Research Square

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Altered Gut Microbiota in Infants is Associated with Respiratory Syncytial Virus Disease Severity

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Abstract

Rationale Respiratory syncytial virus (RSV) is the number one cause of lower respiratory tract infections in infants. There are still no vaccines or specific antiviral therapies against RSV, mainly due to the inadequate understanding of RSV pathogenesis. Recent data suggest a role for gut microbiota community structure in determining RSV disease severity.

Objectives Our objective was to determine the gut microbial profile associated with severe RSV patients, which could be used to help identify future at-risk patients and develop therapeutically protective microbial assemblages that may stimulate immuno-protection.

Methods We enrolled 58 infants hospitalized with RSV, 5 were admitted to the pediatric intensive care unit and thus considered severe, 53 were admitted to the pediatric ward and considered moderate, and 37 healthy controls collected from infants during “well-baby checkups” from 2012 to 2015. We evaluated the composition of gut microbiota within 72 hours of enrollment in these patients via 16s sequencing of fecal DNA.

Measurements and Main Results There was a significant enrichment in S24_7, Clostridiales, Odoribacteraceae, Lactobacillaceae, and Actinomyces in RSV vs. controls. Patients with severe RSV disease had slightly lower alpha diversity (richness and evenness of the bacterial community) of the gut microbiota compared to patients with moderate RSV and healthy controls. Beta diversity (overall microbial composition) was significantly different between all RSV patients (severe and moderate) compared to controls and had significant microbial composition separating all three groups (control, moderate RSV, and Severe RSV).

Conclusions Collectively, these data indicate that a unique gut microbial profile that is associated with severe RSV disease. More mechanistic experiments are needed to determine whether the differences observed in gut microbiota are the cause or consequences of severe RSV disease.

2 Background

Respiratory syncytial virus (RSV) is a ubiquitous respiratory virus infecting the majority of the human population by 1 year of age (1). While most infants develop mild upper respiratory tract infections (URTI), 0.5-2% develop severe, lower respiratory tract infections (LRTI), including bronchiolitis and pneumonia, requiring hospitalization (2). Each year, RSV causes 33 million cases and 3.4 million hospitalizations worldwide in children under 5 years of age (3). In the U.S. alone, RSV causes 85,000 to 144,000 hospitalizations (4), 14,000 deaths (2), and ~$2.6 billion in medical care costs each year (5). Despite the substantial RSV-associated medical and economic burden worldwide, a vaccine for RSV currently remains elusive. The only prevention for infants at high-risk for complications (i.e., premature or underlying immune/cardiopulmonary diseases) is in the form of a monoclonal antibody, palivizumab. In view of the fact that RSV is a “significant unmet medical need” (6) with no known vaccine, there is a necessity to find therapeutic approaches to prevent and treat severe RSV infections.

The recently discovered relationship between respiratory health and gut microbiome dysbiosis has attracted growing interest for therapeutic manipulation to improve vaccine effectiveness and to allow for alternative therapies for viruses with no known vaccines, like RSV. Clinical studies demonstrate that prebiotic and probiotic supplementation alters gut microbiota (7), reduces the incidence of rhinovirus infections in preterm infants (8), and reduces rhinovirus infection duration and severity in adults (9). In mice, gut microbial dysbiosis induced by antibiotic treatment inhibits pulmonary type I interferons (IFNs) and T cell responses, resulting in increased lung viral loads after infection with influenza virus (10, 11). On the other hand, supplementing mice with Lactobacillus plantarum promotes type I IFNs and reduces influenza viral load in the lung (12). In mice, both RSV and influenza virus infection alters the gut microbiome and provides preferential growth environments for the S24_7 family showing a correlation between S24_7 family and RSV infection, although the mechanism is still unknown (13). Several reports have studied the association of airway microbiota with RSV severity (14, 15); nevertheless, there have been no studies that directly investigated the role of gut microbiota in the severity of RSV infections.

We hypothesize that gut microbiota could play an essential role in the pathogenesis of RSV in infants. This study characterizes the gut microbiome in infants hospitalized with RSV infection differentiating severe infections requiring infants to be put into pediatric intensive care unit (PICU) and moderate infections of infants in the general ward. Our results demonstrate that there is a relationship between RSV infection and gut microbiome that could conceivably be interrogated to develop a useful therapeutic target to prevent severe RSV disease.

