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Airway microbiome, host immune response and recurrent wheezing in infants with severe respiratory syncytial virus bronchiolitis

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Abstract

Background: Early interactions between respiratory viruses and microbiota might modulate host immune responses, and subsequently contribute to later development of recurrent wheezing and asthma in childhood. We aimed to study the possible association between respiratory microbiome, host immune response, and the development of recurrent wheezing in infants with severe respiratory syncytial virus (RSV) bronchiolitis.

Methods: Seventy-four infants who were hospitalized at Beijing Children's Hospital during an initial episode of severe RSV bronchiolitis at 6 months of age or less were included and followed for up to the age of 3 years. Sputum samples were collected, and their microbiota profiles, LPS and cytokines were analyzed by 16S rRNA-based sequencing, ELISA, and multiplex immunoassay, respectively.

Results: Twenty-six (35.1%) infants developed recurrent wheezing by the age of 3 years and 48 (64.9%) did not. The relative abundance of *Haemophilus*, *Moraxella* and *Klebsiella* was higher in infants who later developed recurrent wheezing than those who did not (LDA score >3.5). Airway levels of LPS ($P=0.003$), CXCL8 ($P=0.004$), CCL5 ($P=0.029$), IL-6 ($P=0.004$), and IL-13 ($P<0.001$) were significantly higher in infants who later developed recurrent wheezing than those who did not. Moreover, high airway abundance of *Haemophilus* was associated with CXCL8 ($r=0.246$, $P=0.037$) level, and that of *Moraxella* was associated with IL-6 level ($r=0.236$, $P=0.046$) and IL-10 level ($r=0.266$, $P=0.024$).

Conclusion: Our study suggests that higher abundance of *Haemophilus* and *Moraxella* in airway microbiome might modulate airway inflammation during severe RSV bronchiolitis in infancy, potentially contributing to the development of subsequent recurrent wheezing in later childhood.

Key words:

Respiratory microbiome, Respiratory syncytial virus, Airway inflammation, Lipopolysaccharide, Recurrent wheezing, Children

Introduction

Worldwide, respiratory syncytial virus (RSV) is the primary pathogen identified in children with acute lower respiratory tract infection during the first year of life, and is the most common cause of hospital admission in winter season [1]. Prospective studies conducted in different countries have shown that RSV bronchiolitis during infancy is highly associated with the development of recurrent wheezing and asthma later in childhood [2-4], and the risk is higher among infants with severe bronchiolitis requiring hospitalization [3].

It has been shown that there is an initial strong neutrophil response to RSV infection, which is mainly mediated by CXCL8 and positively correlated with disease severity [5, 6]. Other chemokines (particularly CCL-5 and CXCL10) and cytokines (particularly IL-17A, IL-33) may contribute to pathogenesis and post-RSV bronchiolitis-induced asthma [4, 5, 7]. In addition, Th1/Th2 imbalance has also been proposed as a key event in the inflammation process [5]. It is known that excessive inflammatory response to RSV, can lead to long-lived alterations to airway structural and resident immune cells that render the host susceptible to subsequent environmental incursions [8]. Thus, the initial host response to RSV may represent a tipping point in the balance between long-term respiratory health or chronic diseases (e.g. recurrent wheezing).

Directly after birth, respiratory mucosal surfaces are rapidly colonized with a variety of bacteria and undergo a maturation process during early childhood [9]. Emerging evidence suggests that early interactions between respiratory microbiota and viruses might modulate host immune responses which can potentially affect the pathogenesis, severity and asthma-related outcome of acute respiratory tract infections [10, 11], and that bacterial structural ligands can regulate host

immune response in respiratory tract [12].

However, systematically researches on airway microbiome, RSV infection and development of recurrent wheezing in infants are limited. Therefore, we aimed to study the possible association between respiratory microbiome, airway inflammatory response and the development of recurrent wheezing in infants with severe RSV bronchiolitis by age of 3 years.

Materials and Methods

Study design and data collection

This was a prospective observational study. Infants were recruited from Beijing Children's Hospital during two study periods: October 2014 to May 2015 and October to December 2015. We included infants less than 6 month of age diagnosed with a severe bronchiolitis who were positive for RSV and had their first wheezing episode. Severe bronchiolitis was defined as difficulty in breathing with indrawing of the lower chest wall, requiring of oxygen supplement and hospital admission. Respiratory severity score of these patients was also shown [13]. Exclusion criteria were the presence of an ALRI caused by other pathogens, history of previous wheezing, regular use of anti-gastroesophageal reflux medication, prematurity (gestational age <37 weeks), or any chronic disease (lung, cardiac, renal, or hepatic disease). The following was used to exclude bacterial infections: two sputum samples culture positive for bacteria, a peripheral blood cell count of more than 15,000/ μ l or serum C-reactive protein of more than 20 mg/l. In addition, specific PCRs were used to exclude other viral infections.

At enrollment, parents were asked to fill in an extensive questionnaire to obtain information on the infant's demographic factors, environmental exposures, birth and family history, and respiratory health. After discharge contact with the parents/caregivers was scheduled for 1 month, 3 months and then every 3 months to monitor children's respiratory symptoms (date, duration and use of medication) and any change in environmental exposure. The children were followed up to age 3 years. Visits to the study clinic were conducted at 12 months, 24 months and age 3 years; other contacts were conducted by telephone or visit to study site.

We defined the occurrence of a wheezing episode each time a parent/caregiver answered

“Yes” to the question “Has your child had wheezing, and required corticosteroid inhalation?”. Pediatricians’ records were also reviewed for all intercurrent outpatient clinic visits, emergency department visits, and hospitalizations for respiratory symptoms. Recurrent wheezing was defined as the occurrence of 3 or more subsequent episodes of wheezing diagnosed by a pediatrician.

The study was approved by the Ethics committee of Beijing Children’s Hospital, Beijing, China. A written consent was obtained from parents of the study subjects.

