ONLINE SUPPLEMENT

Association of nasopharyngeal microbiota profiles with bronchiolitis severity in infants hospitalized for bronchiolitis

Supplemental Methods

SUPPLEMENTAL METHODS

16S rRNA Gene Sequencing and Compositional Analysis

16S rRNA gene sequencing methods were adapted from the methods developed for the NIH-Human Microbiome Project (1, 2). As nasopharyngeal aspirate samples had a low bacterial biomass, we processed all samples with a low-biomass extraction protocol to avoid sample loss and degradation and to maximize yield. Bacterial genomic DNA was extracted using MO BIO PowerSoil DNA Isolation Kit (Mo Bio Laboratories; Carlsbad, CA) (3), with lowering the amount of buffers C1 (60 µl), C2 (50 µl), C3 (50 µl), C4 (500 µl), and C6 (50 µl). The 16S rDNA V4 region was amplified by PCR and sequenced in the MiSeq platform (Illumina; SanDiego, CA) using the 2x250 bp paired-end protocol yielding pair-end reads that overlap almost completely. The primers used for amplification contain adapters for MiSeq sequencing and single-end barcodes allowing pooling and direct sequencing of PCR products (4, 5).

Sequencing read pairs were demultiplexed based on the unique molecular barcodes, and reads were merged using USEARCH v7.0.1090 (6), allowing zero mismatches and a minimum overlap of 50 bases. Merged reads were trimmed at the first base with a Q5 quality score. We calculated the expected error after taking into account all Q-scores across all the bases of a read and the probability of an error occurring (7). Additionally, a quality filter was applied to the resulting merged reads and reads containing above 0.05 expected errors were discarded. Rarefaction curves of bacterial operational taxonomic units (OTUs) were constructed using sequence data for each sample to ensure coverage of the bacterial diversity present. Samples with suboptimal amounts of sequencing reads were re-sequenced to ensure that the majority of bacterial taxa were encompassed in our analyses.

16S rRNA gene sequences were clustered into OTUs at a similarity cutoff value of 97%

using the UPARSE algorithm (8). OTUs were determined by mapping the centroids to the SILVA database (9) containing only the 16S V4 region to determine taxonomies. A custom script constructed a rarefied OTU table (rarefaction was performed at only one sequence depth) from the output files generated in the previous two steps for downstream analyses of alpha-diversity (e.g., Shannon index) and beta-diversity (e.g., weighted UniFrac) (10, 11). Shannon diversity index is a quantitative measure that takes into account not only richness but also proportion of each bacteria (evenness) within the local community. The weighted UniFrac algorithm calculates the distance between microbial communities based on the phylogenetic relatedness of lineages and relative abundance in each sample.

Quality Control

The processes involving microbial DNA extraction, 16S rRNA gene amplification, and amplicon sequencing included a set of controls that enabled us to evaluate the potential introduction of contamination or off-target amplification. Non-template controls (extraction chemistries) were included in the microbial DNA extraction process and the resulting material was subsequently used for PCR amplification. Additionally, at the step of amplification, another set of non-template controls (PCR-mix) was included to evaluate the potential introduction of contamination at this step. Similarly, a positive control comprised of known and previously characterized microbial DNA was included at this step to evaluate the efficiency of the amplification process. Before samples (unknowns) were pooled together, sequencing controls were evaluated and the rejection criteria were the presence of amplicons in any of the non-template controls or the absence of amplicons in the positive control. In the present study, no

amplicons were observed in the non-template controls and a negligible amount of raw reads were recovered after sequencing.

Microbiota Association Network Analysis

As the presence and/or abundance of an individual genus likely interacts and influences other genera in the microbial community, we displayed the microbiota association network on the basis of the approach of Faust *et al.* (12) using the CoNet software (13). To obtain this network, we first filtered out genera which do not appear in at least 10% of the samples. We then selected the top 50 and bottom 50 relationships for each of four methods: Spearman correlation, Pearson correlation, Bray-Curtis dissimilarity, and Kullback-Leibler dissimilarity. The final network included the edges that were significant ($P<0.05$) in any of the four methods used.

