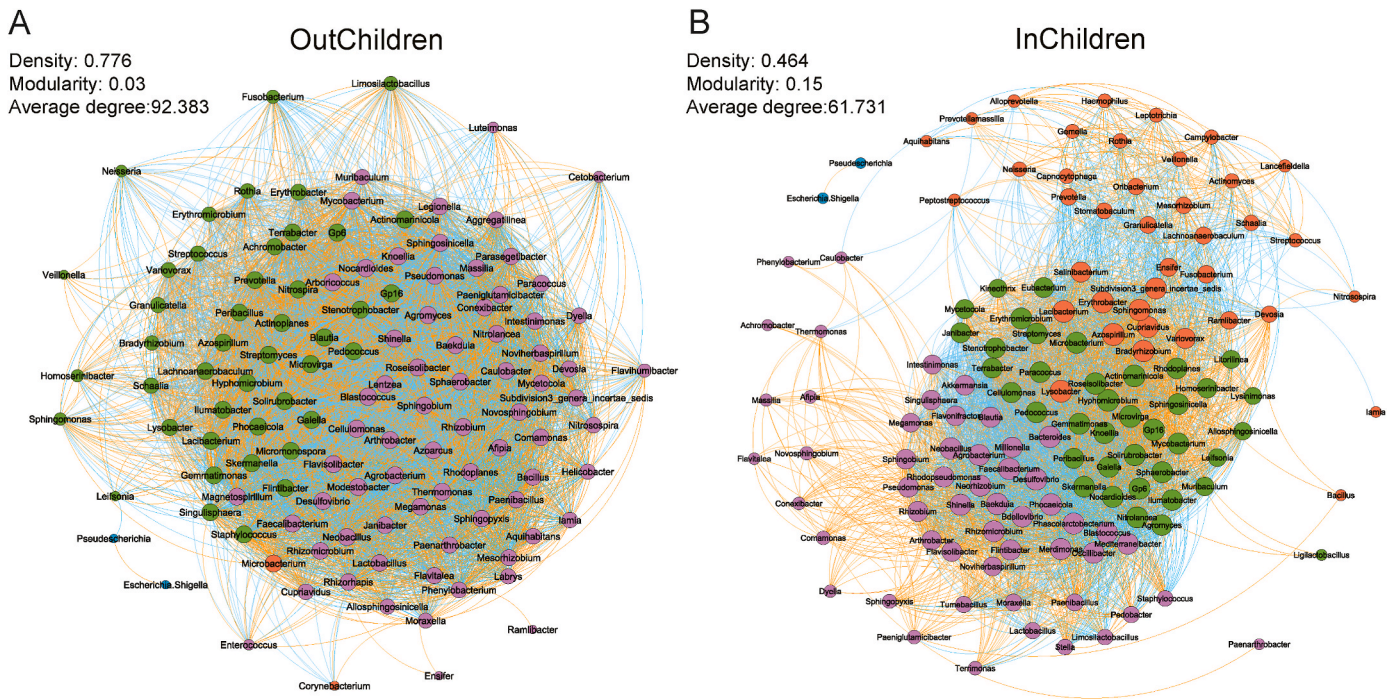

Fig. 3. Co-occurrence network at the genus level. (A) Co-occurrence network for pediatric outpatients. (B) Co-occurrence network for pediatric inpatients. Nodes represent bacterial genera, and edges represent the statistically significant associations between nodes. Yellow edges indicate positive correlations, while blue edges indicate negative correlations.

3.5. The differences in host responses between inpatients and outpatients are notably distinct in both children and adults

Based on the finding that the composition of the pharyngeal microbiota differed between the children's subgroups but not between the adult subgroups, we hypothesized that the host response might associate with this phenomenon. Consequently, we compared the differences in host responses between inpatients and outpatients in both children and adults.

