Hypertension

ORIGINAL ARTICLE

Respiratory Microbiome Profile of Pediatric Pulmonary Hypertension Patients Associated With Congenital Heart Disease

Ting Wang,* Yue Xing,* Bingming Peng, Kai Yang, Chenting Zhang, Yuqin Chen, Gang Geng, Qubei Li, Jian Fu, Mi Li, Zhengxiu Luo, Zhou Fu, Jian Wang

BACKGROUND: Pulmonary hypertension (PH) associated with congenital heart disease (CHD) is the most common type of PH in pediatric patients. The airway microbiome profile in CHD-PH patients remains rarely studied.

METHODS: A total of 158 children were recruited for collection of oropharyngeal swabs to sequence the *16S* ribosomal RNA (*16S* rRNA) V3-V4 region of respiratory microbiome, to establish a correlation between these bacterial groups and echocardiography indicators in CHD-PH patients.

RESULTS: Bacterial α- and β-diversity of the airway microbiome indicated a significantly lower richness in the CHD-PH group and compositional differences associated with the specific taxa and their relative abundances in the upper respiratory tract. Principal coordinate analysis showed that the pharynx microbiota composition in the CHD-PH group varied from that in the CHD or control group. The linear discriminant analysis effect size also highlighted an increased presence of *Streptococcus* and *Rothia* in pediatric CHD-PH patients. Comparison of microbial composition between pediatric and adult PH patients showed significant differences and separation of microbiota. The correlation between bacterial abundance and transthoracic echocardiography indexes in CHD-associated PH indicated that different groups of microbiomes may be related to different PH grades.

CONCLUSIONS: In summary, our study reported the systematic definition and divergent profile of the upper respiratory tract microbiota in pediatric PH patients, CHD and reference subjects, as well as between pediatric and adult PH patients. **(*Hypertension*. 2023;80:214–226. DOI: 10.1161/HYPERTENSIONAHA.122.19182.)** • **Supplemental Material**

Key Words: *16s* rRNA ■ children ■ congenital heart disease ■ microbiota ■ pulmonary hypertension

Pulmonary hypertension (PH) is a clinical syndrome caused by a variety of etiologies, sharing similar pathologic characteristics of increased pulmonary arterial resistance and vascular remodeling.[1] It is defined by cardiac catheterization as the mean pulmonary arterial pressure >20 mmHg at rest in children, as the same in adults.[2,3] The most common type of PH diagnosed in pediatrics is PH associated with congenital heart disease (CHD).[3] CHD includes numerous kinds of birth anomalies of cardiovascular system and imposes significant impact on airway and pulmonary parenchymal function, which predisposes patients to the development of pulmonary complications. It has been estimated that ≈ 10% of children with CHD will eventually develop PH,[4] which is the result of a left-to-right shunt in most cases, with a 5-year survival reported to be 71%.[5] Early

Correspondence to: Jian Wang, State Key Laboratory of Respiratory Disease, The First Affiliated Hospital of Guangzhou Medical University, National Clinical Research Center for Respiratory Disease, Guangdong Key Laboratory of Vascular Disease Guangzhou 510030, Guangdong, China, Email jiw037@health.ucsd.edu or Zhou Fu, Department of Respiratory, Children's Hospital of Chongqing Medical University, National Clinical Research Center for Child Health and Disorders, Chongqing 400014, China, Email 360412157@qq.com

*T. Wang and Y. Xing contributed equally.

Supplemental Material is available at https://www.ahajournals.org/doi/suppl/10.1161/HYPERTENSIONAHA.122.19182

For Sources of Funding and Disclosures, see page 225.

NOVELTY AND RELEVANCE

What Is New?

The congenital heart disease-pulmonary hypertension (PH) microbiome was associated with remarkably reduced bacterial diversity and alterations in taxonomic composition. The enrichment of several genera in the upper respiratory tract was linked to PH severity.

What Is Relevant?

Changes in upper airway bacterial populations may influence the cardiovascular structure, which leads to changes in transthoracic echocardiography parameters in pediatric congenital heart disease-PH patients.

Clinical/Pathophysiological Implications?

The respiratory microbiome is significantly associated with the progression of PH in the development of congenital heart disease, and the distinctive signature of respiratory microbiome may be useful as a noninvasive and adjuvant biomarker to evaluate PH in congenital heart disease.

Nonstandard Abbreviations and Acronyms

CHD	congenital heart disease
PASP	pulmonary artery systolic pressure
PH	pulmonary hypertension
TTE	transthoracic echocardiography

identification and intervention of PH-associated CHD (CHD-PH) are desirable to achieve better clinical management of these patients.

Accumulating evidence has shown that the microbiome plays a crucial role in the maintenance of immune health, homeostasis, and the development of multisystem diseases.[6] Imbalance of microbial communities, also called dysbiosis, has been suggested as a potential contributor to pediatric lung diseases,[7] as well as cardiovascular and respiratory diseases such as systemic arterial hypertension, heart failure, asthma, and pulmonary hypertension.[8–10] A report showed that pediatric patients with CHD displayed significant gut dysbiosis and gut barrier dysfunction, which is worsened by cardiopulmonary bypass during surgery via increased proinflammatory bacteria, suggesting that the intestinal tract could mediate the migration from resident microbes to the microvasculature.[11] Interestingly, previous studies on patients or experimental animal models with PH have linked the altered host microbiota composition, at either the gastrointestinal tract[8,12,13] or respiratory tract,[14] to the development of pulmonary hypertension. We have previously described a distinct profile of the respiratory microbiome in patients with pulmonary hypertension versus donor controls, finding that a higher proportion of specific genera of *Streptococcus*, *Lautropia,* and *Ralstonia* distinguishes patients with PH.[14] This evidence strongly indicates that PH is a systemic disease that might be influenced by the host-microbiota interaction.

Dysbiosis is thought to play a pivotal role in development in both animals and humans. As a main component of the microbial community, the respiratory microbiota is also considered relevant in the morphogenesis of alveoli, lung development, barrier functions, and pulmonary vessels.[15–18] Gut dysbiosis has been identified in patients with CHD. However, there is a paucity of profiles identifying CHD-PH-specific changes in respiratory microbial communities. In this study, we hypothesize that the respiratory microbiota could be associated with the development of PH in children. Herein, we focused on a comprehensive comparison of the microbial composition among pediatric CHD and CHD-PH patients and controls, as well as between pediatric and adult PH patients, aiming to provide novel insights into the relationship between the host microbiome and PH pathogenesis in both pediatric and adult populations.

METHODS

Data Availability

The *16S* rRNA Sequence data associated with this project are available from the NCBI Sequence Read Archive (SRA) database with accession number PRJNA893086.

Recruitment of Pediatric Participants

A total of 158 participants aged under 14 years old were enrolled in this study, including 69 patients with an initial diagnosis of CHD-PH, 54 patients with CHD, and 35 reference subjects. The detailed baseline characteristics are listed in the Table 1. CHD patients with left-to-right shunt were diagnosed by transthoracic echocardiography (TTE), which was performed by an experienced sonographer. PH was defined as pulmonary artery systolic pressure (PASP) >35 mmHg.[19] PASP was estimated by Doppler TTE calculating the right ventricular to right atrial pressure gradient during systole. The simplified Bernoulli equation is used to assess velocity within the tricuspid regurgitation jet (PASP=4×[tricuspid regurgitation]^2+mean right atrial pressure).[20,21] Meanwhile, 35 healthy children with no respiratory symptoms, abnormalities on chest X-ray, or known lung diseases were recruited. Subjects who met any of the following criteria were excluded: (1) right-to-left shunt CHD and congenital cardiopulmonary vascular malformation; (2) any surgery history before diagnosis with CHD; (3) use of oral/nasal corticosteroids or antibiotics within 4 weeks before enrollment; (4) premature birth and test tube birth; (5) use of