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Figure E1. Cluster Silhouette Plot, MARC-35 Cohort

We evaluated the number of clusters using the cluster silhouette plot method as implemented in the R package *cluster*. The silhouette plot presents the silhouette width for each observation in each cluster group. The silhouette width measures the within cluster to between cluster dissimilarity properties of each observation and higher values are indicative of strong within cluster similarity. Silhouette analysis also provides an average silhouette width as a property of each cluster within the overall clustering outcome. We evaluated clustering with k=3, 4, and 5 groups. The k=4 was chosen because it obtained the highest within cluster mean silhouette widths.

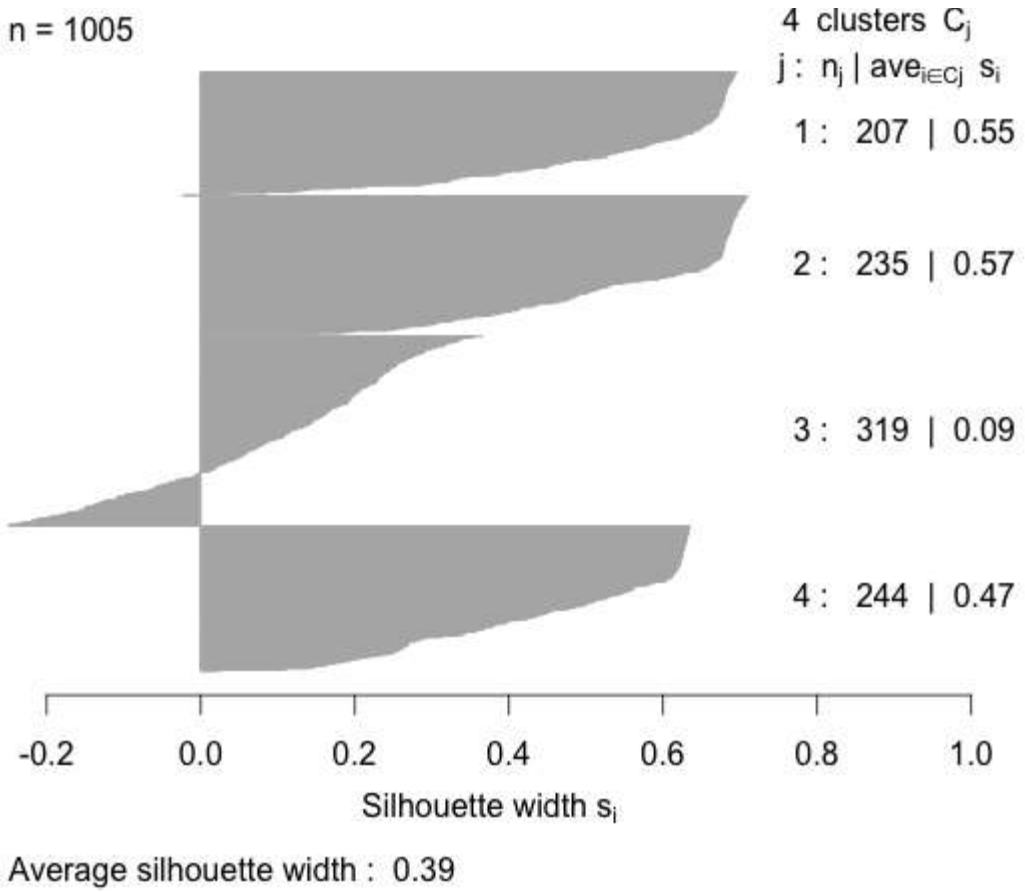

Figure E2. Microbiota Association Network

Nasopharyngeal microbiota network is shown. Green edges indicate co-occurrence, while red edges indicate mutual exclusion. Also note that the graphical distance between nodes is not an actual reflection of the mathematical distance. *Haemophilus*, *Moraxella*, and *Streptococcus* genera were negatively associated with each other, which is consistent with the observed *Haemophilus*-, *Moraxella*-, and *Streptococcus*-dominant profiles. *Streptococcus* genus was positively associated with *Veillonella*, which was also positively associated with *Prevotella* and *Alloprevotella.* This community structure was consistent with the mixed microbiota profile.