In adult inpatients, 41 DEGs were upregulated and 21 DEGs were downregulated compared to adult outpatients. These DEGs were predominantly enriched in immune- and inflammation-related pathways, as indicated by the red symbols in Fig. 5A. Fig. 5B presented all the DEGs (IL36G, IL36RN, IL23A, IL36A, RNASE7, SPRR2A, and S100A7), which were associated with immune and inflammation-related pathways based on previous studies. Functionally, IL36G, IL36A, IL23A, and S100A7 mainly exerted pro-inflammatory effects, while IL36RN and RNASE7 had anti-inflammatory effects. SPRR2A, which was highly expressed in AECs, played a role in maintaining epithelial barrier integrity, resisting pathogen infection, and reducing inflammation and tissue damage. This suggested that the immune response in adult patients might be homeostatic.

Compared to adult patients, there were more DEGs between pediatric inpatients and outpatients. A total of 921 DEGs were identified, with 344 up-regulated and 577 down-regulated DEGs in pediatric inpatients compared to pediatric outpatients. These DEGs were more significantly enriched in immune and inflammation-related pathways compared to adult patients, particularly in pathways associated with neutrophil and granulocyte chemotaxis and migration (Fig. 5C). All DEGs and their associated pathways were presented in Fig. 5D. Hub genes were identified based on protein-protein interactions of DEGs and MCC scores. The top 10 hub genes were CXCL10, CCL4, IL1B, CXCR2, CCR1, CXCR1, CXCL5, CXCL11, CXCL16, and IL1RN (Fig. 5E). After GLM adjustment, the hub genes that remained significantly different were CXCL5, CXCL11, CXCL10, IL1RN, CXCL16, and CCL4 (Supplementary Table 4). These genes were chemokines, chemokine receptors, a pro-inflammatory cytokine, and an anti-inflammatory cytokine. Inflammation-suppressing genes were less common in pediatric patients than in adults. These chemokines were primarily involved in the chemotaxis and activation of immune cells. Therefore, DEGs between pediatric inpatients and outpatients were enriched in pathways related to chemokines and immune cells.

3.6. RSV significantly altered the pharyngeal microbiota structure and transcriptional profiles in children through the involvement of chemokines and neutrophils

Based on the observation that pediatric patients exhibited more activated anti-infective immune pathways and stronger host responses compared to adults, we hypothesized that significant alterations in the pharyngeal microbiome interact with host regulation of transcripts. Differential abundant bacteria were the cover results across both LEfSe, STAMP, and MaAsLin2 analyses (Fig. 1D–G, Fig. 2B–D, Supplementary Fig. 1A, Supplementary Fig. 2J). A Spearman correlation analysis was conducted to assess the relationship between hub genes and the differential bacteria in pediatric patients (Fig. 6A). CXCL10 and CXCL11 exhibited a negative correlation with oral anaerobes, specifically. The correlation coefficients were −0.32 for CXCL10 and *Prevotella*, −0.38 for CXCL10 and *Veillonella*, −0.49 for CXCL11 and *Prevotella*, and −0.51 for CXCL11 and *Veillonella*. However, CXCL10 and CXCL11 were positively correlated with microbes containing many bacterial pathogens, such as *Alphaproteobacteria*. The correlation coefficients were 0.32 for CXCL10 and *Alphaproteobacteria*, 0.23 for CXCL10 and *Actinobacteria*, 0.38 for CXCL11 and *Alphaproteobacteria*, and 0.28 for CXCL11 and *Actinobacteria*. Some common pathogenic bacteria and opportunistic pathogens were among the differential bacteria mentioned above, such as *Sphingomonas* in *Alphaproteobacteria*, and *Actinomyces* in *Actinobacteria*. CIBERSORT analysis showed a higher percentage of granulocytes in pediatric inpatients compared to the pediatric outpatients, such as neutrophils and mast cells (Fig. 6B). No significant differences were observed between adult inpatients and outpatients (Fig. 6C). Meanwhile, we obtained consistent neutrophil results with xCell