Table 1. Personal and TTE Features of Participants

Descriptive measurements	CHD-PH (n=69)	CHD (n=54)	Control (n=35)	*P* Value
Sex, male (%)	30 (43.5%)	24 (44.4%)	22 (57.9%)	0.476
Age, mo; median (IQR)	12.0 (6.0–30.5)	17.0 (5.0–35.0)	16.5 (6.0–33.8)	0.846
Diagnosis				0.002
ASD	13 (18.8%)	17 (31.5%)		
VSD	18 (26.1%)	21 (38.9%)		
PDA	7 (10.1%)	9 (16.7%)		
ASD+VSD	20 (29.0%)	4 (7.4%)		
ASD+PDA	5 (7.2%)	1 (1.8%)		
VSD+PDA	4 (5.8%)	2 (3.7%)		
ASD+VSD+PDA	1 (1.5%)	0		
Scimitar syndrome	1 (1.5%)	0		
Delivery mode				0.88
Spontaneous labor	31 (45%)	25 (46.3%)		
Cesarean section	38 (55%)	29 (53.7%)		
TTE variables				
LA (long axis, mm)	19.45±4.37			
LVD, mm	32.92±6.57			
LVS, mm	20.67±4.22			
AO, mm	14.53±2.92			
DAO, mm	1.34±0.46			
AV, cm^2	1.14±0.39			
TV, cm^2	0.79±0.17			
MV, cm^2	1.19±0.28			
PV, cm^2	1.38±0.42			
MPAD, mm	18.36±3.66			
EF	0.68±0.05	0.68±0.04		0.811
FS	0.37±0.04	0.37±0.03		0.831
IRT, ms	56.71±8.77	58.90±7.74		0.159
RV (long axis, mm)	13.63±2.84			
RVOT, mm	16.98±3.20			
PAP, mmHg	62.45±17.84			

Values are median (interquartile range) or n (%) or mean±SD. Data were compared using the χ^2 test, or Student *t* test. ASD indicates atrial septal defect; DAO, descending aorta diameter; EF, ejection fraction; FS, fractional shortening; IQR, interquartile range; IRT, isovolumic relaxation phase; LA, left atrium; LVD, left ventricular end-diastolic; LVS, left ventricular end-systolic; MPAD, main pulmonary artery; MV, mitral valve area; PAP, pulmonary artery pressure; PDA, patent ductus arteriosus; RV, right ventricular; RVOT, right ventricular outflow tract; TV, tricuspid valve area; TTE, transthoracic echocardiography; and VSD, ventricle septal defect.

immunosuppressive medications; (6) oral or pulmonary infection 4 weeks before enrollment; and (7) known diseases associated with pulmonary hypertension, including autoimmune disease, liver and renal diseases, and hematologic disorders.

The study was approved by the ethics committee of Children's Hospital Affiliated to Chongqing Medical University and performed according to the Declaration of Helsinki. All parents or legal guardians of pediatric participants provided written informed consent.

Collection of Oropharyngeal Swabs

Oropharyngeal swabs were collected on the same day as TTE was performed for patients by inserting the sterilized cotton swabs toward the pharynx, and then the swabs were rotated at least 3× on the back wall of the pharynx for 5 seconds, avoiding contact with the tongue, buccal mucosa, and saliva swab. Then, the head swab was immediately cut off and placed into a sterilized cryopreservation tube. The specimens were stored at −80 °C for further processing within 2 weeks.

DNA Extraction and Sequencing of *16S* rRNA

Swabs were collected in a sterilized cryopreservation tube, snap-frozen in liquid nitrogen, and stored at −80 °C. Microbial DNA was extracted using a DNA isolation kit following the manufacturer's instructions (Qiagen, Redwood City, CA) and quantified for concentration by 1.0% agarose gel electrophoresis and a NanoDrop ND-2000 spectrophotometer (Thermo Scientific, Inc). Then, PCR amplification was performed on an ABI GeneAmp 9700 PCR thermocycler (ABI, CA, United States) using the hypervariable region V3-V4 of the bacterial 16S rRNA gene

with primer pairs 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). The amplification protocol was as follows: predenaturation at 95 °C for 3 minutes, followed by 29 cycles of 30 s at 95 °C, 30 s at 53 °C, and extension at 72 °C for 45 s, with a final 10 min at 72 °C and stabilization at 4 °C. The PCR product was purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA) to remove the nonspecific products and quantified using a Quantus Fluorometer (Promega). Purified 16S rRNA amplicons were pooled in equimolar amounts and paired-end sequenced on an Illumina MiSeq PE300 platform (Illumina, San Diego) according to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China).

Respiratory Microbiome Data Availability of Adult PH Patients

Oropharyngeal swab samples of adult PH patients were previously collected by our previous study.[14] The composition and characteristics of the airway microbiome in adult PH patients were previously described.[14]

Analysis of Respiratory Microbiome Sequencing Data

Raw FASTQ files were de-multiplexed using an in-house perl script, and then quality-filtered by fastp version 0.19.6[22] and merged by FLASH version 1.2.7.[23] The sequences were filtered and clustered into operational taxonomic units using UPARSE 7.1 with 97% similarity. The taxonomy of each operational taxonomic unit representative sequence was analyzed by RDP Classifier version 2.2 against the *16S* rRNA gene database (Silva v138, http://www.arb-silva.de) using confidence threshold of 70%.

Statistical Analysis

Based on the operational taxonomic units, the α-diversity was measured with the Chao, Ace, Sobs, Shannon, Simpson, and Invsimpson indexes. The results were displayed in Mothur software (version v.1.30.1). To compare the bacterial richness and diversity across samples, the β-diversity was estimated by computing the Bray-Curtis and visualized using principal coordinate analysis, and the results were plotted using Vegan v2.5-3 package. The linear discriminant analysis effect size (http://huttenhower.sph.harvard.edu/LEfSe) was performed to identify the significantly abundant taxa of bacteria among the different groups, and linear discriminant analysis scores >3.0 ($P<0.05$) are displayed for taxonomy. The variance inflation factor (VIF) for each TTE variable was estimated using the vif function in the car package (https://cran.r-project.org/web/packages/car/car.pdf) to eliminate multicollinearity problem among the TTE parameters. Correlations between the microbiota and TTE parameters were established using Pearson correlation analysis. An OUT subset based on the key associated genera from linear discriminant analysis effect size in the respective groups was established. Receiver operating characteristic curve was conducted to evaluate the discriminatory ability of the OUT subset, and area under curve was calculated using plotROC R package. The functional profiles from the *16S* rRNA sequence were predicted by Phylogenetic Investigation of Communities in the form of III Kyoto Encyclopedia of Genes and Genomes database pathway, which was visualized by STAMP software. Comparisons between 2 groups were evaluated by student's *t* test or wilcoxon rank-sum test follow by Benjamini-Hochberg false discovery rate (FDR) correction. Comparisons among 3 groups were evaluated by 1-way ANOVA followed by Tukey multiple comparison test. The χ^2 test was used for categorical data. The data were graphically plotted using GraphPad Prism 8.0. $P<0.05$ was considered statistically significant.

RESULTS

Characteristics of Participants

According to the inclusion and exclusion criteria, oropharyngeal swab samples were collected from 54 patients with CHD, 69 patients with CHD-PH, and 35 healthy subjects. As seen in Table 1, there were no significant differences in sex or delivery mode among the CHD, CHD-PH and control groups. The age and diagnosis differed between CHD and CHD-PH patients.

Dysbiosis of the Airway Microbiome in CHD Patients With or Without PH

Sequencing of *16S* rRNA amplicon libraries generated using microbial DNA resulted in a total of 8.04 million high-quality sequences and a mean of 49955±17190 sequences per sample. As shown in Figure 1A and 1B, CHD showed no significant difference of community diversity compared with the control. The Ace, Chao, and Sobs indexes revealed that community richness was significantly decreased in CHD-PH patients compared with CHD patients and control group. Meanwhile, significantly lower Shannon indexes and increased Simpson indexes in the CHD-PH group, which collectively indicating a decreased community evenness, displayed a strong decrease in α-diversity (Figure 1B). The Invsimpson index slightly decreased in the CHD-PH group with no significance.

To analyze the β-diversity, we performed principal coordinate analysis and partial least squares discriminant analysis. Principal coordinate analysis based on the Bray-Curtis dissimilarity showed a significant difference with respect to the overall phylogenetic distance of airway microbiome composition among the CHD, CHD-PH, and control groups (Figure 1C, PERMANOVA, $P<0.05$). Similarly, partial least squares discriminant analysis represented a distinct microbiome profile of the 3 groups (Figure 1D). Overall, the structures of the bacterial communities of the 3 groups were significantly different, and samples could be grouped according to the categories.

