In infants with severe bronchiolitis: Dual-transcriptomic profiling of nasopharyngeal microbiome and host response

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To the Editor

Bronchiolitis is the leading cause of hospitalizations among infants in the U.S.(1) Two major respiratory viruses—respiratory syncytial virus (RSV) and rhinovirus (RV)—account for 85% of severe bronchiolitis (bronchiolitis resulting in hospitalization).(2) While bronchiolitis has been considered a single disease entity and currently-available management

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does not vary by causative virus,(3) emerging evidence suggests heterogeneity in the clinical manifestations and pathobiology of bronchiolitis by virus. For example, epidemiological research has shown that causative viruses are associated with distinct risks of both acute (e.g., illness severity) and chronic (e.g., incident asthma) outcomes in childhood.(1) Recent data also suggest an interplay between respiratory viruses, airway microbiome, and host response.(1) For example, studies have shown virus-specific (e.g., RSV- and RV-specific) microbiome composition (through 16S rRNA gene sequencing)(4) and host response (through transcriptomics(5–7) and metabolomics(8)) in children with acute respiratory infection, including bronchiolitis. Despite the clinical and research importance of bronchiolitis, no study to date has reported an integrated investigation of the between-virus differences in microbiome composition (*beyond* 16S rRNA gene sequencing) and function, along with host response, in the airway of infants with bronchiolitis. In this context, we performed a pilot study using a dual-transcriptomic approach(9,10) —simultaneous profiling of microbe's transcriptome (metatranscriptome) and host transcriptome—to test the hypothesis that, in infants with severe bronchiolitis, RSV and RV infection have distinct nasopharyngeal airway microbiome composition and function as well as host response profiles.

Details of the study design, setting, samples, measurement, and analysis may be found in the Online Supplement (Supplemental Methods [online]). Briefly, in the current pilot study, we performed dual-transcriptomic profiling of the nasopharyngeal samples from five infants with RSV infection and five infants with sole RV infection (i.e., no co-infecting RSV) as part of an ongoing cohort study. This multicenter prospective cohort study—the 35th Multicenter Airway Research Collaboration (MARC-35)—enrolled infants (aged <12 months) hospitalized with bronchiolitis at 17 sites across 14 U.S. states during the 2011–2014 winter seasons. Bronchiolitis was defined by the American Academy of Pediatrics guidelines.(3) In addition to phenotypic data measurements, investigators collected nasopharyngeal aspirates within 24 hours of hospitalization using a standardized protocol. These samples underwent 1) real-time reverse transcription PCR to test for respiratory viruses—e.g., RSV and RV—and 2) dual-transcriptomics through RNAseq to profile the microbiome composition and function, as well as host function, in the nasopharyngeal airway.

The bioinformatic processing and analysis are detailed in the Supplemental Methods (online). Firstly, we used microbial RNA to infer microbial taxonomic composition through PathoScope.(11–13) Then, to compare compositional differences (α-diversity indices and individual species abundances) between the RSV and sole RV infection groups, we used linear regression models and Wald test with Cook's distance correction, respectively. We also used the Benjamini-Hochberg method to correct for multiple testing. Secondly, we used metatranscriptomic contigs to infer microbial function and Gene Ontologies, and compared their between-group differences. Thirdly, we also computed host gene expression profiles for each sample and examined differential expression. Lastly, we also performed pathway enrichment analysis to examine the virus-specific host response.

The ten infants with severe bronchiolitis had a median age of 7 (IQR, 3–9) months. Between the virus groups, there was no significant difference in the baseline characteristics (all

P>0.10; Supplemental Table S1 [online]). All RNAseq samples had sufficient sequence depth (mean, 74 million pair-end reads/sample) to obtain a high degree of sequence coverage. The metatranscriptomic analysis obtained 22,115,512 merged sequences and identified 2,780 microbial lineages after singleton removal.