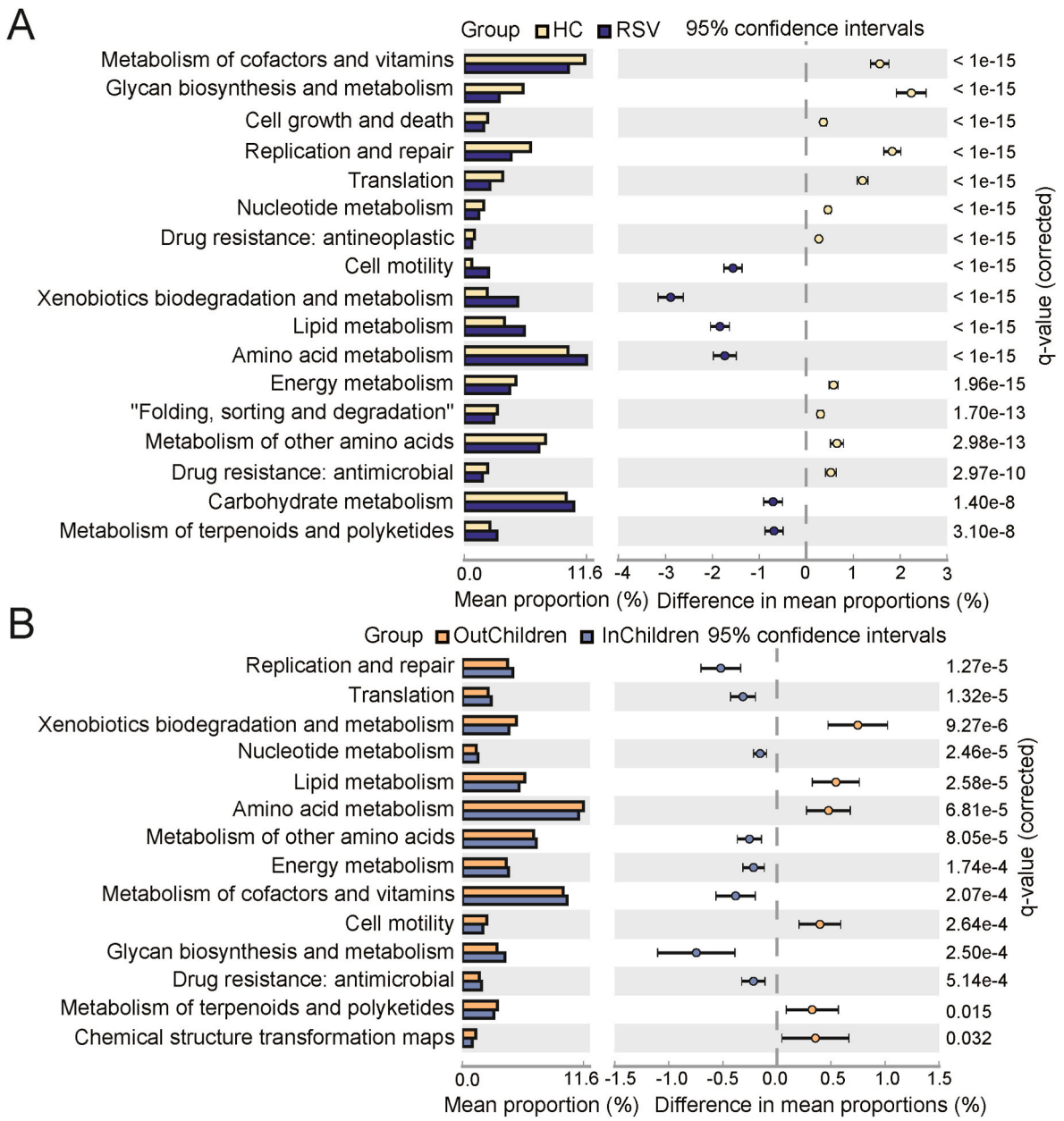

Fig. 4. PICRUST2 functional predictive analyses based on the KEGG database. (A) Pathways (L2) with significant differences between HC and RSV. (B) Pathways (L2) with significant differences between pediatric inpatients and outpatients.

(Supplementary Fig. 3A and B). In summary, these results suggested that *Alphaproteobacteria* and *Actinobacteria* were positively correlated with the expression of CXCL10 and CXCL11 in pediatric inpatients, which may have contributed to the recruitment of more neutrophils, leading to a more severe inflammatory response in children with RSV.