Landscape of Microbiota Composition in the Upper Respiratory Tract in Health and Disease

The differentially enriched bacterial taxa of each cohort are shown in Figure S1A. In the CHD-PH group, the analysis at the phylum level showed that Firmicutes was the most abundant phylum, followed

ORIGINAL ARTICLE

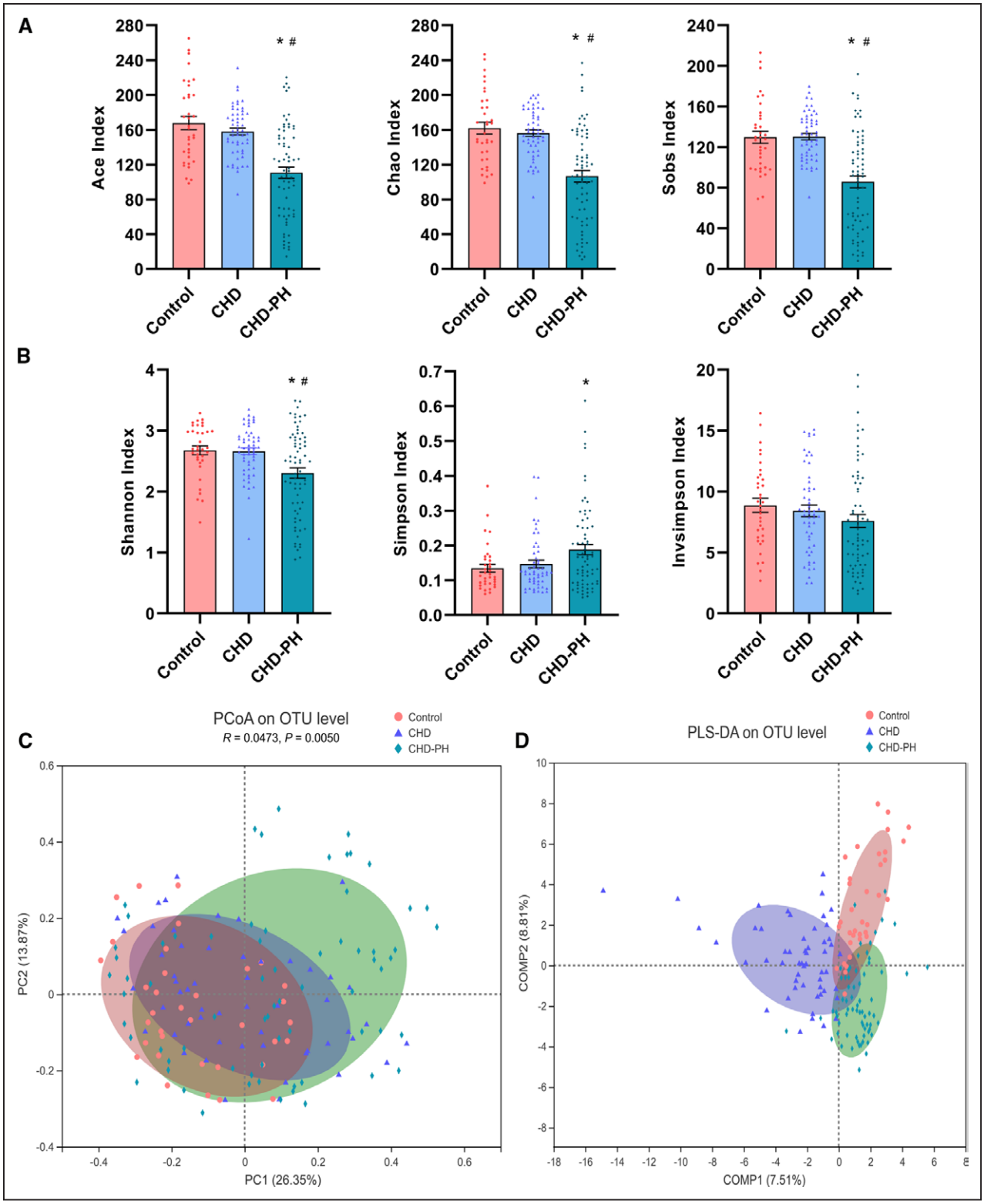

Figure 1. Microbial α-diversity and β-diversity in patients with congenital heart disease (CHD) or pulmonary hypertension (PH) associated with CHD-PH, as well as pediatric reference subjects (pediatric-control).

The α-diversity was analyzed by the Ace (**A**) Ace, Chao, Sobs, (**B**) Shannon, Simpson, and Invsimpson indexes. The β-diversity was shown by principal coordinate analysis (PCoA) (**C**) based on Bray-Curtis Dissimilarity index (PERMANOVA, R=0.0473, P=0.005) and partial least squares discriminant analyses (PLS-DA) (**D**). Data are shown as the mean±SEM and analyzed by 1-way ANOVA with Tukey multiple comparison test. *P<0.05 vs control group; #P<0.05 vs CHD group.

by Actinobacteriota, Bacteroidta, Proteobacteria, Fusobacteria, and Patescibacteria (Figure 2A). The 6 phyla with the richest abundance were shown in Figure 2B. The proportions of Patescibacteria and Fusobacteria were significantly decreased in the CHD-PH groups compared with the CHD and control group (Figure 2B). At family level, CHD-PH patients were characterized by a lower relative abundance of *Veillonellaceae* and *Actinomycetaceae*, and a higher relative abundance of *Micrococcaceae* (Figure 2C and 2D). The microbiota composition at genus level was represented in Figure 2E. The proportion of *Streptococcus* and *Rothia* was statistically larger, while *Veillonella* and *Actinomyces* were statistically smaller in the CHD-PH group than in the control group (Figure 2F).

Specific Upper Airway Microbiota in CHD-PH Varies Between CHD and Control Groups

The differences of the bacterial composition among the 3 groups were compared and analyzed respectively. The differentially abundant microbiota at the phylum and genus levels in CHD-PH versus control (Figure 3A and 3B), CHD versus control (Figure S1B and S1C) and among the 3 groups (Figure S2) were shown. Similarly, the proportions of *Streptococcus* and *Veillonella* in CHD and CHD-PH patients were different from those in the control group. The greatest differences in upper respiratory microbiota taxa between the CHD-PH and control groups were analyzed by a linear discriminant analysis effect size algorithm (linear discriminant analysis score