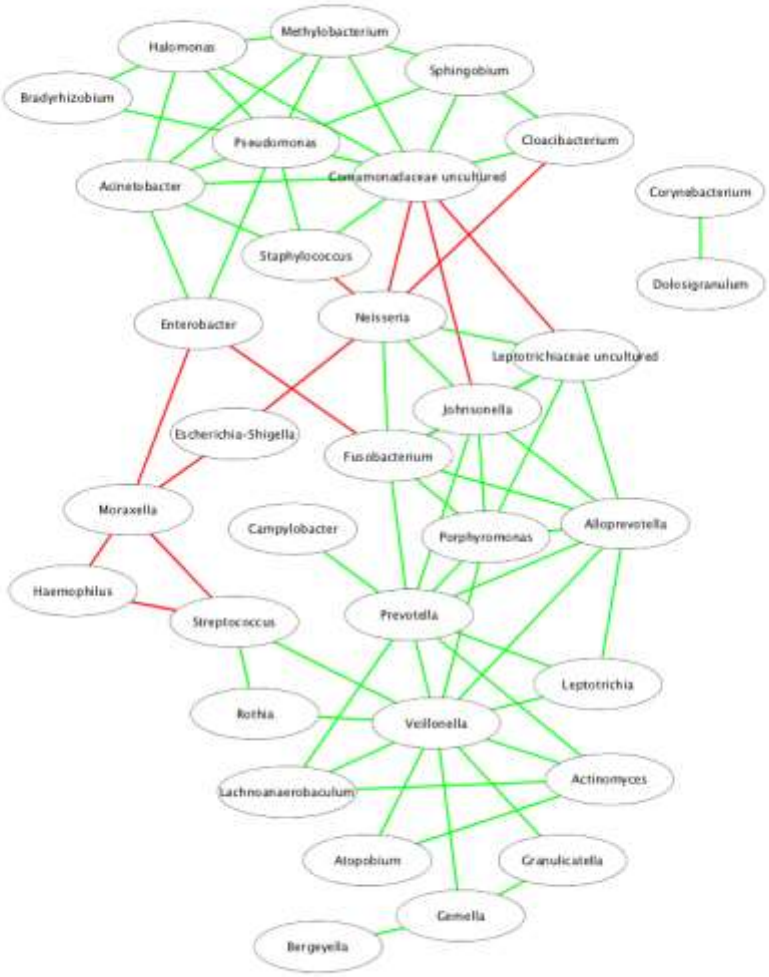

Figure E3. Clustering and Composition in Nasopharyngeal Microbiota of 307 Infants Hospitalized for Bronchiolitis, MARC-30 Substudy

All microbiota profiles of infants were clustered using partitioning around medoids clustering method with weighted UniFrac distance. Colored bars indicate 4 microbiota profiles: *Haemophilus*-dominant profile (green), *Moraxella*-dominant profile (red), *Streptococcus*-dominant profile (yellow), and mixed profile (blue). To obtain further information about the bacterial composition of samples within microbiota profiles, we displayed the 10 most abundant genera present in an adjacent heatmap. HDP = *Haemophilus*-dominant profile; MDP = *Moraxella*-dominant profile; SDP = *Streptococcus*-dominant profile; MP = mixed profile.