4. Discussion

The respiratory microbiota plays a critical role in the development and progression of respiratory infections, and alterations in its composition and function can directly impact host immune responses and disease progression [31,32].In this study, we characterized the dysbiosis of the pharyngeal microbiota in patients infected with RSV. Our findings revealed no significant differences in throat microbiota composition between adult inpatients and outpatients. However, a significant difference was observed between pediatric inpatients and outpatients, potentially attributed to a stronger host response and an increased presence of neutrophils in children.

In the present study, we observed that the pharyngeal microbiota of RSV patients was characterized by a decreased relative abundance of oral anaerobes compared to CAP patients and HC. A study found that *Haemophilus* was more frequently detected in nasopharyngeal swabs of hospitalized patients with RSV, and that a higher relative abundance of *Haemophilus* in the nasopharynx of children was associated with increased viral loads [33,34]. Compared to the controls, RSV-infected children had a higher relative abundance of *Actinomyces* and *Sphingomonas* in their gut microbiota [35,36]. However, there was no significant difference in the relative abundance of *Sphingomonas* between pediatric inpatients and pediatric outpatients. We hypothesize that the microbiota may vary across different stages of the disease and in different regions of the body. It has been reported that RSV viral loads in nasal wash samples are higher in outpatients than in inpatients [37]. RSV infection triggers inflammatory responses and cytokine production, creating an unfavorable respiratory microenvironment for oral