Composition and function of the nasopharyngeal microbiome

Nasopharyngeal microbiome was dominated by *M. catarrhalis* (32%), followed by other bacteria including *S. mitis* (7%), *S. pneumoniae* (6%), *Prevotella* spp. (5%), *P. melaninogenica* (5%), and *H. influenzae* (4%). Comparing the α-diversity between the two groups, Shannon and Simpson indices were generally higher in the RSV group but there were no significant differences (P>0.05; Supplemental Figure S1 [online]). By contrast, at the individual taxon level, the abundance of 52 microbial species were significantly different between the virus groups (q<0.05; Supplemental Figure S2 [online]), including several representatives of the some genera, *Moraxella* and *Staphylococcus.*

The functional profiling identified 18 differentially-expressed microbial genes between the virus groups (q<0.05; Figure 1A [online]). In the RSV group, two genes were upregulated—*coaA* and *mazF*, which are involved in pantothenate kinase and nucleic acid metabolism activities, respectively. By contrast, in the sole RV group, 16 genes were upregulated (e.g., *ddc* involved in carboxylyase activity). Similarly, the GO-level analysis demonstrated different top ontology terms between the virus groups (Figure 1B and Supplemental Table S2 [online]). While some GO terms were shared between the virus groups (e.g., nucleobase biosynthetic process), most of the enriched terms were exclusive to each group, indicating functional differences in the microbiome by infecting virus. Indeed, the RSV group was enriched with nucleic acid-related metabolism (e.g., adenine metabolic process [GO: 0046083], purine-nucleobase compound salvage [GO:0043096]; q<0.05). By contrast, the sole RV group was enriched with acylation and acetylation activities (e.g., C-acyltransferase activity [GO: 0016408], C-acetyltransferase activity [GO: 0016453] activities; q<0.05).

Host gene expression in the nasopharyngeal airway

The unsupervised hierarchical clustering using the transcriptomics data segregated infants from each viral group (Figure 1C). Between the virus groups, 269 genes were differentially expressed (q<0.05; Supplemental Table S3 [online]). For example, compared to the RSV group, the RV group had higher expression of *DEFA1* and *MAP3K14*, both of which are involved in innate immunity. Likewise, the pathway enrichment analysis revealed differentially-enriched signaling and metabolic pathways between the groups (Supplemental Table S4 [online]). For example, the RV group had 49 significantly-enriched pathways (q<0.05)—e.g., Toll-like receptor (hsa04620), NOD-like receptor (hsa04621), RIG-I-like receptor (hsa04622), T cell receptor (hsa04660), and JAK-STAT (hsa04630) signaling pathways—suggesting the virus-specific differential activation of innate immunity with a transition to adaptive immunity in infants with bronchiolitis.

In this dual-transcriptomics profiling of infants with severe bronchiolitis, RSV and sole RV infection had distinctly different nasopharyngeal microbiome composition and function profiles (e.g., enriched nucleic acid metabolism in the RSV group vs. acylation/acetylation

function in the RV group), as well as differences in host transcriptome profile (e.g. upregulated innate immunity pathways in the RV group). These observations lend an additional support to the emerging concept that bronchiolitis pathobiology is heterogenous, (1) at least between the two major viruses (RSV and RV).

Consistent with the current study, an earlier analysis using 16S rRNA gene sequencing demonstrated that the nasopharyngeal microbiome composition differs by virus (e.g., more-abundant Firmicutes bacteria in RSV infection) in infants with severe bronchiolitis.(4) Beyond the composition of the microbiome, our data also suggest differences in its function. For example, infants with RSV infection had enriched nucleic acid metabolism, which may be explained by, at least partially, increased cell turn-over secondary to severe airway inflammation.(14) Additionally, the RV-related enrichment of acylation/acetylation function is in line with recent metabolomics research showing virus-specific signatures—e.g., increased abundance of N-acetyl amino acids in infants with RV bronchiolitis.(8) Taken together, these data suggest potential cross-talk between respiratory viruses and airway microbiome, with downstream functional effects in the airway.

Furthermore, in agreement with the observed up-regulation of host innate immunity signaling (e.g., Toll-like receptor, NOD-like receptor, RIG-I-like receptor signaling) and T cell receptor signaling in the RV group, an earlier analysis of nasal airway microRNA and mRNA in infants with bronchiolitis also showed that RV infection had unique microRNA profile upregulating NFκB signaling(7)—a key regulator for innate and adaptive immune response. Another study also reported that, within children with RSV infection, unique nasopharyngeal airway microbiome profile (through 16S rRNA gene sequencing) is associated with differential regulation of genes related to Toll-like receptor in blood.(5) The current study utilizing a novel dual-transcriptomic approach corroborates these earlier studies, and extends them by simultaneously determining the virus-specific microbiome function and host response signatures in the airway of infants with bronchiolitis.