Sputum collection, processing, and storage

Sputum was obtained by blind nasotracheal aspiration. A soft suction catheter was introduced through the nostril into the trachea when children cough or breath, then at least one ml of sputum was obtained by mechanical suction into a sterile trap. Suction was applied only once the catheter was in place and not applied during removal of the catheter to avoid aspirating anterior nasal contents [14, 15]. An equal volume of sterile dithiothreitol was added to aliquot of sputum (at least 500 µl), and the sample was mixed vigorously on a plate shaker for 15 to 20 minutes to solubilize the mucus. The sputum was then centrifuged, and the supernatant was collected and stored at -80°C for further use. Another aliquot of 200 µl was stored at -80°C for bacterial DNA extraction.

Bacterial DNA extraction and 16S rRNA sequencing

Bacterial DNA was extracted using QIAamp DNA Stool Mini Kit following the manufacturer’s instructions (Qiagen, Hilden, and Germany). The V4-V5 region of the 16S rRNA gene was amplified using specific primers (515F and 907R). The barcoded amplicons were quantified, pooled to construct the sequencing library, and then sequenced on an Illumina HiSeq 2500 platform to generate pair-ended 250 bp reads. Sequencing data analysis was performed by QIIME version 1.7.0. [16]. The Silva Database (Version132) was used for Species annotation.

Detection of cytokines and LPS

Cytokines in sputum supernatants were analyzed with a multiplex immunoassay (Invitrogen, Vienna, Austria). The lower limits of detection (LOD) for these cytokines were: CXCL-8, 2.25

pg/mL; CCL5, 0.76 pg/mL; CXCL-10 1.56 pg/mL, IFN- γ , 14 pg/mL; IL-12p40, 1.35 pg/mL; IL-6, 8.01 pg/mL; IL-10, 1.87 pg/mL; IL-13, 2.66 pg/mL; IL-17, 1.88 pg/mL; IL-33 2.69pg/ml. INF- α was measured with Human IFN α ELISA Kit (Invitrogen, Vienna, Austria), and the LOD was 7.8 pg/ml. Sputum LPS was measured with Human Lipopolysaccharides (LPS) ELISA Kit (Cusabio, Wuhan, China), the assay sensitivity was 1.56 pg/ml. Values lower than LOD were reported as being half of the LOD.

Microbiome and statistical analysis

Alpha diversity was estimated using the number of observed species, Chao1 estimator, and Shannon index. Nonmetric multidimensional scaling (NMDS) was applied to explore the microbiome data structure on the basis of dissimilarity measurement (Bray-Curtis dissimilarity) between 2 samples. Comparison of microbiome structure between different variables was tested using permutational multivariate ANOVA (PERMANOVA) [17]. Linear discriminant analysis effect size (LEfSe), was used to determine taxa that best characterize each study group [18]. In order to achieve high specificity, we considered taxa with linear discriminant analysis (LDA) score >3.5 to be significant. All the analyses, described above, were performed in R (version 2.15.3).

The Mann-Whitney *U* test was used to compare the continuous variables between infants with and without recurrent wheezing. For comparison of categorical variables, the chi-square test was used. Spearman rank correlation coefficients were calculated to determine the strength of the correlations. These analyses were performed using SPSS (version 19.0). A two sided *P* <0.05 was considered statistically significant.

A detailed description of the methods section is available in Supplementary Methods.

Results

Baseline characteristics of study subjects

During the study period, 80 infants were enrolled. Of them, 78 completed the full follow-up, and two were lost before 2 years. Of the 78 children who completed the follow-up, four

experienced only 1 or 2 subsequent wheezing episodes and thus were not included in the subsequent analysis. Of the 74 children who completed the study and fulfilled inclusion criteria, 26 (35.1%) developed recurrent wheezing (3 or more subsequent episodes) by the age of 3 years, and 48 (64.9%) did not. All patients lived in Beijing or its surrounding areas, and 93.2% were Han nationality.

The detailed information on 74 study subjects was shown in Table 1. Of 26 infants who later developed recurrent wheezing, 18 (69.2%) were boy; of 48 infants who did not develop recurrent wheezing later, 36 (75%) were boy. No difference was found in number of infants who have received antibiotics between the two groups as well as between the duration of antibiotic use before sampling. No significant differences were observed in age, sex, race, delivery mode, feeding type, exposure to antibiotics, or parental history of asthma between the two groups.

Airway microbiota structure of study subjects

Sputum samples from the 74 infants underwent 16S rRNA gene sequencing, and two samples were excluded for analysis because of insufficient sequence reads depth. Altogether, 5,866,967 raw tags were generated from 72 samples. After the filtering of low-quality tags and the removing of the chimera sequence, 4,997,592 (median, 71144; IQR, 64481-75944, per sample) effective tags were used for subsequent analyses and clustered into 19,273 OTUs (median, 189; IQR, 138-263, per sample).

Overall, four dominant phyla were detected: Firmicutes (mean relative abundance, 84.5%), Proteobacteria (9.1%), Bacteroidetes (2.5%) and Actinobacteria (1.9%), accounting for 98.0% of the total sequences. At the genus level, the sputum microbiota was dominated by *Streptococcus* (80.0%), *Haemophilus* (3.6%), *Moraxella* (1.8%), unidentified *Prevotellaceae* (1.6%), unidentified *Clostridiales* (1.0%). (Figure 1). The relative abundances of top 30 genera including the common bacteria e.g. *Corynebacterium* and *Staphylococcus* are shown in Table E1.

The bacterial composition analyzed by NMDS generally formed two clusters, with a partial overlap between the clusters (Figure 2A). Consistent with this, PERMANOVA testing confirmed no statistically significant differences in the overall bacterial community structure ($P=0.149$). By

measuring alpha diversity, we found that infants who later developed recurrent wheezing possessed a trend of greater airway microbiota richness and diversity (The number observed species, $P=0.023$; Chao1 estimator, $P=0.040$), although the Shannon index was not statistically significant ($P=0.311$) (Figure 2B).