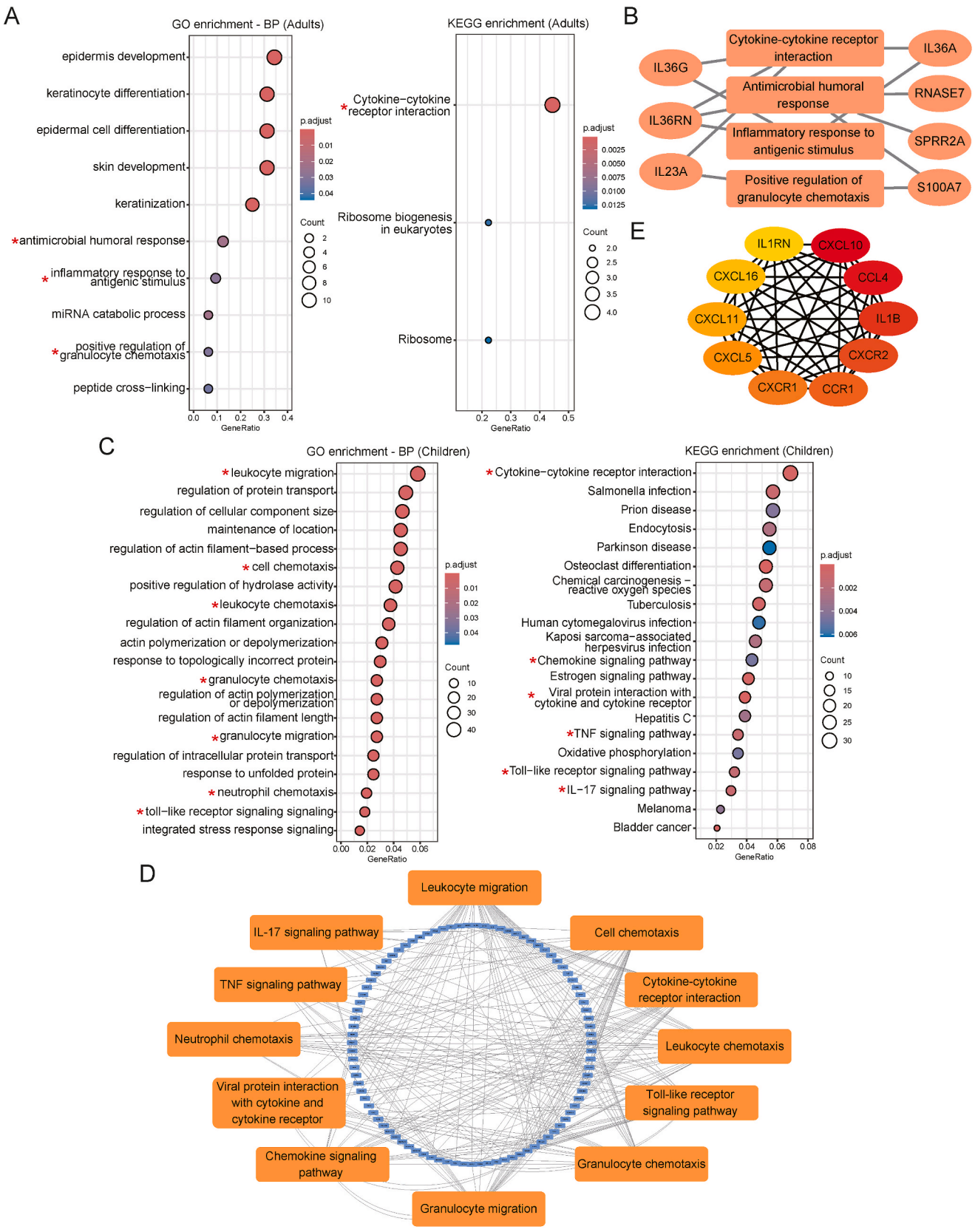

Fig. 5. Transcriptomes analyses. (A) The GO and KEGG functional enrichment analyses of DEGs between adult inpatients and outpatients. (B) Immune and inflammation-related pathways and genes in adults. (C) GO and KEGG functional enrichment analyses of DEGs between pediatric inpatients and outpatients. Immune- and inflammation-related pathways were marked by the red symbols. (D) Immune and inflammation-related pathways and genes in children. (E) Immune and inflammation-relate hub genes in children.