The present study has several potential limitations. First, bronchiolitis involves inflammation of the lower airways in addition to the upper airways. While our analyses relied on nasopharyngeal samples, studies have shown that the upper airway sampling provides reliable representation of the lung microbiome(15) and transcriptome profiles.(16) Second, the limited sample size of this current proof-of-concept study precluded us from evaluating the role of RSV/RV co-infection compared to sole infection and from making robust inferences. Third, we did not have a "healthy control" group. Yet, the study objective was *not* to evaluate the role of metatranscriptome and transcriptome on the development of bronchiolitis (yes/no), but to examine virus-specific (RSV vs. RV) pathobiology within this high-risk population. Lastly, our inferences may not be generalizable beyond infants hospitalized for bronchiolitis. Yet, our findings are directly relevant for infants with severe bronchiolitis which accounts for 130,000 hospitalizations in the U.S. each year.(1)

In summary, based on the dual-transcriptomics analysis in infants with bronchiolitis, we found that RSV and sole RV infection had distinct microbiome composition and function, as well as host response, profiles. In conjunction with earlier studies, our findings suggest that the bronchiolitis pathobiology differs between RSV and RV infection. The current pilot

study lends support to the use of dual-transcriptomic approach, which has a potential to delineate the integrated contributions of the airway microbiome and host to bronchiolitis pathobiology. In conjunction with existent evidence—the data should not only inform future adaptive clinical trials (e.g., the use of microbiome and host data as biomarkers) but also facilitate investigations into the development of treatment strategies (e.g., modification of microbiome, immunomodulators) in infants with bronchiolitis.

