

Letter to the Editor

Serum cathelicidin, nasopharyngeal microbiota, and disease severity among infants hospitalized with bronchiolitis*To the Editor:*

Bronchiolitis is a common acute respiratory infection and the leading cause of hospitalizations in US infants.¹ Although bronchiolitis has been considered virus-induced inflammation of small airways,¹ emerging evidence indicates that the pathogenesis involves a complex interplay among viral agents, airway microbiota, and the innate immune system.²⁻⁴

Among the multiple components of the innate immune system, cathelicidins are a family of host defense peptides with both direct microbicidal and immunomodulatory properties.⁵ In previous studies, serum cathelicidin level was inversely associated with disease severity in children with bronchiolitis,⁶ and administration of LL-37 (the main active form of human cathelicidin) altered the microbiota in animal models.⁷ We have previously demonstrated that, in a cohort of infants with severe bronchiolitis (bronchiolitis requiring hospitalization),⁴ infants with a *Haemophilus*-dominant nasopharyngeal microbiota profile had higher severity; however, host immune responses were not examined. In the present study, we sought to determine interactions between serum LL-37 levels and nasopharyngeal microbiota profiles with regard to disease severity by using data from a prospective cohort of infants with severe bronchiolitis.

Details of the study design, setting, population, testing, and analysis may be found in the Online Repository (see this article's [Methods](#) section at www.jacionline.org). Briefly, this prospective cohort study, the 35th Multicenter Airway Research Collaboration,⁴ enrolled infants (age <1 year) at 17 sites across 14 US states (see [Table E1](#) in this article's Online Repository at www.jacionline.org) during the 2011-2014 winter seasons. Bronchiolitis was defined per the American Academy of Pediatrics guidelines.¹ In addition to clinical data, investigators collected blood samples and nasopharyngeal aspirates within 24 hours of hospitalization by using a standardized protocol. We quantified serum LL-37 concentration by using an ELISA (see this article's [Methods](#) section). To examine the structure and composition of nasopharyngeal microbiota, we sequenced the 16S rRNA gene V4 region on the Illumina MiSeq platform. As previously described,⁴ by using an unbiased clustering approach, we derived 4 distinct microbiota profiles: *Haemophilus*-dominant, *Moraxella*-dominant, *Streptococcus*-dominant, and mixed profiles.

For the current analysis, to simplify data presentation and interpretation, we dichotomized serum LL-37 levels, using the median LL-37 level, to classify patients into 2 groups: low LL-37 (≤ 46 ng/mL) and high LL-37 (> 46 ng/mL). The outcome of interest was *intensive care use*, defined as admission to intensive care unit and/or use of mechanical ventilation. To determine heterogeneity of microbiota-outcome associations by LL-37 status, we fit a random-effects model adjusting for 12 potential confounders (age, sex, race/ethnicity, gestational age, history of breathing problems, daycare attendance, siblings at home, breast-feeding, lifetime history of antibiotic and corticosteroid use, use of antibiotics during prehospitalization visit, and respiratory viruses) for each stratum (low and high LL-37 strata).

Of 1016 enrolled infants, 1005 (99%) met the 16S rRNA gene sequence quality control requirements for analysis. The median age was 3.2 months (interquartile range [IQR], 1.6-5.9 months), 60% were males, and 43% were non-Hispanic white. The median LL-37 level was 46 ng/mL (IQR, 34-60 ng/mL) and that of serum 25-hydroxyvitamin D (25(OH)D) was 27 ng/mL (IQR, 18-33 ng/mL). Several patient characteristics differed by LL-37 status ([Table 1](#)). For example, infants with lower LL-37 levels were younger and more likely to have respiratory syncytial virus infection (both $P < .05$), compared with those with higher LL-37 levels. In contrast, there were no significant differences in the microbiota profiles ([Table 1](#)) or abundance of major bacteria genera (see [Table E2](#) in this article's Online Repository at www.jacionline.org) between the groups (all $P \geq .40$). Likewise, 25(OH)D and LL-37 levels were not significantly correlated ($P = .33$; see [Fig E2](#) in this article's Online Repository at www.jacionline.org).