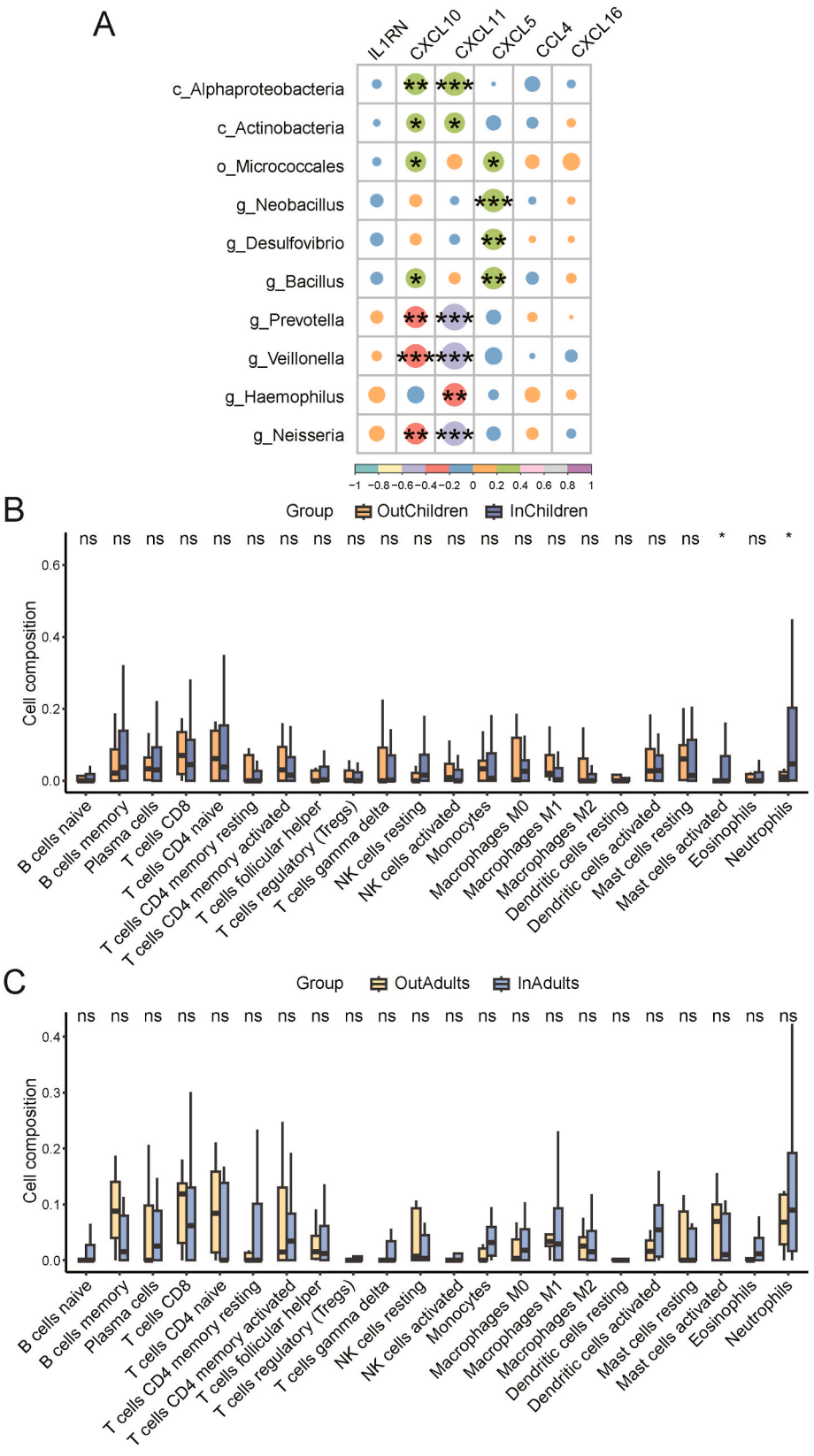

Fig. 6. Integrative analyses between microbiota and transcriptome analysis in pediatric patients. (A) Correlation analysis of immune-related hub DEGs with differential bacteria. (B) CIBERSORT analysis for children. (C) CIBERSORT analysis for adults.

anaerobes colonization and directly or indirectly inhibiting the growth of oral anaerobes. This may provide a plausible explanation for our finding of decreased abundance of *Prevotella* and *Veillonella* in pediatric outpatients compared to pediatric inpatients.

The microbiome acts as a signaling hub, linking environmental factors to the immune system and ultimately influencing the host's immunity and response to infection [38]. In our study, there were no significant differences in the pharyngeal microbiota composition or granulocyte percentages between adult inpatients and outpatients. Consistent with previous reports that the airway microbiome is not linked to RSV susceptibility in adults [39]. DEGs between adult inpatients and outpatients were primarily involved in inflammatory regulation. Immune homeostasis was maintained through a balance of pro- and anti-inflammatory factors. We conclude that adults possess a more stable microbiota and immune system, enabling them to better maintain microbial homeostasis.

Considering the differences in pharyngeal microbiota composition between pediatric inpatients and outpatients, we analyzed the host response and microbiome in an integrated manner. It was revealed that microbial metabolites promote inflammation and upregulate neutrophil-related genes [40]. Increased neutrophils and inflammatory genes have been associated with more severe RSV infections, a finding that aligns with our results [41]. Neutrophils can help clear RSV-infected cells and reduce viral loads [42]. While activated neutrophils can function as antiviral agents, they can also be cytotoxic to host cells [43]. Thus, inappropriate activation of neutrophils following RSV infection can lead to heightened levels of inflammation.

Our results revealed a significant positive correlation between CXCL10 and CXCL11 and both *Alphaproteobacteria* and *Actinobacteria*, which encompass a variety of pathogens. It was reported that *Rickettsia*, belonging to *Alphaproteobacteria*, could induce CXCL10 gene expression [44]. *Mycobacterium bovis*, belonging to *Actinobacteria*, could also increase the level of CXCL10 in human epithelial cells [45]. These findings suggest that CXCL10 and CXCL11 may function as key immune mediators in host responses to diverse infectious diseases. Notably, CXCL10 gene transcription was RSV dependent [46]. Consistent with our findings, CXCL10 was down-regulated in nasal wash samples from pediatric inpatients compared to pediatric outpatients. Lower expression levels of CXCL10 were associated with higher rates of oxygen uptake and longer hospital stays [37]. CXCL10 expression patterns may be closely related to the microbial composition and metabolic activity. In pediatric outpatients, high viral loads reduced the abundance of oral anaerobes, relieving their suppression of CXCL10 and enhancing CXCL10-dependent dendritic cell and $CD8^+$ T cell antiviral activity [37,47,48]. In pediatric inpatients, the enrichment of pathogenic bacteria may suppress CXCL10 secretion via microbial metabolites, impairing viral clearance while simultaneously activating neutrophil-related genes and exacerbating inflammation [40]. Furthermore, the antibody-dependent upregulation of CXCL11 observed in pediatric outpatients may be related to a B cell response driven by high viral loads [46]. However, the specific mechanisms underlying this relationship remain to be elucidated.