Since most of infants who have received antibiotic treatment before this time of sampling, we analyzed possible effects of antibiotic use on airway bacterial structures between infants who later developed recurrent wheezing and who did not. As shown in Table 1, the median duration of antibiotic use before this time of sample was 3 days. We next compared the bacterial structures between infants who received antibiotics more than 3 days and those who had antibiotics less than or equal to 3 days. No significant difference was observed (PERMANOVA, $P=0.169$).

Distinct microbiota members during RSV bronchiolitis between infants with and without subsequent recurrent wheezing

To identify bacterial taxa associated with the development of recurrent wheezing, we performed LEFSe analysis. When LDA scores > 3.5 were used, 2 phyla, 2 classes, 3 orders, 2 families and 4 genera were found to be significantly different between infants who later developed recurrent wheezing and those who did not (Figure 3A, B). In infants who later experienced recurrent wheezing, an enrichment in Proteobacteria phyla was observed, while the Firmicutes was enriched in infants without recurrent wheezing. The most discriminating bacterial genera were *Haemophilus*, *Moraxella* and *Klebsiella*. Further, *Haemophilus* and *Klebsiella* were detected in almost all of the samples. However, *Moraxella* was found in 24 (92.3%) and 32 (77.8%) in infants who later developed recurrent wheezing and those who did not ($P=0.026$). The relative abundances of the top 30 genera in infants who later developed recurrent wheezing and those who did not were shown in the Table E2.

Difference in level of airway LPS between infants with and without subsequent recurrent wheezing

Of 26 infants who later developed recurrent wheezing, 24 (92.3%) had detectable LPS,

whereas of 46 infants who did not develop recurrent wheezing later, 35 (76.1%) had detectable LPS ($P=0.162$). A significant difference in the level of LPS was found between the two groups of patients (median, 11.4 pg/ml; IQR, 4.1-19.4 vs. median, 3.7 pg/ml; IQR, 1.6-7.5, $P=0.003$) (Figure 4).

Difference in level of airway inflammatory cytokines between infants with and without subsequent recurrent wheezing

A total of 11 chemokines and cytokines were measured in 72 sputum samples. Significantly higher levels of CXCL8 ($P=0.004$), CCL5 ($P=0.029$) IL-6 ($P=0.004$) and IL-13 ($P<0.001$) were found in infants who later developed recurrent wheezing than those who did not (Figure 5A). However, no such differences were observed in CXCL10, IFN- γ , IL-10, IL-12p40, IL-17A and IL-33 (Figure 5B). When the ratios of characteristic Th2 and Th1 cytokines IL13/IFN- γ was compared, significantly higher levels were found in infants who later developed recurrent wheezing than those who did not ($P<0.001$, Fig 5C). For IFN- α measured by ELISA, six (8.3%) samples had detectable IFN- α . Of them, one (3.8%) was found in 26 infants who later developed recurrent wheezing and five (10.9%) in 48 infants who did not develop recurrent wheezing later ($P=0.186$) (Figure E1).

Relationship between discriminative bacterial species, mucosal inflammatory response and LPS

The relative abundance of *Haemophilus* was positively associated with levels of CXCL8 ($r=0.246$, $P=0.037$). The relative abundance of *Moraxella* was positively associated with IL-6 ($r=0.236$, $P=0.046$) and IL-10 ($r=0.266$, $P=0.024$). Airway level of LPS was positively associated with the level of IL-17A ($r=0.260$, $P=0.028$).

Discussion

In this prospective study, we demonstrated that during the first episode of severe RSV bronchiolitis in infants < 6 months of age the relative abundance of *Haemophilus*, *Moraxella* and

Klebsiella was higher in infants who later developed recurrent wheezing by the age of 3 years than those who did not. Moreover, in infants who later developed recurrent wheezing, elevated and Th2 biased inflammatory responses were observed, which seemed to be associated with the higher abundance of these potential bacterial pathogens. To our best knowledge, this is the first study to show that airway microbiota may influence the pattern of airway inflammatory responses during RSV bronchiolitis in infancy, potentially contributing to the development of subsequent recurrent wheezing.

During the first episode of severe RSV bronchiolitis in Chinese infants the respiratory microbiota was dominated by four phyla, Proteobacteria, Firmicutes, Bacteroidetes and Actinobacteria, and the three most abundant genera were *Streptococcus*, *Haemophilus* and *Moraxella*. These findings are in line with those reported in industrialized countries [11, 19]. We also observed a general higher abundance of *Streptococcus* genera, and a general lower abundance of *Moraxella*, *Corynebacterium* and *Staphylococcus* genera in our samples compared to those reported in the previous studies [9-11, 19]. Possible reasons for these discrepancies observed included differences in study populations, severity and onset of disease, use of antibiotics as well as collection of samples. It has been shown that severity of RSV infections is positively associated with abundance of *Streptococcus* in the respiratory tract [11]. Indeed, the patients included in this study had severe RSV bronchiolitis and they all required hospitalization. In addition, it should be kept in mind that in this present study sputum samples were used, whereas in other studies nasopharyngeal (NP) swabs or aspirates were used. Man et al, recently found that three common constituents of the nasopharyngeal microbiota: *Staphylococcus*, *Corynebacterium* and *Dolosigranulum* spp were almost exclusively present in NP samples and absent from endotracheal aspirates [20]. Earlier studies have shown that there are differences in bacterial structures between anatomical sites of respiratory tract. By using culture and 16S rRNA sequencing Stearns et al compared bacterial composition between nasopharynx and oropharynx among children younger than 19 months of age and found that there is a significant difference in bacterial community between the two anatomical sites [21]. In oropharynx of young children a high proportion of *Streptococcus* was detected. However, Child nasopharynx had larger proportions of Proteobacteria

such as *Moraxella*, Enterobacteriaceae and *Haemophilus*, as well as the Firmicute *Enterococcus*[21]. Similar differences in bacterial communities were also reported in children when samples taken from nasopharynx, oropharynx and bronchoalveolar lavage were compared [22]. It seems that the bacterial community identified in sputum of infants included in our study represented a mixture of these bacteria reported in oropharynx and sputum. It is known that it is not possible to put catheter blindly to all children. Therefore, it is likely that the sputum extracting catheters used did not reach to trachea as described in the method section, instead it reached to oropharynx in some patients.