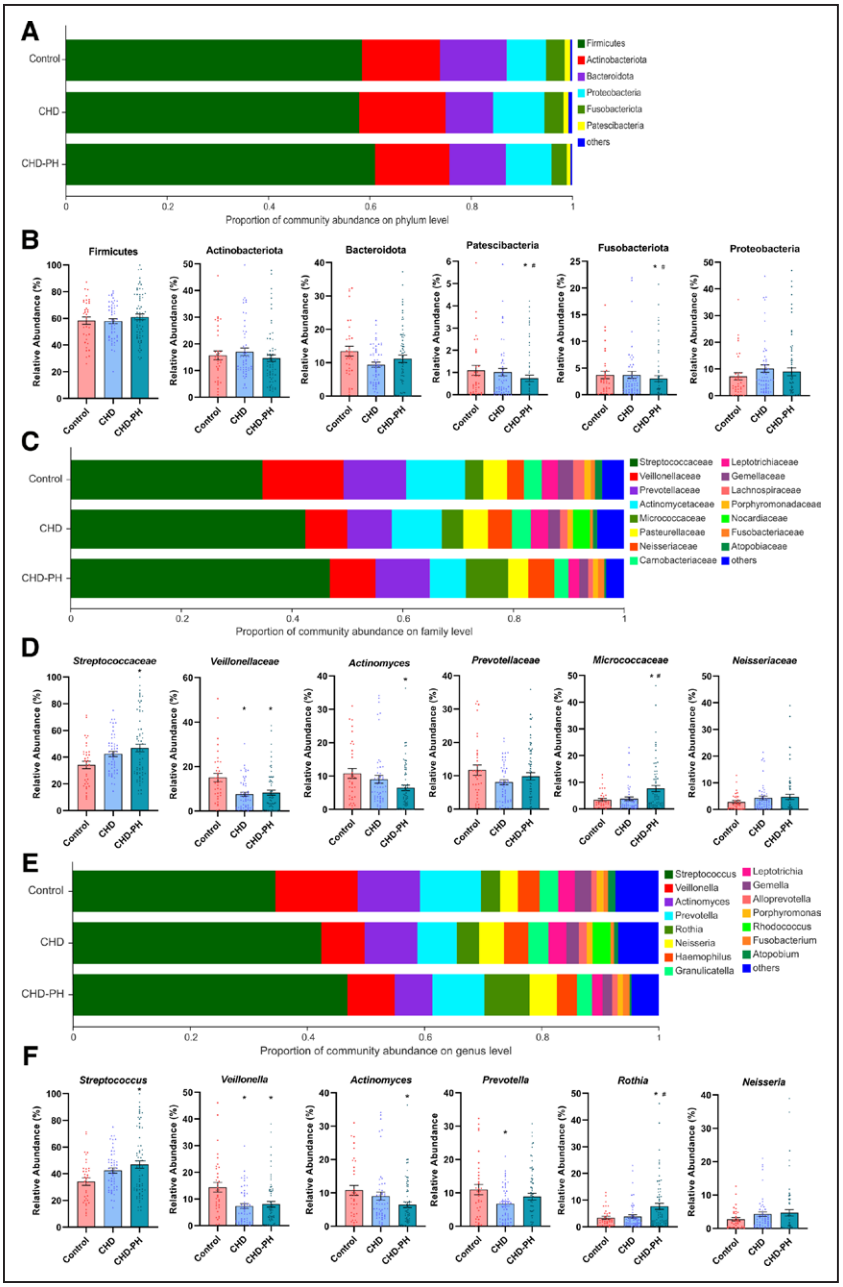

Figure 2. Relative abundance of upper airway microbiota in the congenital heart disease (CHD) group, CHD-pulmonary hypertension (PH) group, and pediatric controls.
Microbial composition bars at the (**A**) phylum level, (**C**) family level, and (**E**) genus level in the upper airway microbiota in the 3 groups. The abundance and differences in the top 6 (**B**) phyla, (**D**) families, and (**F**) genera are also presented. Those with a relative abundance <1% were classified as others. Data are shown as mean±SEM and analyzed by 1-way ANOVA with Tukey multiple comparison test. *P<0.05 vs control group; #P<0.05 vs CHD group.

ORIGINAL ARTICLE

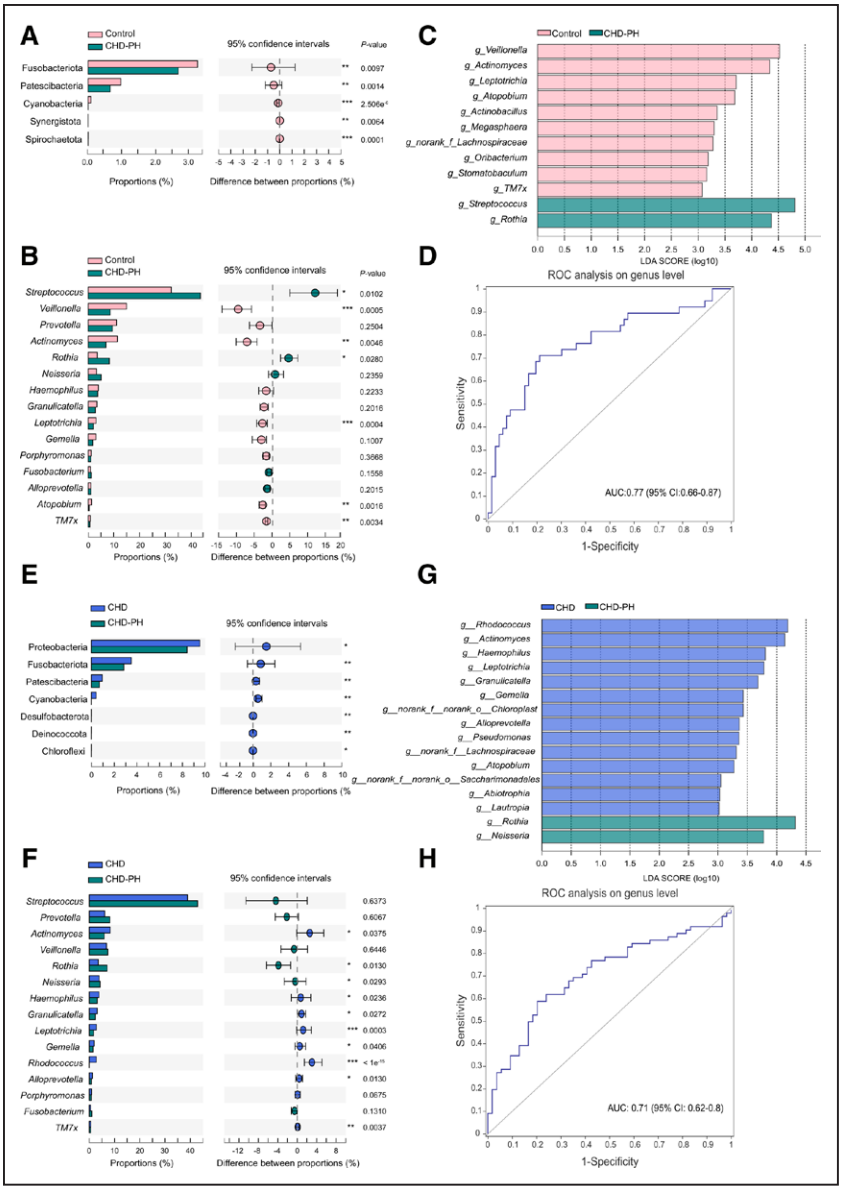

Figure 3. Characterized airway microbiome distinguished congenital heart disease (CHD)-pulmonary hypertension (PH) from CHD and healthy controls.
Comparison of the relative abundance of upper airway microbiota between CHD-PH and control group at (**A**) phylum and (**B**) genus level. Data are analyzed by wilcoxon rank-sum test with Benjamini-Hochberg false discovery rate multiple test correction. **C**, Linear discriminant analysis (LDA) effect size (LefSe, LDA score ≥3) showing the most discriminant microbial genera between the CHD-PH and control groups. **D**, Prediction of distinct genera for CHD-PH from the control group. Receiver operating characteristic (ROC) curve for *Streptococcus* + *Rothia*, AUC=0.77 (95% CI, 0.66–0.87). Comparison of the relative abundance of upper airway microbiota between CHD-PH and CHD at (**E**) phylum and (**F**) genus level. Data are analyzed by wilcoxon rank-sum test with Benjamini-Hochberg false discovery rate. **G**, LefSe analysis between CHD-PH and CHD. **H**, Prediction of distinct genera for CHD-PH from CHD. ROC for *Neisseria*+*Rothia*, AUC=0.71 (95% CI, 0.62–0.8). Prefix: f_ family, and g_ genus. **P*<0.05, ***P*<0.01, and ****P*<0.001.

[log10] >3), identifying *Streptococcus* and *Rothia* as the most enriched in CHD-PH patients (Figure 3C). An OUT subset based on the associated genera identified by linear discriminant analysis effect size was established, which was assessed through receiver operating characteristic curve analysis showing an area under the parametric curve value of 0.77 ([95% CI, 0.66–0.87]; Figure 3D). While the associated genera identified by CHD versus control group had an area under the parametric curve value of 0.61 ([95% CI, 0.49–0.73]; Figure S1D and S1E).

When compared with CHD patients, those patients with PH also displayed a relative difference of distinct microbiota. At the phylum level, CHD-PH showed a decrease in Proteobacteria, Fusobacteriota, Patescibacteria, and Cyanobacteria (Figure 3E). At genus level, the proportions of *Actinomyces*, *Haemophilus*, *Granulicatella*, *Leptotrichia*, *Gemella*, *Rhodococcus*, *Alloprevotella*, and *TM7x* were significantly smaller in PH (Figure 3F), while *Rothia* and *Neisseria* were significantly enriched in CHD-PH (Figure 3G). Receiver operating characteristic curve analysis based on enriched genera in CHD versus CHD-PH showed an area under the parametric curve value of 0.71 ([95% CI, 0.62–0.8]; Figure 3H).