We observed a significant interaction between LL-37 status and microbiota profiles with regard to risks of intensive care use ($P_{\text{interaction}} = .02$), indicating that microbiota-severity associations differ by LL-37 status. For example, among infants with lower LL-37 levels, the *Haemophilus*-dominant profile was associated with higher risks of intensive care use (adjusted odds ratio, 4.14; 95% CI, 1.63-10.5; $P = .003$) compared with the *Moraxella*-dominant profile ([Fig 1](#)). In contrast, among infants with higher LL-37 levels, there were no significant associations between microbiota profiles and risks of intensive care use (all $P > .05$).

These findings are concordant with our previous single-center study of 82 children with bronchiolitis reporting that serum 25(OH)D levels were not correlated with cathelicidin levels in the setting of acute infection, but that cathelicidin was associated with viral etiology.⁶ The current multicenter study of 1005 infants with severe bronchiolitis corroborated these earlier findings and extended them by demonstrating interactions between cathelicidin and airway microbiota with regard to disease severity. The mechanism underlying the observed relationships—that is, an association between *Haemophilus*-dominant microbiota profile and higher severity only among infants with low LL-37 levels—is beyond the scope of our data. However, the association may be causal—that is, LL-37 contributes, through alterations in airway microbiota, to the bronchiolitis morbidity. Alternatively, LL-37 cathelicidin in conjunction with specific microbiota, potentially via facilitating neutrophil extracellular traps formations,⁸ might have contributed to host antiviral response. In addition, another mechanism—that is, specific composition and function of the microbiota upregulates/downregulates the expression of LL-37 cathelicidin locally and/or systemically, thereby contributing to bronchiolitis severity—is also possible. Furthermore, these possibilities are not mutually exclusive.

Although the clinical significance is not yet clear, our findings are of scientific significance with regard to the apparent interrelation between the innate immune response and microbiota. Recent studies are beginning to delineate the mechanisms by which innate immune systems interact with microbes in nonrespiratory niches, such as the gastrointestinal tract.⁷ The present study demonstrates, for the first time, an integrated role of LL-37 antimicrobial peptides (an important component of the innate immune system) and the airway microbiota in the pathogenesis of airway disease. Our data should

TABLE I. Characteristics of 1005 infants hospitalized for bronchiolitis, according to serum LL-37 level