However, there are some limitations to this study. First, 16S rRNA sequencing data could not specify species or strains. Future studies should use metagenomic sequencing to detect microbiome variations at a more detailed level. Second, metabolomics analyses were not performed, but these could be integrated into future research. Third, the sample size for RSV patients was relatively small, so larger sample sizes should be considered in subsequent studies. Fourth, healthy children were not included as a control group, and they should be recruited in future studies. Fifth, this study was a cross-sectional analysis, which could introduce reverse causality. Sixth, because LM22 is derived from peripheral blood immune cells, it may not fully capture the transcriptomic context of throat swab samples dominated by epithelial cells. Nevertheless, cross-validation with xCell yielded results consistent with CIBERSORT, indicating that our findings are not dependent on the choice of deconvolution method. Finally, no distinction was made between the acute and recovery phases of RSV infection, which could be a valuable area for further investigation.

5. Conclusion

RSV patients had a lower abundance of oral anaerobes compared to CAP patients and HC. Few differences were observed in the pharyngeal microbiota composition between adult outpatients and inpatients. Pediatric RSV patients showed greater pharyngeal dysbiosis and a stronger host response than adults, with inpatients having a reduced abundance of oral anaerobes and higher levels of neutrophils compared to pediatric outpatients. Our research indicates that the composition and metabolites of the pediatric pharyngeal microbiome are associated with chemokine-mediated recruitment of inflammatory cells. This study contributes to our understanding of the observed changes and associations between the host transcriptome and microbiome during disease, and may provide a basis for future mechanistic investigations and potential treatment strategies.

CRediT authorship contribution statement

Qi Wang: Writing – original draft, Visualization, Software, Methodology, Formal analysis, Data curation. **Yulin Zhang:** Writing – original draft, Resources, Investigation, Funding acquisition, Data curation. **Xiaoxuan Yao:** Writing – original draft, Validation, Software. **Chun Wang:** Writing – original draft, Validation, Software. **Shibin Chen:** Writing – original draft, Validation. **Bo Liu:** Writing – review & editing, Resources, Funding acquisition. **Lin Sun:** Writing – review & editing, Resources, Data curation. **Xiaohui Zou:** Writing – review & editing, Software, Methodology, Conceptualization. **Bin Cao:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Data

The raw 16S rRNA gene sequencing data have been deposited in the Genome Sequence Archive [49] in National Genomics Data Center [50], China National Center for Bioinformation/Beijing Institute of Genomics, Chinese Academy of Sciences (GSA-Human: HRA013133) that are publicly accessible at https://ngdc.cncb.ac.cn/gsa-human. Raw counts of transcriptome analysis and all analysis scripts used in this study are available at GitHub (https://github.com/QiWangWQ/RSV).

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micpath.2025.108075.

Abbreviations

RSV	Respiratory syncytial virus
AECs	Airway epithelial cells
TLR	Toll-like receptor
DEGs	Differentially expressed genes
HC	Healthy controls
CAP	Community-acquired pneumonia
ASVs	Amplicon sequence variants
PCoA	Principal coordinate analysis
LEfSe	Linear discriminant analysis effect size
LDA	Linear discriminant analysis
KEGG	Kyoto Encyclopedia of Genes and Genomes
GLM	Generalized linear model
FC	Fold change
FDR	False discovery rate
GO	Gene Ontology
STRING	Search Tool for the Retrieval of Interacting Genes
MCC	Maximum clique centrality

Data availability

I have shared the link to my data/code at the Attach File step.

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