Comparison of Microbial Composition Between Children and Adult PH Patients

Comparison of the upper respiratory microbiome of pediatric CHD-PH patients with that of adult PH patients also demonstrated a thoroughly different microbial community between children and adult in both healthy individuals and PH patients. At phylum level, Firmicutes and Actinobacteriota exhibited a dominant increase in pediatric PH patients than in adults (Figure 4A), and the genus *Streptococcus* was much more abundant in pediatric

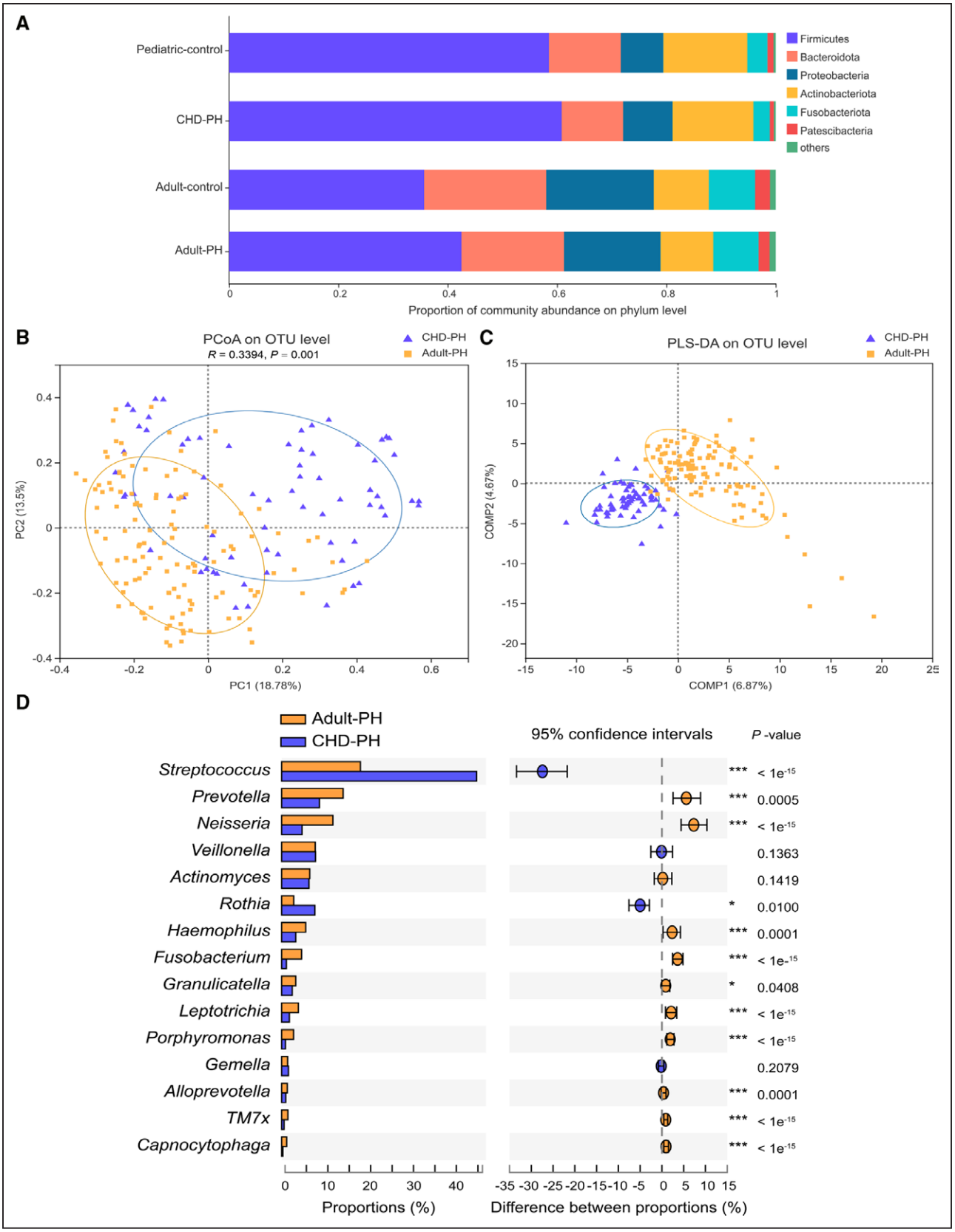

Figure 4. Alterations in upper airway microbiota between adults and pediatric patients with pulmonary hypertension (PH). **A**, Composition bar at the phylum level of the adult PH, adult healthy control, congenital heart disease (CHD)-PH, and pediatric control groups. **B**, PCoA (PERMANOVA, R=0.3394, *P*=0.001) and (**C**) PLS-DA were performed to compare the grouped microbiome profiles between the adult and pediatric patients with PH. **D**, The comparison of relative levels of upper airway microbiota between the adults and pediatric patients with PH at the genus level. Data are analyzed by wilcoxon rank-sum test with Benjamini-Hochberg false discovery rate. *P<0.05 and ***P<0.001.

patients than in adults (Figure S3). Bacteroidota, Actinobacteriota, Fusobacteriota, and Patescibacteria shared a similar descending trend in both pediatric and adult PH patients compared with the respective control group. Principal coordinate analysis and partial least squares discriminant analysis showed significant separation of the microbiota of the children with PH from that of the adults (Figure 4B and 4C).

Moreover, at genus level, the microbial structure showed a dramatic difference, with a higher proportion of *Streptococcus* and *Rothia* in the pediatric CHD-PH patients. *Prevotella*, *Neisseria*, *Haemophilus*, *Fusobacterium*, *Granulicatella*, *Leptotrichia*, *Porphyromonas*, *Alloprevotella*, TM7x, and *Capnocytophaga* were relatively prevalent in the adult PH group (Figure 4D).

Correlation Between Bacterial Abundance and TTE Indexes in CHD-Associated PH

A Pearson correlation analysis was performed between upper airway microbiome taxa and TTE indicators of CHD-PH patients to identify deeper level taxa associations (Figure 5).

For overall CHD-PH patients (Figure 5A), the genus *Streptococcus* was negatively correlated with main pulmonary artery (Pearson correlation coefficient R=−0.33), right ventricle (R=−0.38), and right ventricular outflow tract (R=−0.33), which were representative of right heart function. In contrast, multiple bacteria were positively associated with right heart function. There was a positive association between *Megasphaera* and TTE indexes, including main pulmonary artery (R=0.51), right ventricular outflow tract (R=0.34), and tricuspid valve area (R=0.35). Right ventricle was found positively associated with *Prevotella* (R=0.43) and *Alloprevotella* (R=0.41). *TM7x, Atopobium, Leptotrichia,* and some parent taxa within *Firmicutes* were also mildly correlated with right heart function.

Then, CHD-PH patients were divided into mild PH (35 mmHg < PASP <55 mmHg, n=24), moderate PH (55≤PASP<75 mmHg, n=30) and severe PH (PASP≥75 mmHg, n=15) to detect the correlation between the microbiome of PH stratification and TTE indicators. The microbiota composition in each group of PH severity was shown in Figure S4. In mild PH patients (Figure 5B), main pulmonary artery was found positively associated with *Atopobium* (R=0.57), *Megasphaera* (R=0.62), and *Solobacterium* (R=0.53). However, pulmonary artery pressure was negatively associated with *Streptococcus* (R=−0.46). Meanwhile, right ventricle was positively associated with *Prevotella* (R=0.63), *Leptotrichia* (R=0.59), *Alloprevotella* (R=0.79), *Lachnoanaerobaculum* (R=0.71), and *TM7x* (R=0.62) in the moderate PH group (Figure 5C). In the severe PH group (Figure 5D), we observed that main pulmonary artery was positively associated with *Selenomonas* (R=0.73), *Megasphaera* (R=0.74), *Leptotrichia* (R=0.65), and *Veillonella* (R=0.59). right ventricular outflow tract and pulmonary artery pressure were positively associated with *Gemella* (R=0.54) and *Neisseria* (R=0.54), respectively. These results indicated that different groups of microbes may be related to different PH severities.

Microbial Functions Differ Between CHD-PH Patients and Controls in Children

The function of the microbiome in CHD-PH versus control group was predicted by Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) based on analyzing the abundance composition of the Kyoto Encyclopedia of Genes and Genomes pathway (level 3) of the airway microbiome. As seen in Figure 5E, several material metabolism pathways, including galactose, amino sugar and nucleotide sugar, metabolism, peptidoglycan, phenylalanine, tyrosine, and arginine biosynthesis, were significantly higher in the PH group than in the control group, while pathways of microbial metabolism, lipopolysaccharide, glyoxylate and dicarboxylate, histidine, nitrogen, and ascorbate metabolism were significantly lower in the CHD-PH group.