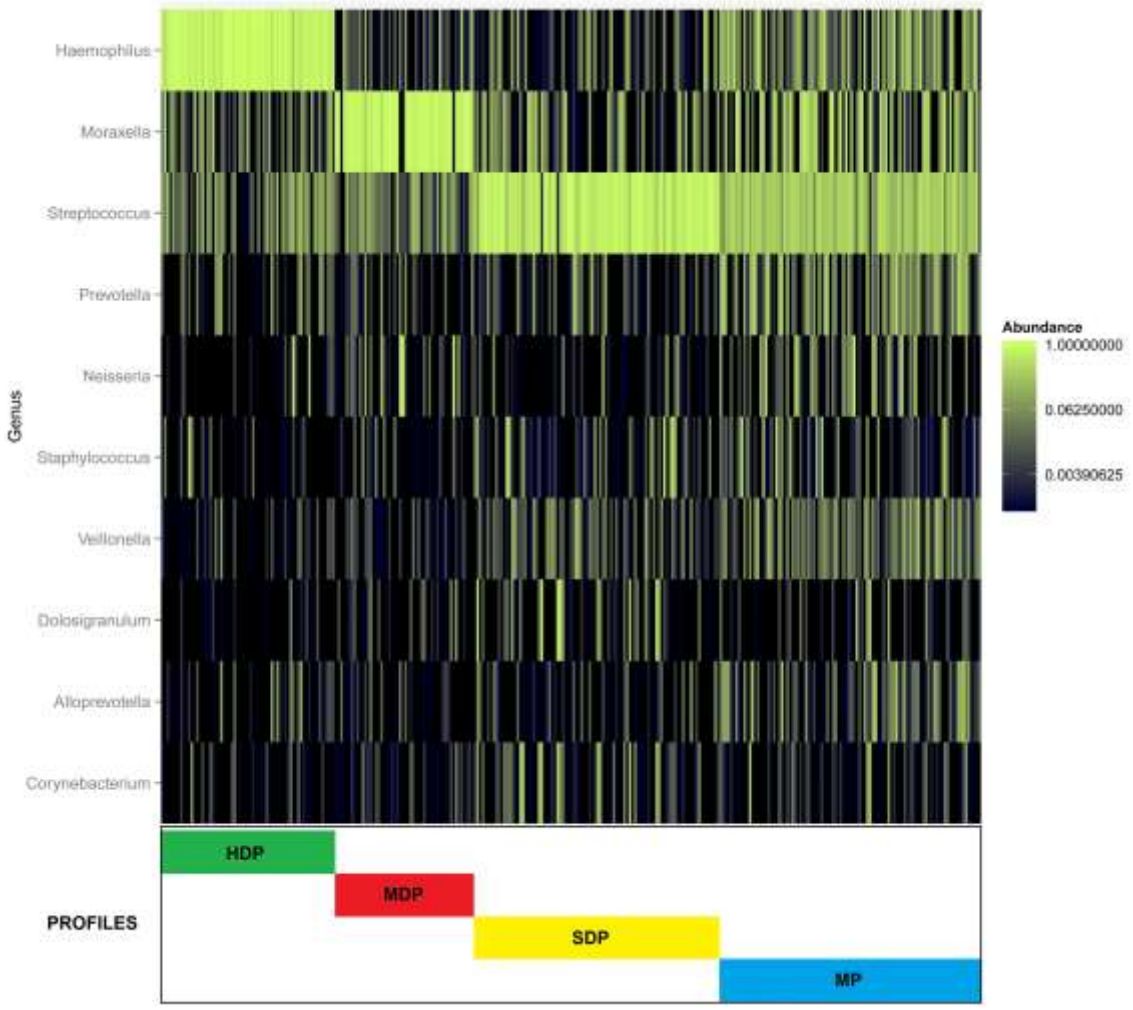

Table E1. Principal Investigators at the 17 Participating Sites in MARC-35

Amy D. Thompson, MD	Alfred I. duPont Hospital for Children, Wilmington, DE
Federico R. Laham, MD, MS	Arnold Palmer Hospital for Children, Orlando, FL
Jonathan M. Mansbach, MD, MPH	Boston Children's Hospital, Boston, MA
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Michelle B. Dunn, MD	Children's Hospital of Philadelphia, Philadelphia, PA
Juan C. Celedon, MD, DrPH	Children's Hospital of Pittsburgh, Pittsburgh, PA
Michael Gomez, MD, MS-HCA and Nancy Inhofe, MD	The Children's Hospital at St. Francis, Tulsa, OK
Brian M. Pate, MD and Henry T. Puls, MD	The Children's Mercy Hospital & Clinics, Kansas City, MO
Stephen J. Teach, MD, MPH	Children's National Medical Center, Washington, D.C.
Richard T. Strait, MD	Cincinnati Children's Hospital and Medical Center, Cincinnati, OH
Ilana Waynik, MD	Connecticut Children's Medical Center, Hartford, CT
Sujit Iyer, MD	Dell Children's Medical Center of Central Texas, Austin, TX
Michelle D. Stevenson, MD, MS	Kosair Children's Hospital, Louisville, KY
Wayne G. Schreffler, MD, PhD and Ari R. Cohen, MD	Massachusetts General Hospital, Boston, MA
Anne K Beasley, MD	Phoenix Children's Hospital, Phoenix, AZ
Thida Ong, MD	Seattle Children's Hospital, Seattle, WA
Charles G. Macias, MD, MPH	Texas Children's Hospital, Houston, TX

Table E2. Richness, Alpha-diversity, and Relative Abundance by Nasopharyngeal Microbiota Profile in MARC-35 Cohort