Supplementary Material

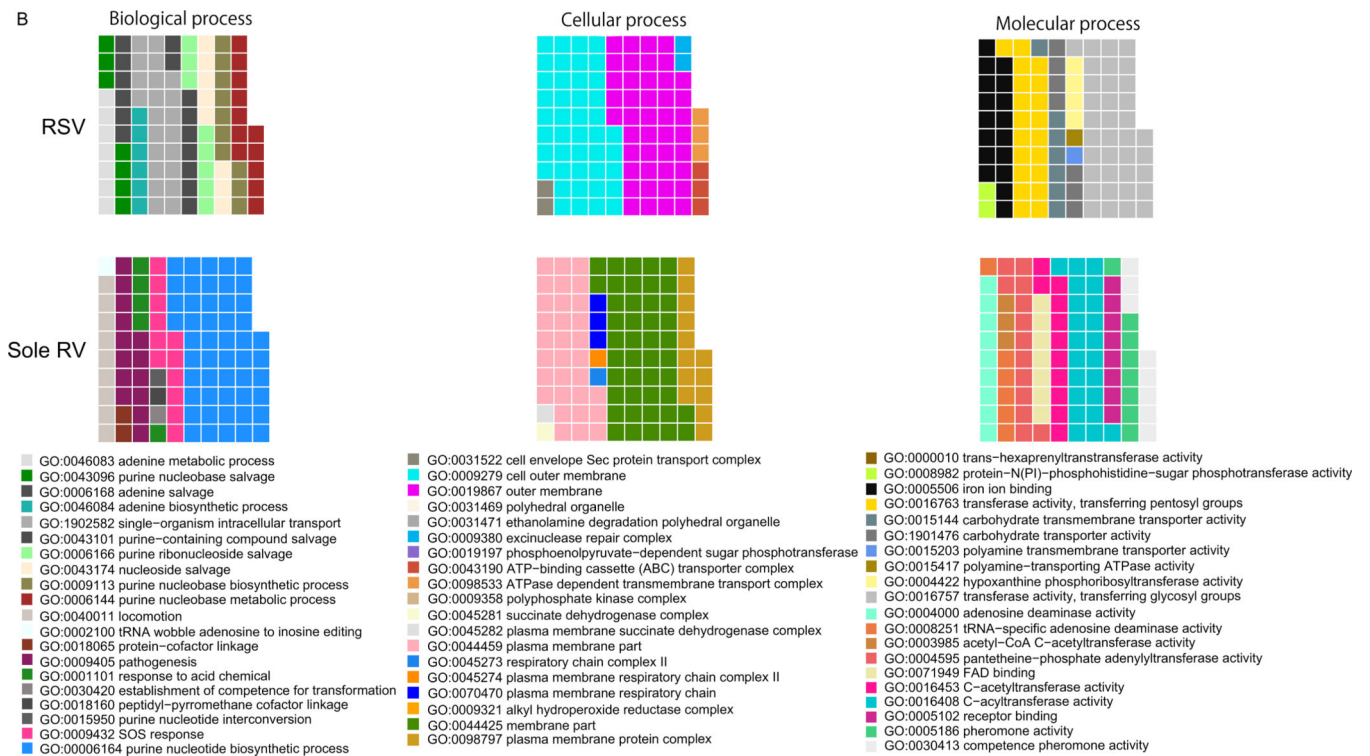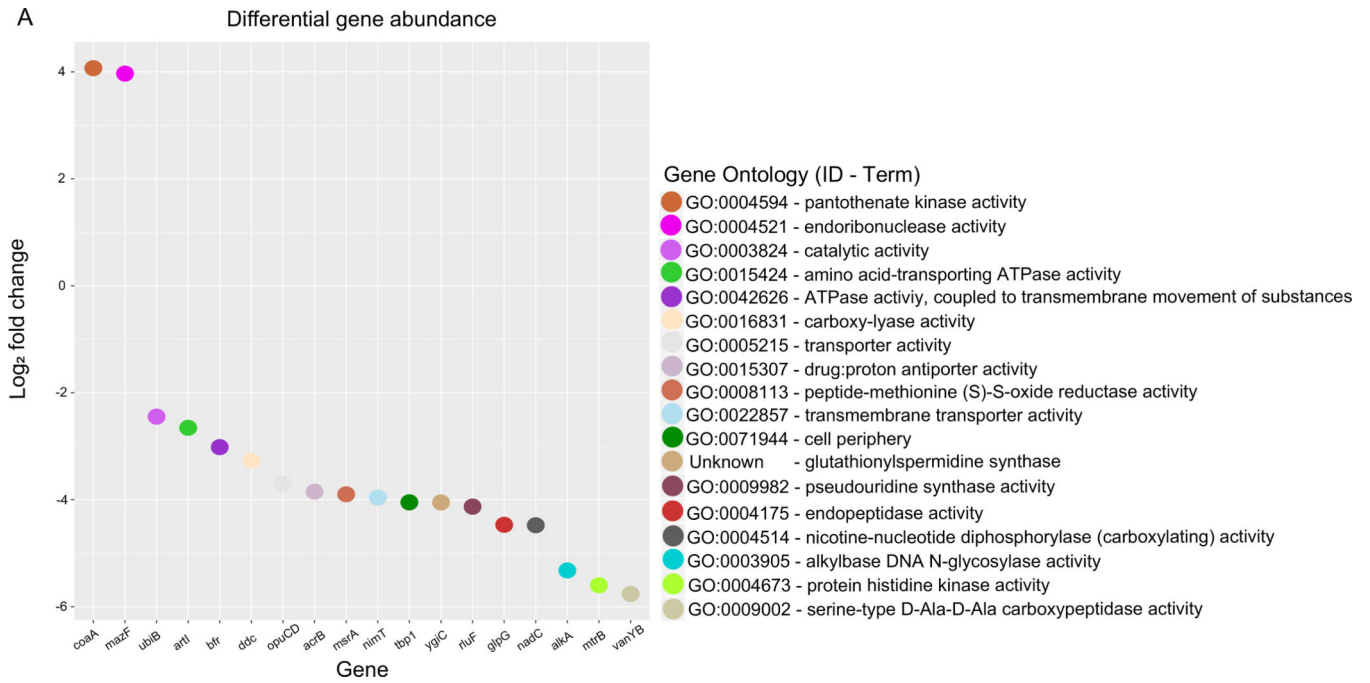Refer to