By measuring alpha diversity using the number of observed species, Chao1 estimator, Shannon index, we found that infant who later developed recurrent wheezing possessed a greater airway microbiota diversity in comparison with infants who did not. Previous studies reported that asthmatic subjects possessed greater airway microbiota diversity than nonasthmatic subjects [23, 24], and there was a relationship between increased airway bacterial diversity and greater bronchial hyperresponsiveness. This is contrary to findings in the gut microbiota, in which inflammatory disorders are associated with decreased bacterial diversity [25].

Several studies reported that in asthmatic patients there were significant changes in the lung microbiota and the most common dysbiotic communities related to an increase in Proteobacteria [24, 26-27]. In this study, we observed that in infants who later developed recurrent wheezing there was a higher abundance of Proteobacteria. Among the phylum of Proteobacteria observed, a significant higher abundance in *Haemophilus*, *Moraxella* and *Klebsiella* genera was found. Our finding is also in agreement with the report by Bisgaard et al in which early life asymptomatic colonization of the upper airway with *H. influenzae* and *M. catarrhalis*, but not *S. aureus* in young infants was associated with a higher risk of developing persistent wheezing and asthma in childhood [28]. Interestingly, in our study, there was no difference in abundance of airway *Streptococcus* between infants who later developed recurrent wheezing and those who did not. A recent study also found that 2 weeks of treatment with Azithromycin during RSV bronchiolitis reduced the abundance of *Moraxella*, and lower *Moraxella* abundance was associated with lower odds of the developing recurrent wheezing over the next 12 months [29].

In this study, higher level of sputum CXCL8, CCL5, IL-6 and IL-13 were observed in infants who later developed recurrent wheezing, showing that these infants had elevated local inflammatory responses. When the ratio of characteristic Th2/Th1 cytokines was examined, an increased Th2 response was noticed in infants who developed recurrent wheezing later. These changes seemed to be associated with the increased abundance of either *H. influenza*, *M. catarrhalis* or both. Ederveen et al, recently reported that in young infants with a RSV infection, the abundance of *Haemophilus* in nasopharyngeal microbiota was clearly associated with production of airway CXCL8 [30]. Although the exact mechanism(s) by which the airway microbiota influences the pattern of airway inflammatory response during RSV bronchiolitis is unclear, it may be partly due to the higher level of LPS produced by these Gram-negative pathogenic bacteria observed in this study. Airway chronic bacterial colonization and endotoxin exposure might induce persistent activation of the innate immune system resulting in the production of pro-inflammatory cytokines in airway [31]. Our finding together with previous reports suggest that alteration in the airway microbiota might influence the pattern of host immune response during RSV bronchiolitis which may contribute to the development of subsequent recurrent wheezing or asthma in childhood. However, it remains to be shown how the airway microbiome influences the immune response and subsequently the severity of viral infection and recurrent wheezing or the immune response among infants who develop recurrent wheezing results in differential colonization. Bellinghausen C et al reported that exposure to common respiratory bacteria can alters the airway epithelial response to subsequent viral infection [32], and exposure of airway epithelial cell to *H. influenzae* before and during RSV-infection synergistically increased the release of IL-6 and CXCL8[32, 33]. Overall, our results support the finding that early interaction between airway microbiota and viral pathogen can modulate host immune responses, potentially influence the development of recurrent wheezing or asthma in childhood.

In addition to *Haemophilus* and *Moraxella*, we also observed a higher abundance of *Klebsiella* in infants who later developed recurrent wheezing. The result has not been previously reported in infants who developed recurrent wheezing after RSV infections. However, the prevalence of *Klebsiella* in sputa of our study subjects is supported by the recent finding that *K.*

pneumoniae has been one of the leading bacterial pathogens causing community-acquired pneumonia in Chinese children [34]. In a study carried out among adults, Huang et al found that there was almost an 8-fold increase in member of *Klebsiella* in patients with severe asthma compared to healthy controls or patients with mild-moderate asthma [27]. However, it remains to be shown whether the presence of *Klebsiella* in airway microbiota could contribute to the pathogenesis of asthma.

There were certain limitations in this study. First, no healthy controls were included. Therefore, the microbiome profiles in healthy Chinese children are not known. Secondly, most of our patients had received antibiotic treatment before sputum sampling. However, our study was focused on comparison of airway microbiome in infants who later developed recurrent wheezing and those did not, and the rate of antibiotic treatments was similar in both groups. Thirdly, we only examined the airway microbiota in patients with an acute stage of RSV bronchiolitis, and did not test their samples collected in convalescent stage of these patients.

In conclusions, our results suggest that higher abundance of *Haemophilus* and *Moraxella* in airway microbiome might modulate airway immune response during severe RSV bronchiolitis in infancy, potentially contributing to the development of subsequent recurrent wheezing in later childhood.

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Table 1. Baseline characteristics of infants who later developed recurrent wheezing and those who did not (N=74).