Variables	LL-37 ≤46 ng/mL n = 515 (51%)	LL-37 >46 ng/mL n = 490 (49%)	P value
Characteristics			
Age (mo), median, (IQR)	2.7 (1.5-5.4)	3.8 (2.1-6.3)	<.001
Male sex	312 (61)	291 (59)	.75
Race/ethnicity			
Non-Hispanic white	231 (45)	197 (40)	.23
Non-Hispanic black	112 (22)	121 (25)	
Hispanic	149 (29)	157 (32)	
Other	23 (5)	15 (3)	
Parental history of asthma	182 (35)	159 (33)	.38
Maternal smoking during pregnancy	85 (17)	59 (12)	.06
C-section delivery	178 (35)	165 (34)	.88
Prematurity (32-37 wk)	99 (19)	84 (17)	.44
Previous breathing problems before the index hospitalization*	87 (17)	116 (24)	.009
History of eczema	69 (13)	77 (16)	.33
Ever attended daycare	98 (19)	132 (27)	.004
Sibling in the household	414 (80)	385 (78)	.53
Mostly breast-fed for the first 3 mo of age	211 (47)	208 (49)	.73
Smoke exposure at home	80 (16)	73 (15)	.85
Antibiotic use before the index hospitalization	149 (29)	163 (33)	.16
Corticosteroid use before the index hospitalization	70 (14)	76 (16)	.44
Clinical presentation			
Weight at presentation (kg), median (IQR)	5.7 (4.5-7.4)	6.5 (5.1-7.9)	<.001
Respiratory rate at presentation (per minute), median (IQR)	48 (40-60)	48 (40-60)	.85
Oxygen saturation at presentation			.25
<90%	46 (9.2)	43 (9)	
90%-93%	76 (15)	77 (16)	
≥94%	381 (76)	360 (75)	
Received antibiotics during prehospitalization visit	100 (20)	75 (15)	.09
Received corticosteroids (systemic or inhaled) during prehospitalization visit	47 (9)	42 (9)	.81
Virology			
Sole RSV infection	323 (63)	257 (52)	
Sole rhinovirus infection	22 (4)	38 (8)	
RSV + rhinovirus coinfection	51 (10)	69 (14)	
RSV + nonrhinovirus pathogens	62 (12)	51 (10)	
Rhinovirus + non-RSV pathogens	11 (2)	20 (4)	
Neither RSV nor rhinovirus	46 (9)	55 (11)	
Viral genomic load (CT value), median IQR			
RSV	22 (20-25)	23 (21-26)	.003
Rhinovirus	28 (26-35)	30 (26-36)	.58
Microbiota profile			
<i>Haemophilus</i> -dominant profile	95 (18)	98 (20)	.62
<i>Moraxella</i> -dominant profile	110 (21)	110 (22)	
<i>Streptococcus</i> -dominant profile	142 (28)	141 (29)	
Mixed profile	168 (33)	141 (29)	
Laboratory data			
Serum LL-37 (ng/mL), median (IQR)	34 (28-40)	62 (54-72)	—
Serum 25(OH)D (ng/mL), median (IQR)	26 (18-33)	27 (19-34)	.32
Serum bioavailable 25(OH)D (ng/mL), median (IQR)	4 (3-7)	4 (3-7)	.15
Food sensitization†	90 (18)	89 (18)	.84
Aeroallergen sensitization‡	4 (1)	9 (2)	.57
Hospital course			
Hospital length of stay (d), median (IQR)	2 (1-4)	2 (1-3)	.06
Intensive care use‡	92 (18)	69 (14)	.12

Data are no. (%) of children unless otherwise indicated. Percentages may not equal 100, because of missingness.

RSV, Respiratory syncytial virus.

*Defined as a child having cough that wakes him or her at night and/or causes emesis, or when the child has wheezing or shortness of breath without cough.

†Defined by having ≥1 positive values for allergen-specific IgE.

‡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.