DISCUSSION

The correlation between the human microbiome and disorders has been thoroughly studied in recent years. Consistent evidences have recently shown that the respiratory microbiome plays key roles in maintaining respiratory physiological function by interacting with the host system.[24,25] In contrast to traditional views, the respiratory tract harbors its own microbiota, which is influenced to some extent by the oral and oropharyngeal cavities.[26] The current study focuses on this question by comparing CHD-PH patients with CHD patients and healthy individuals, as well as pediatric patients with adult PH-specific clustering of microbial communities. First, our results showed a diverse microbiome in the upper respiratory tracts of CHD and CHD-PH patients compared with control subjects. The respiratory dysbiosis observed in CHD-PH patients was characterized by a reduction in commensal bacterial species and richness, which was not prominent in CHD compared with healthy individuals. Meanwhile, we found some specific microbiota constituents in CHD patients who differed from those of healthy children but were similar to those of CHD-PH patients. In addition, the results indicated that pediatric CHD-PH also shared different upper airway microbiota profiles compared with adult PH. These findings indicate that compositional differences were associated with the presence or absence of specific taxa and their relative abundances in the upper respiratory tract. Finally, we found that different specific microbiomes may be related to different PH stratifications, which may become a means of evaluating

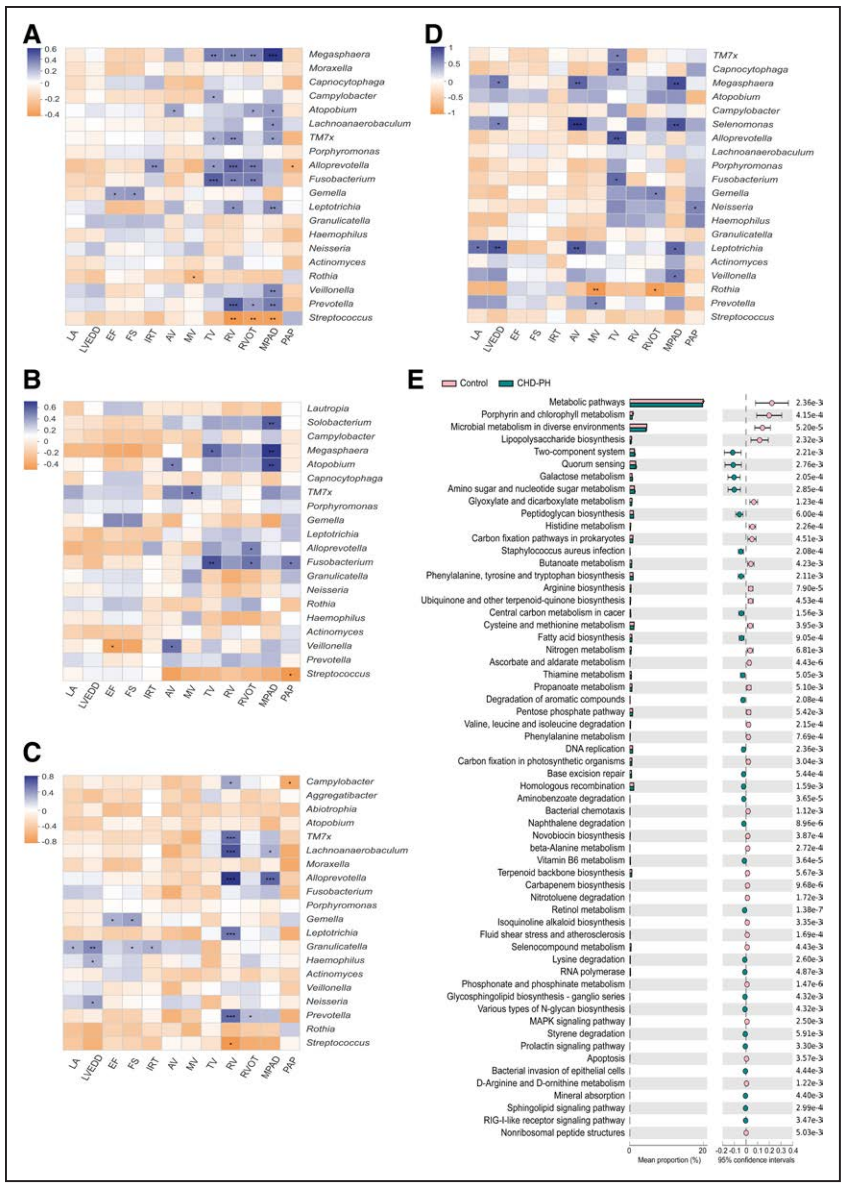

Figure 5. Heatmap of the top 20 bacteria showing the correlation between upper airway bacteria of congenital heart disease (CHD)-pulmonary hypertension (PH) and transthoracic echocardiography (TTE) parameters at the genus level.
A, Overall CHD-PH patients were stratified into (**B**) mild PH (n=24), (**C**) moderate PH (n=30) and (**D**) severe PH (n=15) groups based on the evaluated pulmonary artery systolic pressure. Correlations were calculated by Pearson correlation analysis. Blue represented positive correlation and orange represented negative correlation. **E**, Functional predictions for the microbiome of the CHD-PH and control groups. Significant Kyoto Encyclopedia of Genes and Genomes pathways at level 3 for the microbiome of the 2 groups were visualized by STAMP software using Wilcoxon rank-sum test with multiple test correction of Benjamini-Hochberg false discovery rate (FDR; $P<0.05$). *$P<0.05$, **$P<0.01$, and ***$P<0.001$.

pediatric PH. This study provided the first comprehensive observation of upper respiratory microbial characteristics in CHD-PH and its differences from adult patients with PH. The results of this study provide novel findings from the perspective of the upper microbiota to further investigate the potential cause and preventive targets for PH in the CHD process.

The microbiome is considered one of the environmental factors involved in the pathogenesis of multiple diseases, but little attention has been given to CHD in this regard. In the past 2 years, several studies focused on the relationship between CHD and the gastrointestinal tract have reported that CHD patients had intestinal dysfunction and endotoxemia,[27] which could drive changes in the bacterial community and translocation of gut bacterial antigens by modifying both local microbial composition and increasing circulating bacterial load.[11,28] In terms of the gut, CHD patients remain at high risk of developing gut dysbiosis due to numerous stressors, including abnormal gut perfusion, hypoxemia, and impaired nutrition.[27] From the perspective of the lung, CHD may also be complicated by lung dysbiosis as a result of increased pulmonary blood flow caused by valvar regurgitation and residual cardiac shunts. Although there is currently no direct evidence proving the interaction between the gut and lung microbiota, which is also acknowledged as the "gut-lung axis," some surveys have demonstrated that the coincidence of gut and lung dysbiosis exists in chronic lung diseases such as asthma, pulmonary fibrosis, chronic obstructive pulmonary disease, and PH.[8,14,29–31] Similar to gut microbial alterations, our study strongly suggested that alterations in microbial profiles are also present in the upper respiratory tract. The dominant respiratory microbiota of a healthy individual is mainly characterized by commensal bacteria such as *Prevotella*, *Veillonella*, *Streptococcus,*

and *Neisseria*, which are involved in the development of host immune homeostasis.[24] Nevertheless, the mechanism of action of the above altered respiratory microbiota profile in patients with CHD and CHD-PH still warrants further study.

The results caused by respiratory microbiota imbalance are similar to those involved in PH pathophysiology. Nitric oxide, partly metabolized by bacteria from NO_2^- of the host, alleviates PH by inducing pulmonary arterial endothelial cell relaxation and reducing extracellular calcium influx. In our results, KEGG enrichment identified decreased nitrogen metabolism in CHD-PH patients, and the comparison between CHD-PH and healthy children showed a decreased proportion of *Veillonella* and *Actinomyces* (Figure 3B), which accounted for the largest proportion of NO_2^--producing bacteria in both aerobic and anaerobic conditions in the upper respiratory tract.[32] These results suggest that altered *Veillonella* and *Actinomyces* in the upper respiratory tract might be associated with PH in the development of CHD. However, as the microbiome community is dynamic with multiple factors,[33] to thoroughly understand the association between respiratory bacteria and the onset of PH, longitudinal sampling of donors during the disease process will be needed in future studies.