Characteristics	RW + (n=26)	RW - (n=48)	<i>P</i> value
Age (mon)	2.6 (1.8-3.8)	2.2 (1.7-3.4)	0.468
Male (%)	18 (69.2)	36 (75.0)	0.594
Han nationality (%)	23 (88.5)	45 (93.8)	1.000
Gestational age (wk)	38.9 (37.7-39.5)	38.8 (37.7-39.3)	0.670
Birth weight (g)	3.0 (2.8-3.5)	3.3 (2.9-3.6)	0.181
Birth by cesarean section (%)	15 (57.7)	28 (58.3)	0.957
Mostly breast milk (%)	16 (61.5)	32 (66.7)	0.659
Siblings under age 14 (%)	18 (69.2)	27 (60.4)	0.275
Parental smoking (%)	12 (46.2)	19 (39.6)	0.557
Parental history of asthma (%)	1 (3.8%)	1 (2.1%)	1.000
Historical use of antibiotics (%)	5 (19.2)	10 (20.8)	0.870
Current use of antibiotics before sampling (%)†	23 (88.5%)	45 (93.8%)	0.516
Erythromycin (%)	15 (65.2%)	33 (73.3%)	0.660
Days of use before sampling(d)	3 (3-5)	3 (2-4)	0.238
Onset of disease (d)	6 (5-7)	6 (4-7)	0.266
Respiratory severity score	8.0 (7.0-9.0)	8.0 (6.25-8.75)	0.107
Hospital Stay (d)	7.5 (5.8-10.0)	5.3 (7.0-8.0)	0.198
WBCs (/ul)	8250 (7138-10640)	8690 (6845-11360)	0.396
Neutrophils %	36.1 (25.6-43.6)	33.0 (23.1-41.2)	0.515
Lymphocytes %	53.7 (46.7-61.1)	57.3 (48.6-66.0)	0.223

Monocytes%	9.8 (7.5-12.3)	8.3 (5.8-11.7)	0.143
Eosinophils %	0.1 (0-0.85)	0.25 (0-0.90)	0.393
Serum CRP level (mg/l)	2.48 (1.66-8.64)	2.26 (1.36-6.57)	0.483

RW+ indicates infants who later developed recurrent wheezing; RW- indicates infants who did not develop recurrent wheezing later.

Data are median values (interquartile ranges) or no. (%) of subjects.

P value was assessed by using the Mann-Whitney U test or the χ^2 test as appropriate.

† Most of infants had used erythromycin or cephalosporin.

Figure legends

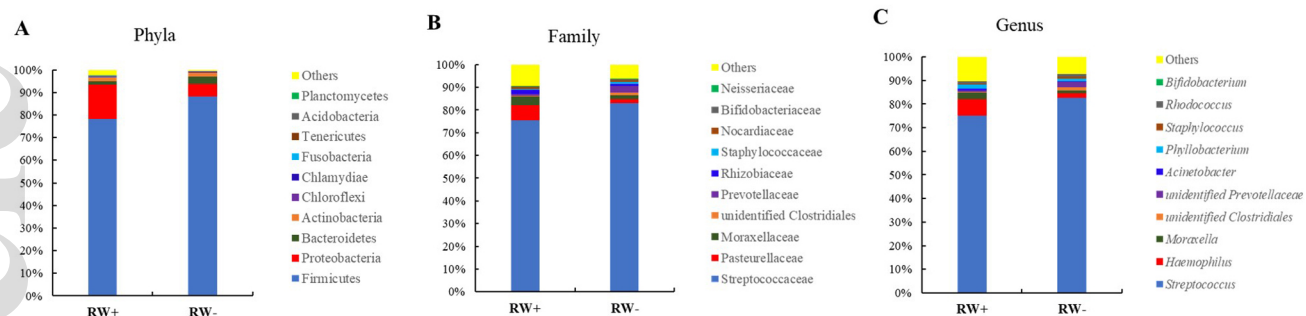
Figure 1. The distributions of the top 10 bacteria (mean relative abundance) in the sputum microbiomes during RSV bronchiolitis of infants who later developed recurrent wheezing and those who did not. Results were shown at the phyla level (A), family level (B), genus level (C) and species level (D). RW+ indicates infants who later developed recurrent wheezing; RW- indicates infants who did not develop recurrent wheezing later.

Figure 2. A, Bacterial community structure during RSV bronchiolitis in infant who later developed without recurrent wheezing and those who did not. In this nonmetric multidimensional scaling (NMDS) plot, each dot represents the microbiome composition of 1 sample. Dots that are closer to each other have a more similar microbiome composition than dots that are farther to each other. B, α -Diversity measures (Observed species, Chao1 index, Shannon index) in infant with and without recurrent wheezing. The median values and interquartile ranges were shown. The P value was assessed by using Mann-Whitney U test. RW+ indicates infants who later developed recurrent wheezing; RW- indicates infants who did not develop recurrent wheezing later.

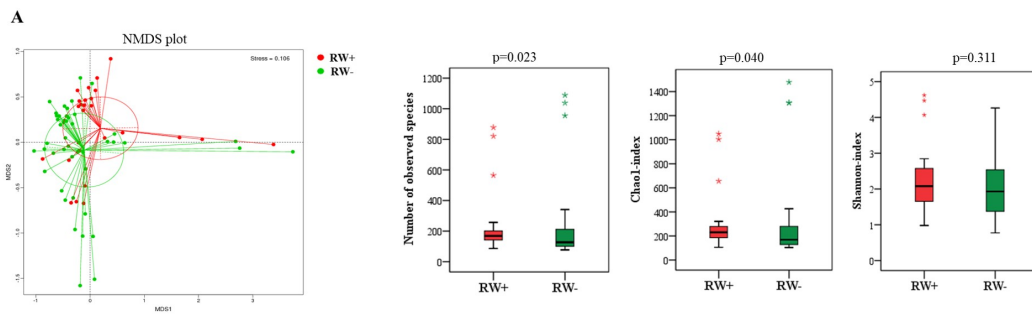
Figure 3. Linear discriminant analysis (LDA) shows distinct sputum microbiota tax during RSV bronchiolitis associated with the development of subsequent recurrent wheezing. A, LDA scores as calculated by LEfSe of taxa differentially abundant in infants with and without recurrent wheezing. B, LEfSe cladogram representing differentially abundant taxa. All taxa were significantly associated with either the development of recurrent wheezing or did not ($P < 0.05$). Only taxa with LDA scores >3.5 are presented. RW+ indicates infants who later developed recurrent wheezing; RW- indicates infants who did not develop recurrent wheezing later.