Indices	***Haemophilus*-dominant profile** n=193 (19.2%)	***Moraxella*-dominant profile** n=220 (21.9%)	***Streptococcus*-dominant profile** n=283 (28.2%)	**Mixed profile** n=309 (30.7%)	**P-value**
Richness					
Number of genera, median (IQR)	14 (7-21)	13 (7-20)	17 (10-26)	20 (12-27)	<0.001
Alpha-diversity, median (IQR)					
Shannon index	0.68 (0.38-1.06)	0.56 (0.25-0.98)	0.88 (0.53-1.39)	1.35 (0.97-1.80)	<0.001
Relative abundance of 10 most abundant genera, mean (SD)					
Streptococcus	0.06 (0.07)	0.06 (0.06)	0.67 (0.24)	0.32 (0.14)	0.003*
Moraxella	0.09 (0.13)	0.82 (0.16)	0.06 (0.10)	0.27 (0.25)	0.003*
Haemophilus	0.76 (0.19)	0.03 (0.08)	0.03 (0.06)	0.13 (0.17)	0.003*
Prevotella	0.01 (0.03)	0.01 (0.02)	0.02 (0.05)	0.04 (0.10)	0.003*
Staphylococcus	0.01 (0.02)	0.00 (0.02)	0.05 (0.16)	0.01 (0.04)	0.003*
Neisseria	0.01 (0.06)	0.02 (0.05)	0.01 (0.03)	0.04 (0.10)	0.003*
Corynebacterium	0.00 (0.02)	0.01 (0.02)	0.03 (0.10)	0.02 (0.06)	0.009*
Alloprevotella	0.01 (0.02)	0.01 (0.02)	0.01 (0.04)	0.03 (0.07)	0.003*
Veillonella	0.00 (0.01)	0.00 (0.01)	0.02 (0.05)	0.02 (0.03)	0.003*
Gemella	0.00 (0.01)	0.00 (0.01)	0.01 (0.03)	0.01 (0.05)	0.003*

Abbreviations: IQR, interquartile range; SD, standard deviation

* Benjamini-Hochberg adjusted P-value accounting for multiple comparisons

Table E3. Richness, Alpha-diversity, and Relative Abundance by Subcluster in Mixed Microbiota Profile, MARC-35 Cohort

Indices	**Subcluster 1** n=158	**Subcluster 2** n=77	**Subcluster 3** n=74	**P-value**
Richness				
Number of genera, median (IQR)	14 (8-22)	21 (16-27)	28 (22-36)	<0.001
Alpha-diversity, median (IQR)				
Shannon index	1.10 (0.83-1.40)	1.37 (1.08-1.69)	2.02 (1.70-2.19)	<0.001
Relative abundance of 10 most common genera, mean (SD)				
Streptococcus	0.36 (0.13)	0.33 (0.13)	0.22 (0.11)	0.002*
Moraxella	0.48 (0.15)	0.07 (0.10)	0.03 (0.05)	0.002*
Haemophilus	0.03 (0.05)	0.38 (0.15)	0.08 (0.08)	0.002*
Prevotella	0.02 (0.05)	0.03 (0.06)	0.12 (0.15)	0.002*
Staphylococcus	0.01 (0.02)	0.02 (0.05)	0.03 (0.05)	0.02*
Neisseria	0.02 (0.06)	0.02 (0.05)	0.11 (0.16)	0.002*
Corynebacterium	0.01 (0.06)	0.01 (0.07)	0.02 (0.07)	0.99*
Alloprevotella	0.01 (0.02)	0.01 (0.02)	0.08 (0.12)	0.002*
Veillonella	0.01 (0.02)	0.02 (0.04)	0.03 (0.04)	0.002*
Gemella	0.00 (0.01)	0.01 (0.06)	0.03 (0.06)	0.01*

Abbreviations: IQR, interquartile range; SD, standard deviation

The number of subclusters for the data was determined using the average silhouette score.