3 Results

Characteristics of Study Population

Our study population included 37 patients enrolled from Le Bonheur Outpatient Clinic during well-baby checkup (control), 53 patients admitted to the general ward (moderate) who tested positive for RSV and negative for influenza by RT-PCR, and 5 patients admitted to the pediatric intensive care unit (PICU) (severe) who tested positive for RSV and negative for influenza by RT-PCR, from 2012 to 2015. Patients had an average age of 93, 94, and 66 days, respectively ($p = 0.28$; Table 1). The majority of moderate and severe patients had O2 supplementation ($p = < 0.001$) and wheezing ($p = 0.009$) compared to the control group. As both wheezing and O2 supplementation is commonly associated with RSV, this correlation is not surprising. However, the only significant difference observed between the RSV groups (moderate and severe) regarding all demographic metadata was in the family history of asthma due to none of the severe patients having a family history of asthma (Table 1).

The Gut Microbiome

Although the role of gut microbiota in regulating the immune system and respiratory infections is being increasingly recognized, the role of gut microbiota in RSV disease severity has never been addressed in infant humans. Here, we analyzed the gut microbiota from infants hospitalized with RSV. The patient

fecal DNA was isolated for microbiome analysis by 16 s rRNA sequencing. Resulting sequencing data was analyzed using Qiime 2 pipeline with a 99% OTU identification by GreenGenes database (Fig. 1B).

Gut microbiome richness is reduced in severe RSV infected patients

To examine the gut microbiota in RSV patients, we determined the α-diversity in the stool samples using Simpson index and Shannon index in QIIME 2. There was no difference in the species richness and evenness (α-diversity) of gut microbiome between RSV patients and control patients (Fig. 2A, B). We further segregated the RSV patients into moderate and severe RSV patients (based on their PICU status) to determine if there are differences in gut microbiome as a result of severity of RSV disease. The patients with severe RSV (PICU) had a slightly reduced species richness and evenness in the gut microbiota compared to that of control and moderate RSV (non-PICU) patients (Fig. 2C, D). This slight decrease of richness and evenness was not significant but could be indicative of a specific taxa abundance change being associated with severe patients compared to control and moderate patients.

Gut microbiome phylogenetic diversity shows significant clustered populations in RSV disease severity

In addition to richness and evenness of gut microbiota, we determined the overall qualitative relatedness of phylogenetic distances (β-diversity). Significant differences were found in microbiome composition between RSV and control group as well as between disease severity. We applied the multivariate method of PERMANOVA with 999 permutations using unweighted UniFrac distance matrix ($P < 0.0001$). In addition, we used multivariate redundant discriminant analysis (RDA) at the OTU level to visualize the separation of RSV and the control groups (Fig. 3A). The patients were further distinguished into groups by disease severity (Fig. 3B). This showed that there were not only statistically significant differences between the control and RSV patients, but there was a further separation between severity of RSV in the patients ($P < 0.05$). To expand our understanding of the phylogenetic differences driving the separation of clusters, we performed Linear Discriminate Analysis of Effect size (LEfSe) analysis to identify enriched taxa that best characterize the alterations in the severe, moderate, and control patients. We were able to identify the key phylotypes that could be used as biomarkers at different phylogenetic levels to discriminate between control, moderate, and severe RSV (Fig. 4; Supplemental Fig. 2, 3). LEfSe comparison of the gut microbiota highlights the distinctive microbial profile of patients with severe disease. The taxa in gray show the specific microbial groups that are enriched in the severe patients when comparing to the moderate and control groups. The blue and the red colors show the specific microbial groups that are enriched in the microbiome for moderate and control patients, respectively. Due to the fact that LEfSe includes biologically informative clades differentiating two or more phenotypes, this may be important for clinical therapeutics. On a family level we see that S24_7 and Odoribacteraceae are associated with severe RSV, while Clostridiales and Lactobacillaceae are characteristic of moderate RSV (Fig. 4A). At the genus level, we observed S24_7, Odoribacter, and Oribacterium being associated with severe RSV and Clostridiales and Coriobacteriaceae being associated with moderate disease (Fig. 4B). The specific microbes associated with genus and OTUs are also shown in Fig. 4C. Furthermore, we used discriminant analysis of principal components at the OTU level in conjunction with one-way ANOVA with