Figure 4. Level of LPS in sputum from infants who later developed recurrent wheezing and those who did not. The median values and interquartile ranges were shown. The P value was assessed by using Mann-Whitney U test. RW+ indicates infants who later developed recurrent wheezing; RW- indicates infants who did not develop recurrent wheezing later.

Figure 5. Comparison of inflammatory mediates levels in sputum from infants who later developed recurrent wheezing and those who did not. **A**, Level of chemokines and cytokines with significant difference identified between the two groups. **B**, Level of chemokines and cytokines without significant difference identified between the two groups. **C**, ratio calculated for Th1/Th2 cytokines. The median values and interquartile ranges were shown. The *P* value was assessed by using Mann-Whitney *U* test. RW+ indicates infants who later developed recurrent wheezing; RW- indicates infants who did not develop recurrent wheezing later.

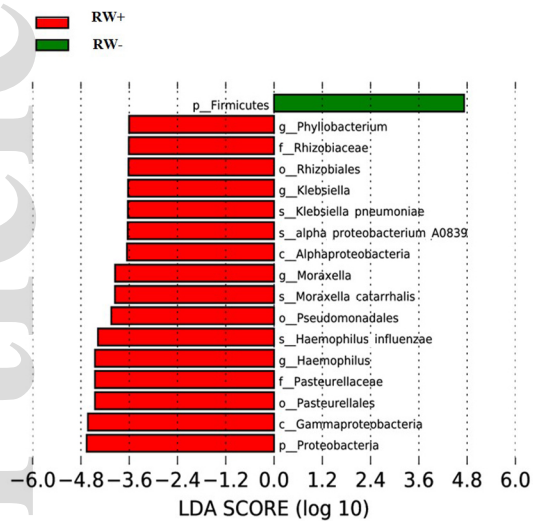


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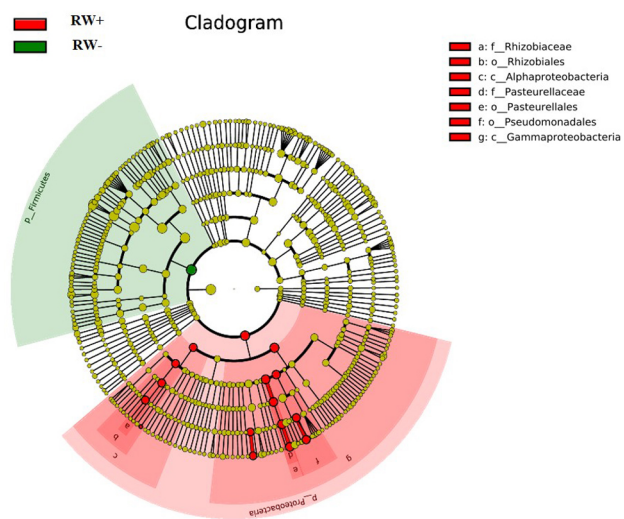


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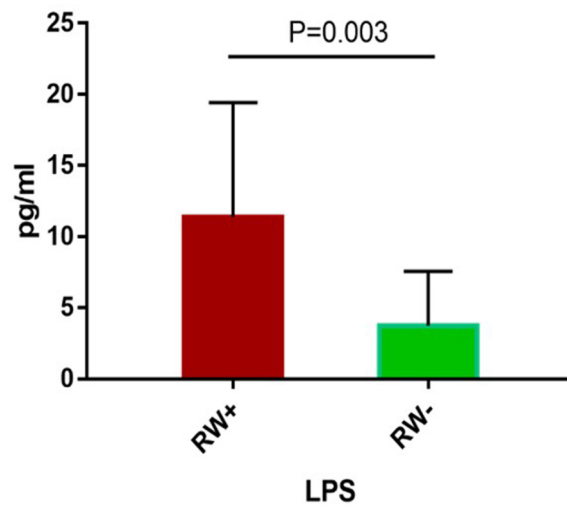
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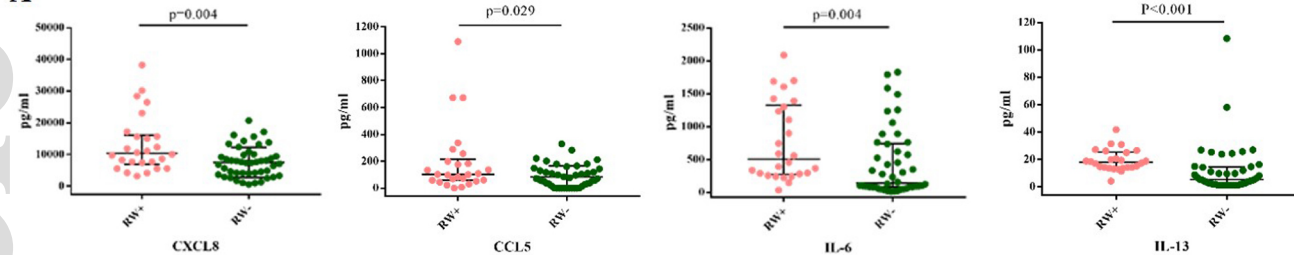