Web version on PubMed Central for supplementary material.
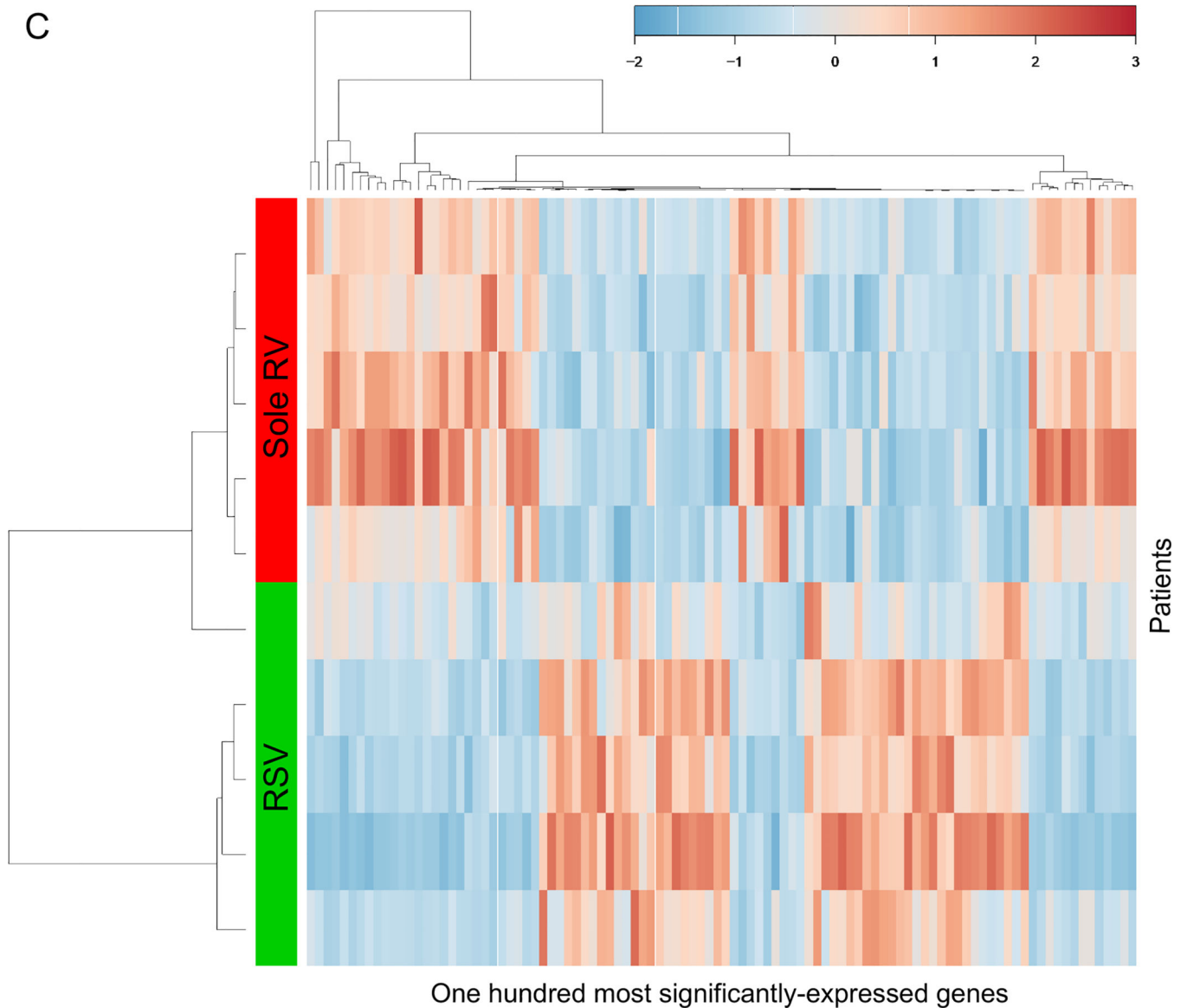
Acknowledgments