Benjamini-Hochberg correction and Tukey's range test to further elucidate the microbial differences between disease severity groups. We showed that Enterococcus, Lactobacillus, Oscillospira, Odoribacter, Tissierella Soehngenia, and S24_7 were among the top bacterial OTUs to separate the specific microbiome characteristics between the groups in a discriminant analysis of principal components (DAPC) cluster graph (Fig. 5A). Looking at the disease severity groups, at the Family level, S24_7 is increased in severe patients compared to control patients while Moraxellaceae is decreased (Fig. 5B). At the genus level, S24_7 is again increased in severe patients compared to control patients while Tissierella Soehngenia is decreased (Fig. 5C). At the OTU level, we observed an increase in relative percent abundance of S24_7 and Odoribacter with a decrease in Tissierella Soehngenia, between severe and control RSV. However, the most significant of these was significant increases in S24_7 in severe patients compared to moderate patients. This coincides with the LEfSe analysis, giving additional data showing that there is an association with S24_7 and severity of RSV disease.

4 Discussion

The lung and gut microbiome axis has increasingly been studied over the last decade to elucidate the relationship between microbiome dysbiosis and immunity in the lung (16–22). The gut-lung microbiome axis has been proposed and studied for its ability to help regulate the immune system (13, 22, 23). Because there are a vast number of microbes residing in the gut, there is no shortage of microorganism-associated molecular patterns (MAMPs) as well as pathogen-associated molecular patterns (PAMPs) that can initiate in the gut and alter immune functions in the lung (24). MAMPs and PAMPS are able to activate Toll Like Receptors (TLR) on dendritic cells, T cells, epithelial cells, macrophages, and B cells. This activation of TLRs can change cytokine, chemokine, and antibody production in the lung, all of which have been shown to be important in viral clearance for RSV (25, 26).

Studies have shown associations between bronchiolitis and the gut microbiome in infants, along with associations between RSV and the gut microbiome of mice. In infants, these studies demonstrated that there was a Bacteroides-dominant profile that was associated with a higher likelihood of bronchiolitis (27). In mice, there was a significant increase in Bacteroidetes and a decrease in Firmicutes phyla abundance (13). However, to our knowledge, our study is the first to directly investigate the role of gut microbiota in RSV disease severity in infants. Our findings indicate that between RSV infected patients and control patients there are no significant changes in the abundance of microbes in the gut (Fig. 2A, B), but there is a significant change in gut microbial composition between RSV infected patients and control patients (Fig. 3A).

Furthermore, there were significant enrichments in the families S24_7, Clostridiales, Odoribacteraceae, Lactobacillaceae, Actinomyces using LEfSe and ANOVA with BH correction between these RSV and control patients. Likewise, our data indicates that when looking at severity (PICU vs. Non-PICU vs. Control), there is only a slight decrease in commensal microbial abundance across the patient groups (Fig. 2C, D). We do, however, see separate phylogenetic clustering between the severe and moderate patients and separation from the control patients (Fig. 3B). This clustering was shown to be dependent

upon Enterococcus, Lactobacillus, Oscillospira, Odoribacter, Tissierella Soehngenia, and S24_7 (Fig. 4). The LEfSe analysis showed us 6 bacterial OTUs that were enriched in the severe group when compared to the moderate and control groups. Specifically, a significant increase in S24_7 OTU 191 coincided with severe versus moderate RSV (Fig. 5D). This data suggests that the bacteria that are driving these differences are encompassed within the Firmicutes and Bacteroidetes phylum but are varied at the family and OTU levels illustrating unknown factors contributing to this dysbiosis of the gut microbiome. Our study demonstrates that the gut microbiome diversification is associated with RSV disease severity and suggests that altering the gut microbiome may have clinical relevance.

Despite the fact that S24_7 has been shown to make up a significant portion of the mouse and human intestinal microbiome (13, 28, 29). A study characterizing 30 population genomes of S24_7 found that 20 of the 30 populations genomes contain a metalloprotease belonging to M6 peptidase family. Peptidases within this family exhibit antimicrobial capabilities as well as degradation abilities for extracellular matrices (ECM). In addition to M6 peptidase in their genomes, 11 of the 30 populations of S24_7 contained IgA degrading peptidase sequences in their genomes (peptidase family M64). This could be crucial to the microbiome's interaction with RSV severity as it has been shown that IgA is vital for mucosal defense in RSV infection and aids in protection to upper respiratory tract infections (30–34). These mucosal antibodies have, similarly, been shown to protect human adults from experimental RSV infection (35, 36). Therefore, IgA is important for protection and disruption of memory IgA can contribute to severity and reoccurrence of RSV. While it is enticing to speculate that elevations in M6 peptidases due to enrichment of S24_7 family members leads to degradation of RSV specific IgA and disease severity, it is yet to be determined whether a gut microbial profile low in diversity and enriched in S24_7 is the cause, or result, of severe RSV disease. Mechanistic studies need to be done in order to address this important question. It is possible that by elucidating the mechanism of S24_7 in RSV disease severity therapeutic targets could be identified.