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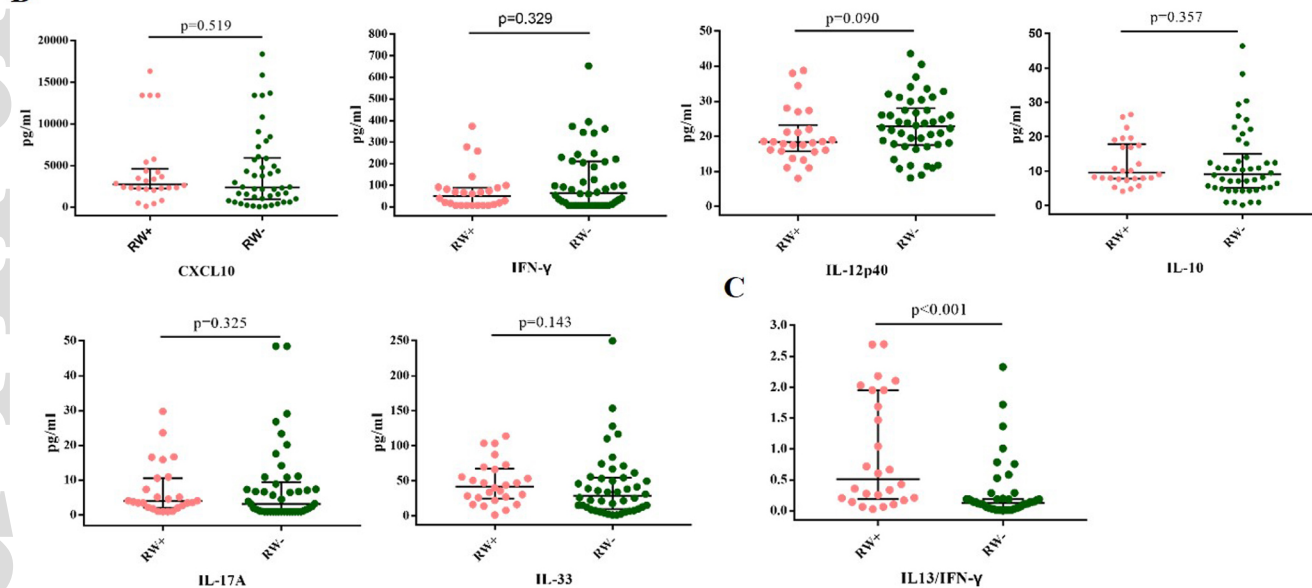


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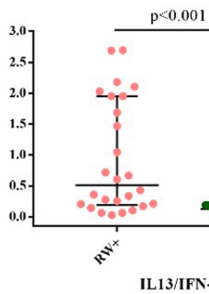
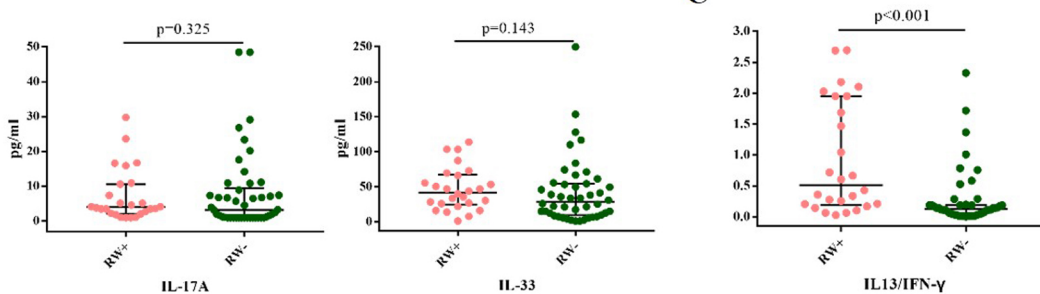
A



B



C



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Supplementary Methods

Bacterial DNA extraction and 16S rRNA sequencing

Bacterial DNA was extracted using QIAamp DNA Stool Mini Kit following the manufacturer's instructions (Qiagen, Hilden, and Germany). The Bacterial DNA was quantified and used for PCR amplification. The V4-V5 hypervariable region of 16S rRNA gene was amplified from sputum DNA by using specific primers 515F (5-GTGCCAGCMGCCGCGGTAA-3) and 907R (5-CCGTCAATTCCTTTGAGTTT-3). The barcoded amplicons were quantified, pooled to construct the sequencing library, and then sequenced on an Illumina HiSeq 2500 platform to generate pair-ended 250 bp reads.

The raw mate-paired files of 16S rRNA gene sequences were first trimmed to dispose bases with high error probability (>0.01) and merged using FLASH (version 1.2.7). The pre-processed data were then analyzed using QIIME (version 1.7.0) software package [E1]. Operational taxonomic units (OTUs) were clustered with 97% sequence similarity using UPARSE (version 7.0.1001). The normalized OUT tables were used for diversity and statistical analyses. The Silva Database (Version132) was used for Species annotation.

Microbiome analysis

Alpha-diversity was estimated using the number of observed species, Chao1 estimator, Shannon index. Nonmetric multidimensional scaling (NMDS) was applied to explore the microbiome data structure on the basis of dissimilarity measurement

(Bray-Curtis dissimilarity) between 2 samples. Comparison of microbiome structure between different variables was tested using permutational multivariate ANOVA (PERMANOVA), a non-parametric test similar to ANOVA but that does not require the data to be normally distributed. PERMANOVA uses a permutation test with pseudo F ratios to generate the P values [E2]. Linear discriminant analysis effect size (LEfSe), a method used for biomarker discovery, was used to determine taxa that best characterize each study group [E3]. In order to achieve high specificity, we considered taxa with linear discriminant analysis (LDA) score >3.5 to be significant. All the analyses, described above, were performed in R (version 2.15.3).

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Supplementary Table

Supplementary Table E1. Relative abundances of top 30 genera in sputum of infants with RSV bronchiolitis.

genus	Mean relative abundance
<i>Streptococcus</i>	0.79977
<i>Haemophilus</i>	0.03617
<i>Moraxella</i>	0.01805
unidentified_ <i>Clostridiales</i>	0.00974
unidentified_ <i>Prevotellaceae</i>	0.01632
<i>Acinetobacter</i>	0.00611
<i>Phyllobacterium</i>	0.01171
<i>Staphylococcus</i>	0.00795
<i>Rhodococcus</i>	0.00963
<i>Bifidobacterium</i>	0.00155
<i>Veillonella</i>	0.00711
<i>Neisseria</i>	0.00254
<i>Klebsiella</i>	0.00541
<i>Alloprevotella</i>	0.00256
<i>Porphyromonas</i>	0.00166
<i>Granulicatella</i>	0.00218
<i>Chlamydia</i>	0.00063
<i>Gemella</i>	0.00680
unidentified_ <i>Enterobacteriaceae</i>	0.00231
<i>Lactobacillus</i>	0.00214
<i>Leuconostoc</i>	0.00106
<i>Ureaplasma</i>	0.00058
unidentified_ <i>Corynebacteriaceae</i>	0.00121
<i>Lactococcus</i>	0.00224
<i>Actinomyces</i>	0.00182
<i>Fusobacterium</i>	0.00049
<i>Anoxybacillus</i>	0.00236
<i>Prevotella</i>	0.00071
<i>Leptotrichia</i>	0.00023
<i>Thermomonas</i>	0.00046