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A

Differential gene abundance

Gene Ontology (ID - Term)

- GO:0004594 - pantothenate kinase activity
- GO:0004521 - endoribonuclease activity
- GO:0003824 - catalytic activity
- GO:0015424 - amino acid-transporting ATPase activity
- GO:0042626 - ATPase activiy, coupled to transmembrane movement of substances
- GO:0016831 - carboxy-lyase activity
- GO:0005215 - transporter activity
- GO:0015307 - drug:proton antiporter activity
- GO:0008113 - peptide-methionine (S)-S-oxide reductase activity
- GO:0022857 - transmembrane transporter activity
- GO:0071944 - cell periphery
- Unknown - glutathionylspermidine synthase
- GO:0009982 - pseudouridine synthase activity
- GO:0004175 - endopeptidase activity
- GO:0004514 - nicotine-nucleotide diphosphorylase (carboxylating) activity
- GO:0003905 - alkylbase DNA N-glycosylase activity
- GO:0004673 - protein histidine kinase activity
- GO:0009002 - serine-type D-Ala-D-Ala carboxypeptidase activity

B

Biological process   Cellular process   Molecular process

RSV

Sole RV

- GO:0046083 adenine metabolic process
- GO:0043096 purine nucleobase salvage
- GO:0006168 adenine salvage
- GO:0046084 adenine biosynthetic process
- GO:1902582 single−organism intracellular transport
- GO:0043101 purine−containing compound salvage
- GO:0006166 purine ribonucleoside salvage
- GO:0043174 nucleoside salvage
- GO:0009113 purine nucleobase biosynthetic process
- GO:0006144 purine nucleobase metabolic process
- GO:0040011 locomotion
- GO:0002100 tRNA wobble adenosine to inosine editing
- GO:0018065 protein−cofactor linkage
- GO:0009405 pathogenesis
- GO:0001101 response to acid chemical
- GO:0030420 establishment of competence for transformation
- GO:0018160 peptidyl−pyrromethane cofactor linkage
- GO:0015950 purine nucleotide interconversion
- GO:0009432 SOS response
- GO:00006164 purine nucleotide biosynthetic process

- GO:0031522 cell envelope Sec protein transport complex
- GO:0009279 cell outer membrane
- GO:0019867 outer membrane
- GO:0031469 polyhedral organelle
- GO:0031471 ethanolamine degradation polyhedral organelle
- GO:0009380 excinuclease repair complex
- GO:0019197 phosphoenolpyruvate−dependent sugar phosphotransferase
- GO:0043190 ATP−binding cassette (ABC) transporter complex
- GO:0098533 ATPase dependent transmembrane transport complex
- GO:0009358 polyphosphate kinase complex
- GO:0045281 succinate dehydrogenase complex
- GO:0045282 plasma membrane succinate dehydrogenase complex
- GO:0044459 plasma membrane part
- GO:0045273 respiratory chain complex II
- GO:0045274 plasma membrane respiratory chain complex II
- GO:0070470 plasma membrane respiratory chain
- GO:0009321 alkyl hydroperoxide reductase complex
- GO:0044425 membrane part
- GO:0098797 plasma membrane protein complex

- GO:0000010 trans−hexaprenyltranstransferase activity
- GO:0008982 protein−N(PI)−phosphohistidine−sugar phosphotransferase activity
- GO:0005506 iron ion binding
- GO:0016763 transferase activity, transferring pentosyl groups
- GO:0015144 carbohydrate transmembrane transporter activity
- GO:1901476 carbohydrate transporter activity
- GO:0015203 polyamine transmembrane transporter activity
- GO:0015417 polyamine−transporting ATPase activity
- GO:0004422 hypoxanthine phosphoribosyltransferase activity
- GO:0016757 transferase activity, transferring glycosyl groups
- GO:0004000 adenosine deaminase activity
- GO:0008251 tRNA−specific adenosine deaminase activity
- GO:0003985 acetyl−CoA C−acetyltransferase activity
- GO:0004595 pantetheine−phosphate adenylyltransferase activity
- GO:0071949 FAD binding
- GO:0016453 C−acetyltransferase activity
- GO:0016408 C−acyltransferase activity
- GO:0005102 receptor binding
- GO:0005186 pheromone activity
- GO:0030413 competence pheromone activity

C

One hundred most significantly-expressed genes

FIGURE 1. Nasopharyngeal airway microbiome function and host response between five respiratory syncytial virus and five sole rhinovirus bronchiolitis

A. *Microbial* gene abundance. Differential microbial gene expression in the nasopharyngeal airway between the RSV and sole RV groups. Upregulated genes in the RSV group are represented by positive $\log_2$ fold changes, while upregulated genes in the sole RV group are represented by negative $\log_2$ fold changes. Color represents Gene Ontology terms associated with the genes.

B. Top Gene Ontology terms of the nasopharyngeal *microbiome*. The waffle plots show the top ontology (biological process, cellular component, and, molecular function) terms within each of the two groups. The number of squares indicates the importance of the function within each group. The majority of the enriched terms differ between the groups.

C. Unsupervised hierarchical clustering of the 100 most differentially-expressed *host* genes. The gene expression profiles separated infants with RSV bronchiolitis from those with sole RV bronchiolitis. The color bar indicates the standardized expression of each gene to a mean of 0. Upregulated genes have positive values and are displayed as red. Downregulated genes have negative values and are displayed as blue. The differences in gene expression between RSV and sole RV are summarized in Supplemental Table S3.

Abbreviations: RSV, respiratory syncytial virus; RV, rhinovirus