Our findings highlight that there is a correlation between RSV infection and dysbiosis of the gut microbiome. We identified disruptions in the abundance of microbes in patients with severe RSV disease as well as characteristic microbiome shifts in all RSV patients. We identified that there were 6 specific, enriched microbes associated with RSV severity. Together, our findings identified changes in phylogenetic diversity in RSV patients and identified specific microbes associated with severity of disease. However, there were limitations to this study. One of the limitations of this study is the small sample size, particularly for severe RSV patients. It is also noteworthy that this study was conducted in Memphis, Tennessee where there is a socioeconomic and racial skew in patient demographics that can be a limitation to the extrapolation of these studies across sites. It is unclear if the changes in gut microbiota are causal or correlated with RSV (or hospitalization). Further studies need to be done to characterize the change in gut microbiota of RSV patients fully. Future work will include additional patient recruitment to more precisely define a gut microbial profile associated with severe RSV disease. These studies could lead to the development of logistic regression models to predict infants at high risk for severe RSV disease based on gut microbiome.

5 Methods

5.1 Ethics Statement

These studies were approved by the Institutional Review Board at the University of Tennessee Health Science Center and Louisiana State University.

5.2 Patient enrollment and sample collection

Patients were enrolled at Le Bonheur Children's Hospital in Memphis, Tennessee, during the RSV season in the years 2012-2015. Written consent was obtained from the parent(s)/guardian(s) of the patients. A total of 58 infants hospitalized with RSV were enrolled in the study (**Table 1**). This work includes samples from 23 patients that were used in a study recently submitted for publication and 33 patients used in another study recently accepted for publication (37). Inclusion criteria were patients less than 12 months old (1 control patient was 368 days old), informed consent from parent or guardian, patients could not have a positive blood culture within 72 hours prior to collection of stool, patients could not be diagnosed with any immunodeficiency, patients could not be on any antibiotics within four weeks prior to enrollment, patients could not have been placed on oxygen for more than 7 days within 3 months prior to the study, patients could not be receiving other investigational immunomodulatory or investigational antiviral agents, patients could not have a hemodynamically significant congenital heart disease, and patients must have tested positive for RSV and negative for influenza, by RT-PCR or be positive for RSV antigens tested by the hospital's diagnostic lab . Among RSV positive patients, 5 infants were admitted to the PICU and thus considered severe; 53 were admitted to the pediatric ward and considered moderate. Healthy controls patients were enrolled from Le Bonheur Outpatient Clinic during well-baby checkups. Within 72 hours of enrollment to the hospital, stool samples from these infants were collected, and 100 mg aliquots of stool were stored at -80 °C until DNA extraction.

5.3 16s sequencing

Fecal DNA was isolated from the 100 mg aliquots of stool using QIAamp PowerFecal DNA Kit (Qiagen) according to the manufacturer's instructions. Fecal DNA was sent to the University of Alabama at Birmingham Center for Clinical and Translational Science for 16s sequencing. Unique barcoded primers were used to amplify the hypervariable V4 regions of the bacterial 16S rRNA gene from each sample. The resulting amplicon libraries were then gel purified using QIAquick Gel Extraction Kit (Qiagen). Finally, the purified libraries were sequenced using Next-generation sequencing performed on the Illumina MiSeq system using 250 bp paired-end reads (38) (**Figure 1A**).