* Benjamini-Hochberg adjusted P-value accounting for multiple comparisons

Table E4. Multivariable Associations of Nasopharyngeal Microbiota Profiles with Bronchiolitis Outcomes, MARC-35 Cohort

	Intensive care use*		Hospital length-of-stay ≥3 days	
	OR (95% CI)	**P-value**	**OR (95% CI)**	**P-value**
Primary Exposure (Microbiota Profile)				
Haemophilus-dominant profile	**1.98 (1.08-3.62)**	**0.03**	**2.47 (1.60-3.83)**	**<0.001**
Moraxella-dominant profile	Reference		Reference	
Streptococcus-dominant profile	1.32 (0.74-2.34)	0.34	1.06 (0.71-1.57)	0.78
Mixed profile	1.19 (0.68-2.09)	0.54	1.01 (0.68-1.48)	0.97
Covariate				
Age (mo)				
<2	Reference		Reference	
2-5.9	**0.33 (0.21-0.51)**	**<0.001**	0.79 (0.57-1.09)	0.15
6-12	**0.29 (0.16-0.52)**	**<0.001**	**0.44 (0.29-0.68)**	**<0.001**
Male (vs. female) sex	0.95 (0.65-1.38)	0.77	0.98 (0.74-1.29)	0.87
Race/ethnicity				
Non-Hispanic white	Reference		Reference	
Non-Hispanic black	0.81 (0.48-1.38)	0.34	0.71 (0.50-1.02)	0.06
Hispanic	1.05 (0.63-1.74)	0.85	0.96 (0.68-1.35)	0.82
Other	1.60 (0.59-4.36)	0.35	0.97 (0.47-2.03)	0.95
Prematurity (32-37 weeks)	1.33 (0.84-2.10)	0.22	1.16 (0.82-1.64)	0.39
Previous breathing problems before the index hospitalization	1.37 (0.81-2.32)	0.23	1.38 (0.96-2.00)	0.09
Ever attended daycare	0.71 (0.40-1.27)	0.25	0.91 (0.64-1.30)	0.61
Sibling at home	1.18 (0.72-1.95)	0.51	1.39 (0.98-1.96)	0.07
Antibiotic use before index hospitalization	0.66 (0.42-1.05)	0.08	0.91 (0.66-1.26)	0.58
Corticosteroid use before index hospitalization	1.91 (1.09-3.33)	0.02	1.31 (0.87-1.97)	0.20
Received antibiotics during prehospitalization visit	**2.32 (1.47-3.66)**	**<0.001**	1.39 (0.96-1.99)	0.08
Virology				
Sole RSV infection	Reference		Reference	
Sole rhinovirus infection	0.80 (0.35-1.81)	0.58	**0.35 (0.18-0.68)**	**0.002**
RSV + rhinovirus coinfection	1.51 (0.86-2.66)	0.15	1.03 (0.68-1.59)	0.85
RSV + non-rhinovirus pathogens	1.04 (0.56-1.95)	0.90	0.83 (0.53-1.31)	0.43
Rhinovirus + non-RSV pathogens	0.17 (0.02-1.37)	0.10	**0.24 (0.09-0.67)**	**0.007**
Neither RSV nor rhinovirus	1.16 (0.61-2.18)	0.65	0.83 (0.52-1.33)	0.45

Abbreviations: CI, confidence interval; OR, odds ratio

Bold results are statistically significant

* Defined as admission to intensive care unit and/or use of mechanical ventilation (continuous positive airway pressure and/or intubation during inpatient stay, regardless of location) at any time during the index hospitalization

Table E5. Multivariable Associations of Nasopharyngeal Microbiota Profiles with Bronchiolitis Outcomes by Viral Pathogen, MARC-35 Cohort*

Outcome by microbiota profile	RSV infection (n=693)† OR (95% CI)	P-value	RSV + rhinovirus coinfection (n=120) OR (95% CI)	P-value	Rhinovirus infection (n=91)‡ OR (95% CI)	P-value	Neither RSV nor rhinovirus (n=101) OR (95% CI)	P-value
Intensive care use								
Haemophilus-dominant profile	2.60 (1.15-5.86)	0.02	3.50 (0.45-27.4)	0.23	3.99 (0.36-44.6)	0.26	4.71 (0.56-39.3)	0.15
Moraxella-dominant profile	Reference		Reference		Reference		Reference	
Streptococcus-dominant profile	1.60 (0.78-3.29)	0.20	1.67 (0.27-10.3)	0.58	0.57 (0.02-17.8)	0.75	1.84 (0.17-19.5)	0.61
Mixed profile	1.81 (0.87-3.75)	0.11	0.74 (0.15-3.67)	0.71	0.29 (0.01-6.4)	0.43	0.85 (0.09-7.90)	0.88
Hospital length-of-stay ≥3 days								
Haemophilus-dominant profile	3.56 (2.02-6.29)	<0.001	1.67 (0.41-6.93)	0.48	1.37 (0.26-7.18)	0.71	2.93 (0.73-11.7)	0.13
Moraxella-dominant profile	Reference		Reference		Reference		Reference	
Streptococcus-dominant profile	1.30 (0.82-2.01)	0.27	0.33 (0.09-1.23)	0.10	0.53 (0.03-8.56)	0.65	1.72 (0.38-7.88)	0.48
Mixed profile	1.14 (0.71-1.83)	0.60	0.45 (0.15-1.40)	0.17	0.69 (0.09-5.02)	0.71	4.55 (1.11-18.6)	0.04