53 **Supplementary Table E2.** Relative abundances of top 30 genera in sputum of infants who later
54 developed recurrent wheezing and those who did not during RSV bronchiolitis.

genus	Infants with recurrent wheezing (median and interquartile range)	Infants without recurrent wheezing (medians and interquartile range)
<i>Streptococcus</i>	0.86854 (0.60502-0.92671)	0.87807 (0.75870-0.93478)
<i>Haemophilus</i>	0.00477 (0.00285-0.00981)	0.00158 (0.00036-0.00670)
<i>Moraxella</i>	0.00051 (0.00026-0.00265)	0.00010 (0.00000-0.00047)
unidentified_ <i>Clostridiales</i>	0.00031 (0.00012-0.00084)	0.00059 (0.00052-0.00556)
unidentified_ <i>Prevotellaceae</i>	0.00157(0.00055-0.00505)	0.00111 (0.00026-0.00475)
<i>Acinetobacter</i>	0.00365 (0.00076-0.00545)	0.00132 (0.00047-0.00295)
<i>Phyllobacterium</i>	0.00579 (0.00416-0.02091)	0.00157 (0.00058-0.00849)
<i>Staphylococcus</i>	0.00220 (0.00141-0.00483)	0.00210 (0.00112-0.00656)
<i>Rhodococcus</i>	0.00223 (0.00005-0.01266)	0.00356 (0.00056-0.00992)
<i>Bifidobacterium</i>	0.00005 (0.00000-0.00014)	0.00012 (0.00000-0.00049)
<i>Veillonella</i>	0.00339 (0.00193-0.00756)	0.00390 (0.00206-0.00824)
<i>Neisseria</i>	0.00009 (0.00003-0.00086)	0.00042 (0.00010-0.00164)
<i>Klebsiella</i>	0.00306 (0.00162-0.00962)	0.00186 (0.00071-0.00348)
<i>Alloprevotella</i>	0.00008 (0.00000-0.00032)	0.00043 (0.00015-0.00223)
<i>Porphyromonas</i>	0.00008 (0.00003-0.00041)	0.00012 (0.00003-0.00077)
<i>Granulicatella</i>	0.00090 (0.00043-0.00222)	0.00097 (0.00034-0.00243)
<i>Chlamydia</i>	0.00000 (0.00000-0.00000)	0.00000 (0.00000-0.00003)
<i>Gemella</i>	0.00367 (0.00230-0.01135)	0.00389 (0.00195-0.00730)
unidentified_ <i>Enterobacteriaceae</i>	0.00085 (0.00041-0.00156)	0.00107 (0.00041-0.00202)
<i>Lactobacillus</i>	0.00132(0.00063-0.00205)	0.00098 (0.00054-0.00216)
<i>Leuconostoc</i>	0.00003(0.00000-0.00005)	0.00003 (0.00000-0.00006)
<i>Ureaplasma</i>	0.00000 (0.00000-0.00000)	0.00000 (0.00000-0.00003)
unidentified_ <i>Corynebacteriaceae</i>	0.00045 (0.00029-0.00111)	0.00042 (0.00021-0.00087)
<i>Lactococcus</i>	0.00065 (0.00035-0.00203)	0.00173 (0.00048-0.00266)
<i>Actinomyces</i>	0.00046 (0.00024-0.00188)	0.00079 (0.00031-0.00170)
<i>Fusobacterium</i>	0.00000 (0.00000-0.00003)	0.00000 (0.00000-0.00006)
<i>Anoxybacillus</i>	0.00060 (0.00015-0.00128)	0.00085 (0.00020-0.00395)
<i>Prevotella</i>	0.00001 (0.00000-0.00016)	0.00003(0.00000-0.00032)
<i>Leptotrichia</i>	0.00000 (0.00000-0.00003)	0.00000 (0.00000-0.00001)
<i>Thermomonas</i>	0.00038 (0.00002-0.00126)	0.00000 (0.00000-0.00003)

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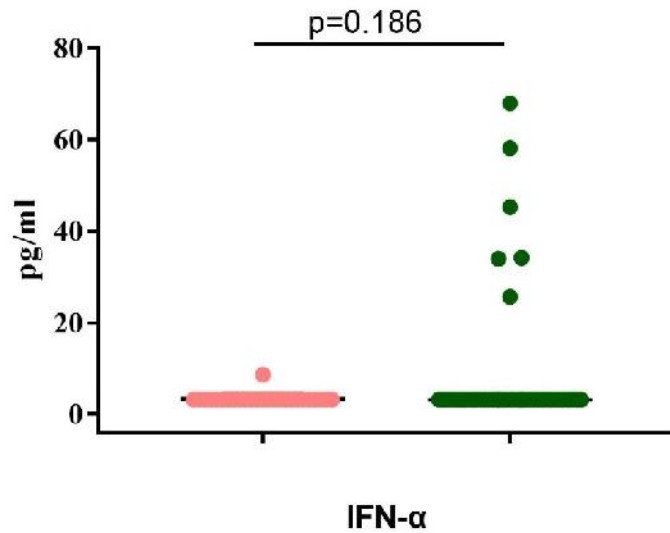
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Supplementary Figure

Figure E1



Level of IFN- α in sputum from infants who later developed without recurrent wheezing and those who did not. The median values and interquartile ranges were shown. The *P* value was assessed by using Mann-Whitney *U* test. RW+ indicates infants who later developed recurrent wheezing; RW- indicates infants who did not develop recurrent wheezing later.