5.4 Bioinformatics

Sequence data with a minimum length of 250 base pairs were processed and analyzed using QIIME 2 version 2018.4.0 (RRID: SCR_008249). Sequences were analyzed using the standard pipeline for QIIME 2, and operational taxonomic units (OTUs) were generated using a 99% identity. The OTUs were then classified by phylogeny using the classifier from GreenGenes database 13_8. Subsequently, alpha diversity measured using Simpson Index and Shannon Index in Qiime2, beta diversity, the community similarity, measured via unweighted unifrac distance matrix and PERMANOVA with 999 permutations was used to identify significant features between groups. Linear discriminant analysis effect size (LEfSe: RRID: SCR_014609) in Galaxy (**Figure 1B**) was used to discover biomarkers specific to each of the patient groups. In Calypso, the data was normalized using cumulative sum scaling (CSS) normalization for multivariate tests. In order to address false discovery rate two-way repeated-measures analysis of variance (ANOVA) in conjunction with Benjamini–Hochberg multiple-inference correction with Dunnett's correction was used to test for significant differences in alpha diversity and differential taxa abundance between groups. Significance was defined as $p < 0.05$ after FDR adjustment (Prism v8.0, GraphPad Software, La Jolla, CA) (Calypso). Quality control of sample data can be found in **supplemental figure 1**. Data are presented as means ± SEM. The datasets generated for this study can be found in the SRA accession database: PRJNA579491.

6 Conclusions

Respiratory syncytial virus (RSV) is a ubiquitous respiratory virus infecting the majority of the human population by one year of age. It is the number one cause of lower respiratory tract infections in infants, yet there are still no vaccines or specific antiviral therapies against RSV. Most patients infected with RSV develop mild upper respiratory tract infections, but a percentage of patients develop severe lower respiratory tract infections eliciting the need for intensive care and sometimes resulting in death. While there have been papers that show the interrelationships between gut and nasal microbiome changes in RSV patients there has been no data that observes the relationship between gut microbiota and severity of RSV disease. This manuscript makes the case that gut microbiome changes are associated with severity of RSV disease and these changes could be possible therapeutic targets that should be investigated in further studies.

7 Declarations

7.1 Ethics Statement

These studies were approved by the Institutional Review Board at the University of Tennessee Health Science Center and Louisiana State University.

7.2 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

7.3 Consent for publication

Not applicable

7.4 Data Availability

The datasets generated for this study can be found in the SRA accession database: PRJNA579491.

7.5 Author Contributions

Dahui You designed the study. Jeffrey Harding executed data management, performed data analyses, and drafted the manuscript. David Siefker enrolled patients and collected clinical data, performed sample processing, and performed data collection. Luan Vu performed data collection. Joseph Pierre performed data analysis and drafted the manuscript. John DeVincenzo and Lisa Harrison enrolled patients and collected clinical data. Stephania Cormier conceptualized and designed the study and drafted the manuscript. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

7.6 Funding

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Abbreviations

RSV – Respiratory syncytial virus

URTI - upper respiratory tract infections

LRTI - lower respiratory tract infections

PICU - pediatric intensive care unit

OTU - operational taxonomic units

LEfSe - Linear discriminant analysis effect size

CSS - cumulative sum scaling

RDA - redundant discriminant analysis

DAPC - discriminant analysis of principal components

PAMP - pathogen-associated molecular patterns

TLR - Toll Like Receptors

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Tables

Due to technical limitations, tables are only available as a download in the supplemental files section

Figures

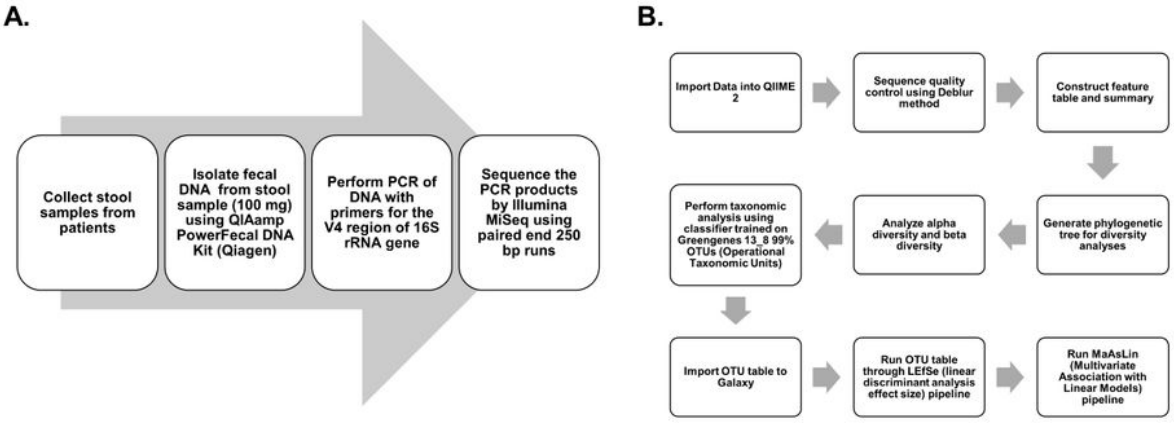

Figure 1

Sample collection process and data analysis workflow. (A) Workflow from sample collection to sequence data. (B) Data analysis workflow in Qiime 2 and Galaxy.