Abbreviations: CI, confidence interval; OR, odds ratio; RSV, respiratory syncytial virus

* Mixed-effects logistic regression model adjusting for 10 patient-level variables (age, sex, race/ethnicity, gestational age, history of breathing problems, daycare attendance, siblings at home, lifetime history of antibiotic use, history of corticosteroid use, and use of antibiotics during the pre-hospitalization visit) and sites as random effect

† RSV with or without non-rhinovirus pathogens

‡ Rhinovirus with or without non-RSV pathogens

Table E6. Unadjusted and Multivariable Associations of Nasopharyngeal Microbiota Profiles with Hospital Length-of-Stay, MARC-35 Cohort

	Unadjusted model		Adjusted model*	
Outcome by microbiota profile	**RR (95% CI)**	**P-value**	**RR (95% CI)**	**P-value**
Hospital length-of-stay (count variable)				
Haemophilus-dominant profile	1.22 (1.10-1.36)	<0.001	1.23 (1.10-1.38)	<0.001
Moraxella-dominant profile	Reference		Reference	
Streptococcus-dominant profile	1.05 (0.94-1.16)	0.39	0.89 (0.80-0.99)	0.03
Mixed profile	0.92 (0.83-1.02)	0.11	0.86 (0.77-0.96)	0.007

Abbreviations: CI, confidence interval; RR, rate ratio

* Mixed-effects Poisson regression model adjusting for 11 patient-level variables (age, sex, race/ethnicity, gestational age, history of breathing problems, daycare attendance, siblings at home, lifetime history of antibiotic use, history of corticosteroid use, use of antibiotics during the pre-hospitalization visit, and respiratory viruses detected by PCR) and sites as random effect

Table E7. Richness, Alpha-diversity, and Relative Abundance by Nasopharyngeal Microbiota Profile in MARC-30 Substudy

Indices	***Haemophilus*-dominant profile** n=65 (21.2%)	***Moraxella*-dominant profile** n=52 (16.9%)	***Streptococcus*-dominant profile** n=92 (30.0%)	**Mixed Profile*** n=98 (31.9%)	**P-value**
Richness					
Number of genera, median (IQR)	9 (4-14)	11 (6-17)	14 (9-19)	21 (15-28)	<0.001
Alpha-diversity, median (IQR)					
Shannon index	0.56 (0.19-0.99)	0.56 (0.27-0.94)	1.03 (0.72-1.32)	1.71 (1.37-2.06)	<0.001
Relative abundance of 10 most common genera, mean (SD)					
Streptococcus	0.06 (0.07)	0.06 (0.07)	0.66 (0.23)	0.28 (0.13)	0.003†
Haemophilus	0.81 (0.16)	0.03 (0.08)	0.04 (0.08)	0.11 (0.14)	0.003†
Moraxella	0.05 (0.09)	0.75 (0.27)	0.06 (0.10)	0.18 (0.23)	0.003†
Prevotella	0.02 (0.04)	0.01 (0.03)	0.02 (0.05)	0.09 (0.13)	0.003†
Neisseria	0.01 (0.06)	0.05 (0.18)	0.01 (0.03)	0.05 (0.11)	0.17†
Staphylococcus	0.01 (0.04)	0.01 (0.02)	0.03 (0.11)	0.03 (0.09)	0.69†
Veillonella	0.00 (0.01)	0.00 (0.01)	0.02 (0.04)	0.04 (0.06)	0.003†
Dolosigranulum	0.00 (0.01)	0.01 (0.02)	0.04 (0.13)	0.01 (0.04)	0.051†
Alloprevotella	0.01 (0.03)	0.00 (0.01)	0.01 (0.02)	0.04 (0.08)	0.009†
Corynebacterium	0.00 (0.01)	0.00 (0.01)	0.03 (0.08)	0.01 (0.05)	0.04†

Abbreviations: IQR, interquartile range; SD, standard deviation

* Subclustering of the mixed profile was summarized in Table E8.