In addition, an altered microbiome was also found to be correlated with illness severity and may help discriminate critical patients with COVID-19.[34] To explore the relationship of respiratory bacteria and PH severity, correlation analyses were performed between the abundance of the top 20 genera and TTE indexes. Along with changes in the airway microbiome, a strong relationship between the disrupted microbiome and TTE variables, especially a strong positive correlation between right heart parameters of moderate-severe PH and *Leptotrichia*, was also observed in CHD-PH in our study.

According to the PICRUSt2 results, the KEGG enrichment function of the upper respiratory microbiota can also predict the development of PH. We found that multiple bacterial functions based on KEGG (level 3) categories were disturbed in CHD patients with PH. The most considerable relationship between differentially colonized bacteria and multiple metabolic pathways was identified. Interestingly, a recent study showed that plasma metabolites were correlated with clinical measurements in response to shunt correction in CHD-PH.[35] Nevertheless, it is still largely unclear how altered respiratory microbiota profiles contribute to PH during the CHD process, suggesting that metabolomics should be considered in the future to further determine the detailed interacting mechanism between PH development and altered lung microbiome.

The current study had several limitations. Above all, we were limited by the use of echocardiography as right heart catheterization is invasive and expensive. Second, BALF might be a better choice to represent the lung microbiome, as oropharyngeal swab collection of upper respiratory tract is likely to be contaminated by oral (or saliva) microbiome. Yet, electronic bronchoscopy was unethical and infeasible for pediatric CHD and PH patients. Further prospective studies will be performed to compare the microbiome deep down from the lung. Microbiota profiling studies, however, represent only the first step in omics research and should be followed by focused studies to quantitatively map "microbiota-cell/microbiota-microbiota" pathways as well as determine the causal link between the host microbiome and PH occurrence in the CHD process.

Taken together, *16S* rRNA sequencing was used in this study to explore the altered microbial characteristics in the upper respiratory tract of pediatric CHD patients with PH compared with control individuals. The findings of the current study revealed that the respiratory microbiota composition was significantly different in CHD-PH patients compared with the controls or pediatric CHD or adult PH patients. Meanwhile, different microbiota may suggest different PH grades. Overall, these data suggest that dysbiosis of respiratory microbiota may play a key role in bridging the linkage between the heart and lung, providing novel insights into the roles of airway microbiota during disease pathogenesis and its significance as a potential preventive or therapeutic target.

CONCLUSIONS

The upper airway microbiota composition of CHD-PH differs from that of healthy individuals and pediatric CHD patients. Our results comprehensively demonstrated a potential correlation between changes in upper airway bacterial populations and cardiovascular structure reflected by TTE parameters in pediatric CHD-PH patients. Prospective studies are warranted to explore whether such microbiota disruption conveys an increased risk of developing PH in pediatric CHD patients.

PERSPECTIVES

The upper airway microbiota composition of pediatric CHD patients with PH differs from that of healthy individuals and adults with PH. This study comprehensively demonstrated a potential correlation between changes in upper airway bacterial populations and cardiovascular structure reflected by TTE parameters in pediatric CHD-PH patients. Prospective studies are warranted to explore whether such microbiota disruption conveys an increased risk of developing PH in pediatric CHD patients.

ARTICLE INFORMATION

Received February 11, 2022; accepted October 30, 2022.

Affiliations

Department of Respiratory Children's Hospital of Chongqing Medical University (T.W., B.P., G.G., Q.L., Z.L., Z.F.), Department of thoracic and Cardiac Surgery Children's Hospital of Chongqing Medical University (J.F.), and Department of Cardiovascular Medicine Children's Hospital of Chongqing Medical University (M.L.), National Clinical Research Center for Child Health and Disorders, Ministry of Education Key Laboratory of Child Development and Disorders, Children's Hospital of Chongqing Medical University, China. State Key Laboratory of Respiratory Disease, National Clinical Research Center for Respiratory Disease, Guangdong Key Laboratory of Vascular Disease, Guangzhou Institute of Respiratory Health, The First Affiliated Hospital of Guangzhou Medical University, China (Y.X., K.Y., C.Z., Y.C., J.W.).

Author Contributions

T. Wang and Y. Xing designed the study, collect specimens, analyzed and interpreted the data, wrote the article, and performed data analysis and revised the article. B. Peng collect specimens and analyzed and interpreted the data. Y. Chen, C. Zhang, and K. Yang designed the study and revised the article. G. Geng, Q. Li, J. Fu, Z. Luo, and M. Li contributed to the provision of throat swab specimens. Z. Fu and J. Wang contributed to study design, provided financial support, performed data analysis and interpretation, wrote, edited, and revised the article, and provided final approval.

Sources of Funding

This work was partially supported by grants from the National Natural Science Foundation of China (82000034, 82120108001, 82170069, 81970057, 8180005, 82170065) and the Chongqing Science and Technology Commission (cxtc2020jcyj-msxmX0261).

Disclosures

None.