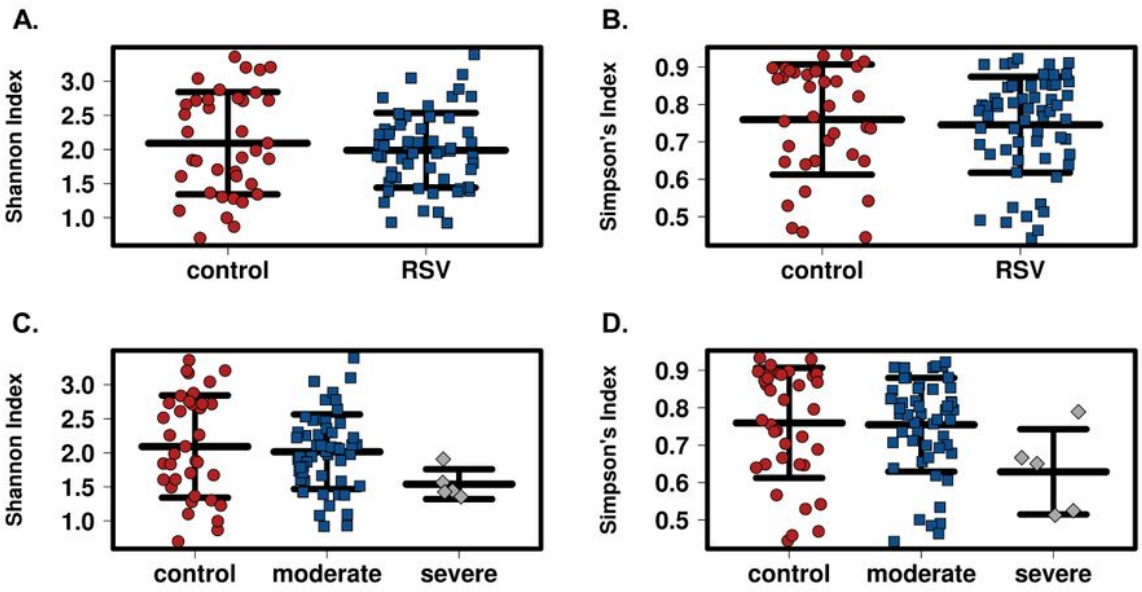

Figure 2

α-diversity is not significantly reduced in severe RSV infected patients compared to healthy controls. (A) Simpson Index of the gut microbiota shows no difference between healthy controls (n=37) and infants with RSV disease (n=58). (B) Shannon Index of the gut microbiota shows no difference between healthy controls (n=37) and infants with RSV disease (n=58). (C) Simpson Index is slightly reduced in severe RSV disease (n=5) compared to healthy controls (n=37) or infants with moderate RSV disease (n=53) but is

not significant. (D) Shannon Index is slightly decreased in severe RSV disease (n=5) compared to healthy controls (n=37) and infants with moderate RSV disease (n=53) but is not significant. Each point represents an individual infant and the mean ± SEM

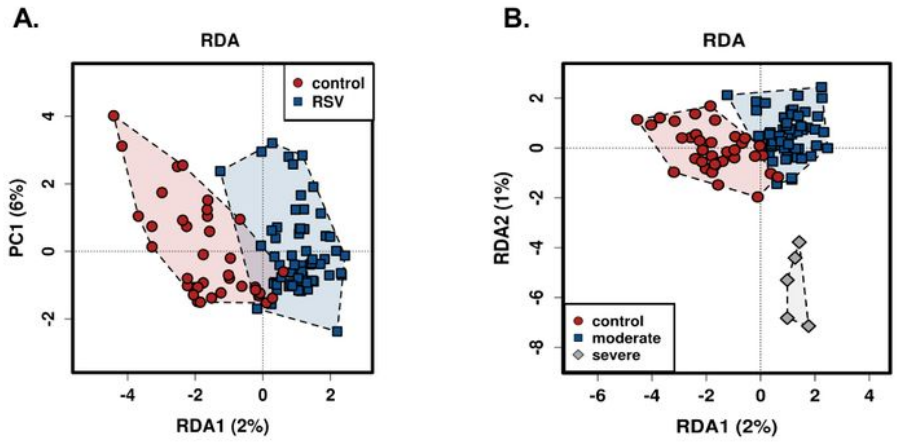

Figure 3

RDA plots show separate phylogenetic clustering for control and RSV patients. Gut microbiome composition in infants infected with RSV and healthy control infants were analyzed using RDA to