† Benjamini-Hochberg adjusted P-value accounting for multiple comparisons

Table E8. Richness, Alpha-diversity, and Relative Abundance by Subcluster in Mixed Microbiota Profile in MARC-30 Substudy

Indices	**Subcluster 1** n=42	**Subcluster 2** n=40	**Subcluster 3** n=16	**P-value**
Richness				
Number of genera, median (IQR)	25 (19-31)	17 (12-23)	18 (16-25)	0.002
Alpha-diversity, median (IQR)				
Shannon index	1.98 (1.70-2.29)	1.41 (1.05-1.78)	1.63 (1.39-1.81)	<0.001
Relative abundance of 10 most common genera, mean (SD)				
Streptococcus	0.23 (0.12)	0.31 (0.14)	0.29 (0.10)	0.49*
Haemophilus	0.10 (0.10)	0.03 (0.04)	0.34 (0.17)	0.003*
Moraxella	0.01 (0.03)	0.42 (0.16)	0.03 (0.08)	0.003*
Prevotella	0.18 (0.15)	0.02 (0.04)	0.02 (0.04)	0.003*
Neisseria	0.09 (0.16)	0.01 (0.03)	0.02 (0.05)	0.20*
Staphylococcus	0.01 (0.02)	0.04 (0.10)	0.06 (0.14)	0.63*
Veillonella	0.07 (0.07)	0.02 (0.03)	0.03 (0.04)	0.003*
Dolosigranulum	0.01 (0.03)	0.02 (0.05)	0.00 (0.00)	0.99*
Alloprevotella	0.07 (0.12)	0.01 (0.02)	0.02 (0.04)	0.08*
Corynebacterium	0.02 (0.06)	0.01 (0.03)	0.00 (0.00)	0.99*

Abbreviations: IQR, interquartile range; SD, standard deviation

The number of subclusters for the data was determined using the average silhouette score.

* Benjamini-Hochberg adjusted P-value accounting for multiple comparisons.

Table E9. Unadjusted and Multivariable Associations of Nasopharyngeal Microbiota Profiles with Bronchiolitis Outcomes, MARC-30 Substudy

	Unadjusted model		Adjusted model*	
Outcome by microbiota profile	**OR (95% CI)**	**P-value**	**OR (95% CI)**	**P-value**
Intensive care use†				
Haemophilus-dominant profile	6.98 (3.13-16.5)	<0.001	5.34 (1.96-14.5)	0.001
Moraxella-dominant profile	Reference		Reference	
Streptococcus-dominant profile	3.64 (1.73-8.06)	0.001	1.95 (0.79-4.83)	0.15
Mixed profile	3.20 (1.54-7.05)	0.003	2.40 (0.96-5.95)	0.06
Hospital length-of-stay ≥3 days				
Haemophilus-dominant profile	7.81 (3.45-18.9)	<0.001	6.70 (2.37-19.0)	<0.001
Moraxella-dominant profile	Reference		Reference	
Streptococcus-dominant profile	3.72 (1.75-8.44)	<0.001	1.89 (0.74-4.83)	0.18
Mixed profile	3.43 (1.63-7.74)	0.002	2.70 (1.05-6.93)	0.04

Abbreviations: CI, confidence interval; OR, odds ratio

* Mixed-effects logistic regression model adjusting for 11 patient-level variables (age, sex, race/ethnicity, gestational age, history of breathing problems, daycare attendance, siblings at home, lifetime history of antibiotic use, history of corticosteroid use, use of antibiotics during the pre-hospitalization visit, and respiratory viruses detected by PCR) and sites as random effect

† Defined as admission to intensive care unit and/or use of mechanical ventilation (continuous positive airway pressure and/or intubation during inpatient stay, regardless of location) at any time during the index hospitalization