REFERENCES

1. Thenappan T, Ormiston ML, Ryan JJ, Archer SL. Pulmonary arterial hypertension: pathogenesis and clinical management. *BMJ*. 2018;360:j5492. doi: 10.1136/bmj.j5492
2. Simonneau G, Montani D, Celermajer DS, Denton CP, Gatzoulis MA, Krowka M, Williams PG, Souza R. Haemodynamic definitions and updated clinical classification of pulmonary hypertension. *Eur Respir J*. 2019;53:1801913. doi: 10.1183/13993003.01913-2018
3. Lammers AE, Apitz C, Zartner P, Hager A, Dubowy KO, Hansmann G. Diagnostics, monitoring and outpatient care in children with suspected pulmonary hypertension/paediatric pulmonary hypertensive vascular disease. Expert consensus statement on the diagnosis and treatment of paediatric pulmonary hypertension. The european paediatric pulmonary vascular disease network, endorsed by ishlt and dgpk. *Heart (British Cardiac Society)*. 2016;102 Suppl 2:ii1–13. doi: 10.1136/heartjnl-2015-307792
4. Haworth SG, Hislop AA. Treatment and survival in children with pulmonary arterial hypertension: The uk pulmonary hypertension service for children 2001-2006. *Heart (British Cardiac Society)*. 2009;95:312–7. doi: 10.1136/hrt.2008.150086
5. Barst RJ, McGoon MD, Elliott CG, Foreman AJ, Miller DP, Ivy DD. Survival in childhood pulmonary arterial hypertension: Insights from the registry to evaluate early and long-term pulmonary arterial hypertension disease management. *Circulation*. 2012;125:113–122. doi: 10.1161/CIRCULATIONAHA.111.026591
6. Kamada N, Chen GY, Inohara N, Núñez G. Control of pathogens and pathobionts by the gut microbiota. *Nat Immunol*. 2013;14:685–690. doi: 10.1038/ni.2608
7. Stricker S, Hain T, Chao CM, Rudloff S. Respiratory and intestinal microbiota in pediatric lung diseases-current evidence of the gut-lung axis. *Int J Mol Sci*. 2022;23:6791. doi: 10.3390/ijms23126791
8. Kim S, Rigatto K, Gazzana MB, Knorst MM, Richards EM, Pepine CJ, Raizada MK. Altered gut microbiome profile in patients with pulmonary arterial hypertension. *Hypertension*. 2020;75:1063–1071. doi: 10.1161/HYPERTENSIONAHA.119.14294
9. Yuzefpolskaya M, Bohn B, Nasiri M, Zuver AM, Onat DD, Royzman EA, Nwokocha J, Mabasa M, Pinsino A, Brunjes D, et al. Gut microbiota, endotoxemia, inflammation, and oxidative stress in patients with heart failure, left ventricular assist device, and transplant. *J Heart Lung Transplant*. 2020;39:880–890. doi: 10.1016/j.healun.2020.02.004
10. Yang T, Santisteban MM, Rodriguez V, Li E, Ahmari N, Carvajal JM, Zadeh M, Gong M, Qi Y, Zubcevic J, et al. Gut dysbiosis is linked to hypertension. *Hypertension*. 2015;65:1331–1340. doi: 10.1161/HYPERTENSIONAHA.115.05315
11. Salomon J, Ericsson A, Price A, Manithody C, Murry DJ, Chhonker YS, Buchanan P, Lindsey ML, Singh AB, Jain AK. Dysbiosis and intestinal barrier dysfunction in pediatric congenital heart disease is exacerbated following cardiopulmonary bypass. *JACC Basic Transl Sci*. 2021;6:311–327. doi: 10.1016/j.jacbts.2020.12.012
12. Sanada TJ, Hosomi K, Shoji H, Park J, Naito A, Ikubo Y, Yanagisawa A, Kobayashi T, Miwa H, Suda R, et al. Gut microbiota modification suppresses the development of pulmonary arterial hypertension in an su5416/hypoxia rat model. *Pulmonary circulation*. 2020;10:2045894020929147. doi: 10.1177/2045894020929147
13. Callejo M, Mondejar-Parreño G, Barreira B, Izquierdo-Garcia JL, Morales-Cano D, Esquivel-Ruiz S, Moreno L, Cogolludo A, Duarte J, Perez-Vizcaino F. Pulmonary arterial hypertension affects the rat gut microbiome. *Sci Rep*. 2018;8:9681. doi: 10.1038/s41598-018-27682-w
14. Zhang C, Zhang T, Lu W, Duan X, Luo X, Liu S, Chen Y, Li Y, Chen J, Liao J, et al. Altered airway microbiota composition in patients with pulmonary hypertension. *Hypertension* 2020;76:1589–1599. doi: 10.1161/HYPERTENSIONAHA.120.15025
15. Yun Y, Srinivas G, Kuenzel S, Linnenbrink M, Alnahas S, Bruce KD, Steinhoff U, Baines JF, Schaible UE. Environmentally determined differences in the murine lung microbiota and their relation to alveolar architecture. *PLoS One*. 2014;9:e113466. doi: 10.1371/journal.pone.0113466
16. Dolma K, Freeman AE, Rezonzew G, Payne GA, Xu X, Jilling T, Blalock JE, Gaggar A, Ambalavanan N, Lal CV. Effects of hyperoxia on alveolar and pulmonary vascular development in germ-free mice. *Am J Physiol Lung Cell Mol Physiol*. 2020;318:L421–L428. doi: 10.1152/ajplung.00316.2019
17. Rofael SAD, McHugh TD, Troughton R, Beckmann J, Spratt D, Marlow N, Hurst JR. Airway microbiome in adult survivors of extremely preterm birth: the epicure study. *Eur Respir J*. 2019;53:1801225. doi: 10.1183/13993003.01225-2018
18. Pammi M, Lal CV, Wagner BD, Mourani PM, Lohmann P, Luna RA, Sisson A, Shivanna B, Hollister EB, Abman SH, et al. Airway microbiome and development of bronchopulmonary dysplasia in preterm infants: A systematic review. *J Pediatr*. 2019;204:126–133.e2. doi: 10.1016/j.jpeds.2018.08.042
19. Lam CS, Roger VL, Rodeheffer RJ, Borlaug BA, Enders FT, Redfield MM. Pulmonary hypertension in heart failure with preserved ejection fraction: A community-based study. *J Am Coll Cardiol*. 2009;53:1119–1126. doi: 10.1016/j.jacc.2008.11.051
20. Lopez L, Colan SD, Frommelt PC, Ensing GJ, Kendall K, Younoszai AK, Lai WW, Geva T. Recommendations for quantification methods during the performance of a pediatric echocardiogram: A report from the pediatric measurements writing group of the american society of echocardiography pediatric and congenital heart disease council. *J Am Soc Echocardiogr*2010;23:465–495. quiz576467. doi: 10.1016/j.echo.2010.03.019
21. Koestenberger M, Apitz C, Abdul-Khaliq H, Hansmann G. Transthoracic echocardiography for the evaluation of children and adolescents with suspected or confirmed pulmonary hypertension. Expert consensus statement on the diagnosis and treatment of paediatric pulmonary hypertension. The european paediatric pulmonary vascular disease network, endorsed by ishlt and d6pk. *Heart (British Cardiac Society)*. 2016;102 Suppl 2:ii14–22. doi: 10.1136/heartjnl-2014-307200
22. Chen S, Zhou Y, Chen Y, Gu J. Fastp: An ultra-fast all-in-one fastq preprocessor. *Bioinformatics (Oxford, England)*. 2018;34:i884–i890. doi: 10.1093/bioinformatics/bty560
23. Magoč T, Salzberg SL. Flash: Fast length adjustment of short reads to improve genome assemblies. *Bioinformatics (Oxford, England)*. 2011;27:2957–2963. doi: 10.1093/bioinformatics/btr507
24. Man WH, de Steenhuijsen Piters WA, Bogaert D. The microbiota of the respiratory tract: Gatekeeper to respiratory health. *Nat Rev Microbiol*. 2017;15:259–270. doi: 10.1038/nrmicro.2017.14
25. Segal LN, Clemente JC, Tsay JC, Koralov SB, Keller BC, Wu BG, Li Y, Shen N, Ghedin E, Morris A, et al. Enrichment of the lung microbiome with oral taxa is associated with lung inflammation of a th17 phenotype. *Nat Microbiol*. 2016;1:16031. doi: 10.1038/nmicrobiol.2016.31
26. Bassis CM, Erb-Downward JR, Dickson RP, Freeman CM, Schmidt TM, Young VB, Beck JM, Curtis JL, Huffnagle GB. Analysis of the upper respiratory tract microbiotas as the source of the lung and gastric microbiotas in healthy individuals. *Mbio*. 2015;6:e00037. doi: 10.1128/mBio.00037-15
27. Pathan N, Burmester M, Adamovic T, Berk M, Ng KW, Betts H, Macrae D, Waddell S, Paul-Clark M, Nuamah R, et al. Intestinal injury and

endotoxemia in children undergoing surgery for congenital heart disease. *Am J Respir Crit Care Med*. 2011;184:1261–1269. doi: 10.1164/rccm.201104-0715OC
28. Dinakaran V, Rathinavel A, Pushpanathan M, Sivakumar R, Gunasekaran P, Rajendhran J. Elevated levels of circulating DNA in cardiovascular disease patients: Metagenomic profiling of microbiome in the circulation. *PLoS One*. 2014;9:e105221. doi: 10.1371/journal.pone.0105221
29. Budden KF, Gellatly SL, Wood DL, Cooper MA, Morrison M, Hugenholtz P, Hansbro PM. Emerging pathogenic links between microbiota and the gut-lung axis. *Nat Rev Microbiol*. 2017;15:55–63. doi: 10.1038/nrmicro.2016.142
30. O'Dwyer DN, Ashley SL, Gurczynski SJ, Xia M, Wilke C, Falkowski NR, Norman KC, Arnold KB, Huffnagle GB, Salisbury ML, et al. Lung microbiota contribute to pulmonary inflammation and disease progression in pulmonary fibrosis. *Am J Respir Crit Care Med*. 2019;199:1127–1138. doi: 10.1164/rccm.201809-1650OC
31. Lai HC, Lin TL, Chen TW, Kuo YL, Chang CJ, Wu TR, Shu CC, Tsai YH, Swift S, Lu CC. Gut microbiota modulates copd pathogenesis: Role of anti-inflammatory parabacteroides goldsteinii lipopolysaccharide. *Gut*. 2022;71:309–321. doi: 10.1136/gutjnl-2020-322599
32. Sato-Suzuki Y, Washio J, Wicaksono DP, Sato T, Fukumoto S, Takahashi N. Nitrite-producing oral microbiome in adults and children. *Sci Rep*. 2020;10:16652. doi: 10.1038/s41598-020-73479-1
33. Byrd AL, Liu M, Fujimura KE, Lyalina S, Nagarkar DR, Charbit B, Bergstedt J, Patin E, Harrison OJ, Quintana-Murci L, et al. Gut microbiome stability and dynamics in healthy donors and patients with non-gastrointestinal cancers. *J Exp Med*. 2021;218: e20200606. doi: 10.1084/jem.20200606
34. Tang L, Gu S, Gong Y, Li B, Lu H, Li Q, Zhang R, Gao X, Wu Z, Zhang J, et al. Clinical significance of the correlation between changes in the major intestinal bacteria species and covid-19 severity. *Engineering (Beijing, China)*. 2020;6:1178–1184. doi: 10.1016/j.eng.2020.05.013
35. He YY, Yan Y, Chen JW, Liu S, Hua L, Jiang X, Xu XQ, Lu D, Jing ZC, Yan FX, et al. Plasma metabolomics in the perioperative period of defect repair in patients with pulmonary arterial hypertension associated with congenital heart disease. *Acta Pharmacol Sin*. 2022;43:1710–1720. doi: 10.1038/s41401-021-00804-3