visualize the phylogenetic dissociations in RSV infected patients compared to healthy controls. (A) Control patients cluster separately from moderate or severe RSV patients on a phylogenetic basis. (B) Severe RSV patients cluster separately from control and moderate RSV patients showing a significantly different phylogenetic composition.

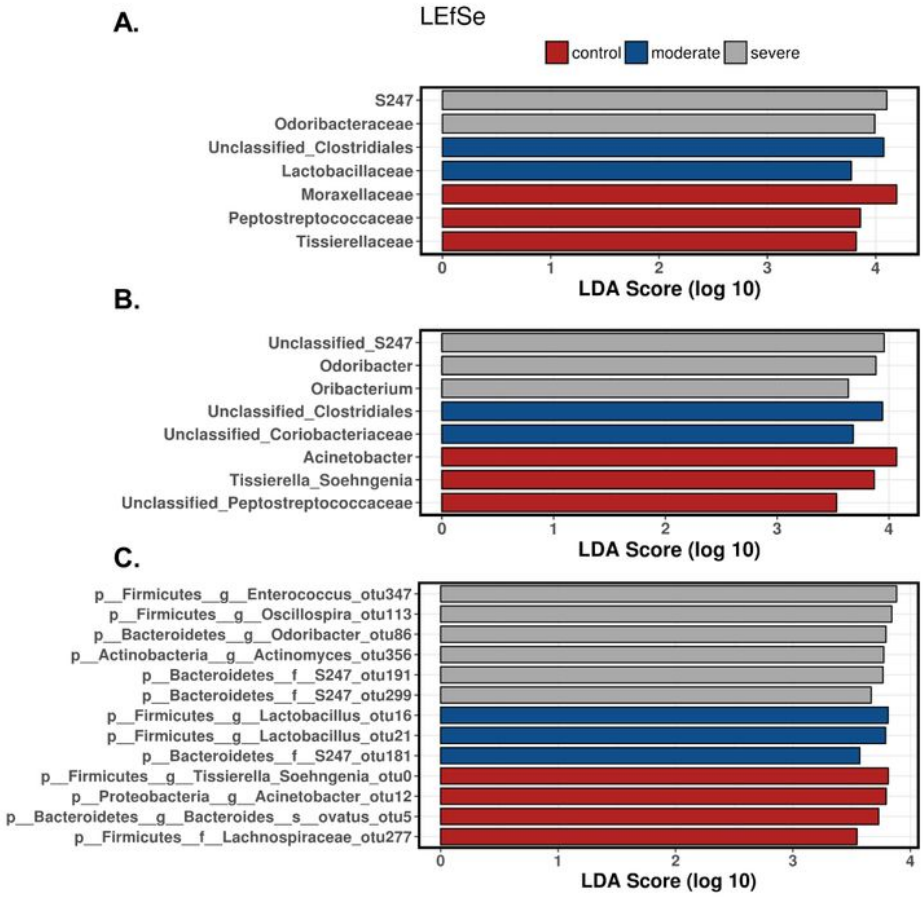

Figure 4

Linear discriminant analysis effect size (LEfSe) analysis of severe, moderate, and control shows six specific bacterial clades that distinguish severe RSV from control or moderate RSV. The LEfSe analysis shows the linear effect size between the samples and calculates the linear discriminant analysis score for each of the Operation Taxonomic Units (OTUs). Taxonomic rank is denoted by the first small letter in the naming. (A) LEfSe at the family level, (B) LEfSe at the genus level, and (C) LEfSe at the OTU level All the phylogenetic clades shown are p< 0.05 following non-parametric factorial Kruskal-Wallis (KW) sum-rank test and Wilcoxon rank-sum test.

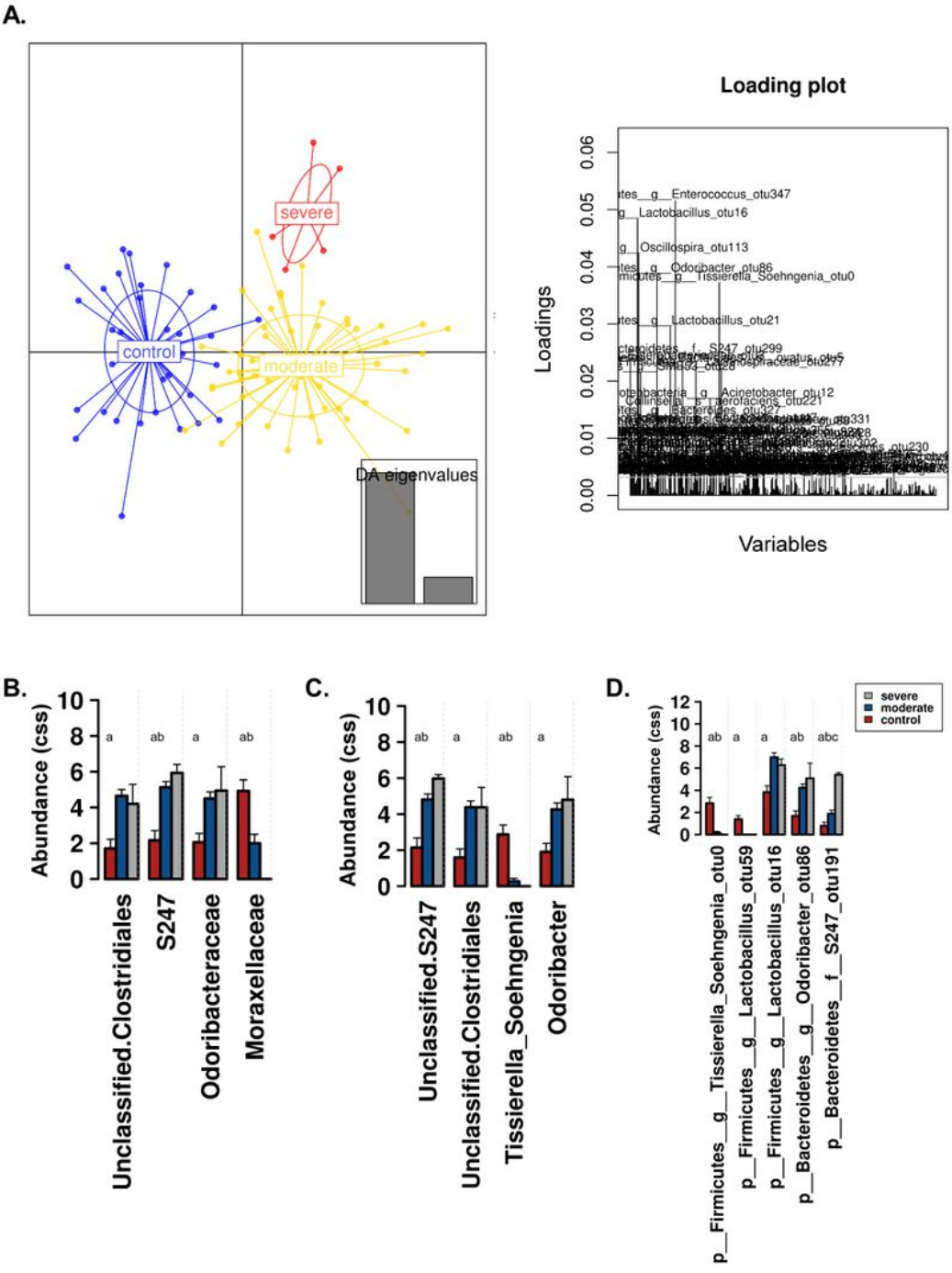

Figure 5

Discriminant analysis of principal components (DAPC) plot (A) at OTU level revealed distinct clustering of severe patients (red), moderate patients (yellow), and control patients (blue). Canonical loading plot stresses the specific OTUs most influential in the separation of clusters. (B) Percent abundance with cumulative-sum scaling (CSS) + log transformation at the family level. (C) Percent abundance with CSS + log transformation at the genus level. (D) Percent abundance with CSS + log transformation at the OTU level. Significance ($p<0.05$) with BH correction between control vs. moderate, control vs. severe, and moderate vs. severe is denoted by a, b, and c, respectively.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1.jpg
- SupplementalFigure1.JPEG
- SupplementalFigure3.JPEG
- Table1.XLSX
- SupplementalFigure2.JPEG