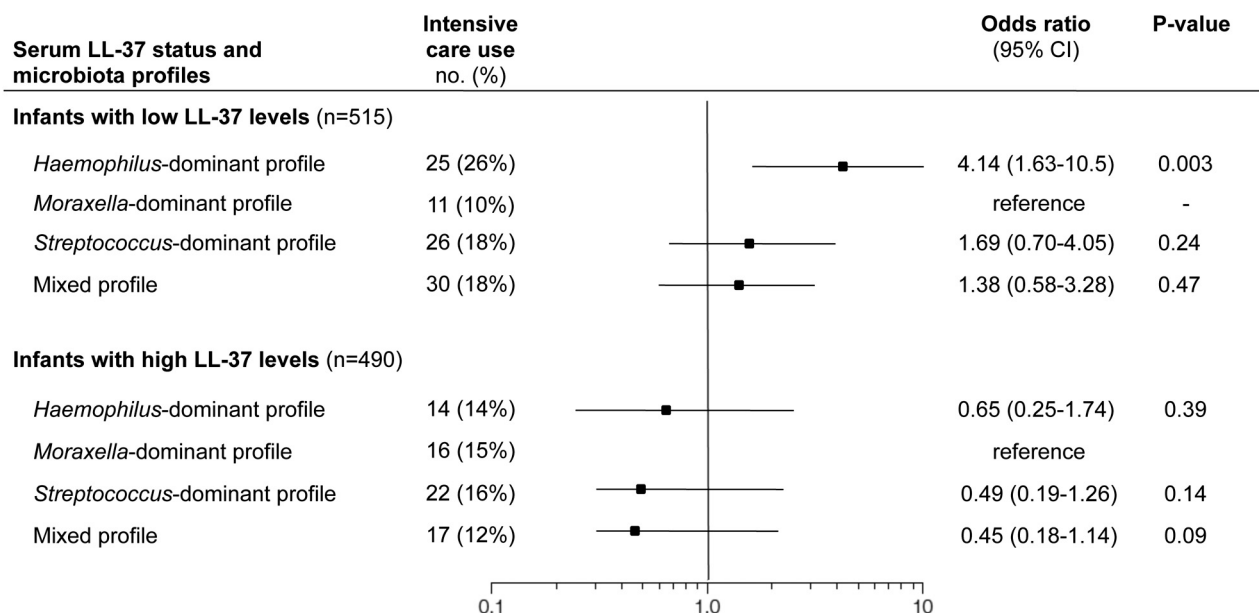


FIG 1. Multivariable associations between nasopharyngeal microbiota profile and risks of intensive care use stratified by serum LL-37 level. We constructed random-effects models for each strata (infants with low LL-37 levels [≤ 46 ng/mL] and those with high LL-37 levels [>46 ng/mL]) with the *Moraxella*-dominant profile as the reference. Models were adjusted for age, sex, race/ethnicity, gestational age, history of breathing problems, daycare attendance, siblings at home, breast-feeding, lifetime history of antibiotic use, history of corticosteroid use, use of antibiotics during the prehospitalization visit, and respiratory viruses detected by PCR. The model also accounted for patient clustering at the hospital level.

facilitate further mechanistic investigations into this complex interplay.

We acknowledge several potential limitations. First, our data were based on serum LL-37 levels and nasopharyngeal microbiota, which may not reflect the cathelicidin activity or microbiota in the lung. Nonetheless, studies have shown that cathelicidin derived from bone marrow rather than airway epithelial cells is responsible for antimicrobial effects in the lung,⁹ and that upper airway microbiota is a reliable representation of that of lower airway in children.¹⁰ Second, 16S rRNA gene sequencing precluded us from examining the function of microbiome. We hope to address this important issue in future work using meta-transcriptomic approaches. Third, this study did not have the information of a “control” group. Yet, the study objective is not to assess the role of microbiome and cathelicidin on the development of bronchiolitis but to determine their relationship with disease severity among infants who have bronchiolitis. Finally, although the study cohorts consisted of racially/ethnically diverse US sample of severe bronchiolitis, our inferences might not be generalizable to those with mild-to-moderate illness.

In sum, on the basis of data from a multicenter cohort of 1005 infants with severe bronchiolitis, we found an interaction between serum cathelicidin (LL-37) and nasopharyngeal microbiota with regard to higher disease severity. Specifically, we observed significant associations between *Haemophilus*-dominant profile and higher risks of intensive care use only in infants with lower LL-37 levels. Our findings should facilitate research into understanding the complex interplay between the innate immunity, airway microbiome, and bronchiolitis pathogenesis in infants.

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METHODS

Study design, setting, and participants

This is an analysis of data from a multicenter prospective cohort study of infants (age <1 year) hospitalized with bronchiolitis (severe bronchiolitis). This study, called the 35th Multicenter Airway Research Collaboration,^{E1} was coordinated by the Emergency Medicine Network,^{E2} a collaboration of 235 participating hospitals.

Using a standardized protocol, site investigators at 17 sites across 14 US states (Table E1) enrolled infants hospitalized with an attending physician diagnosis of bronchiolitis during 3 consecutive bronchiolitis seasons from November 1 to April 30 (2011–2014). Bronchiolitis was defined by the American Academy of Pediatrics guidelines—acute respiratory illness with some combination of rhinitis, cough, tachypnea, wheezing, crackles, and retractions.^{E3} We excluded infants with previous enrollment, those who were transferred to a participating hospital more than 24 hours after the original hospitalization, those who provided consent more than 24 hours after hospitalization, or those with known heart-lung disease, immunodeficiency, immunosuppression, or gestational age of less than 32 weeks. All patients were treated at the discretion of the treating physician. The institutional review board at each of the participating hospitals approved the study. Written informed consent was obtained from the parent or guardian.

Data collection

Investigators conducted a structured interview that assessed patients' demographic characteristics, medical and family history, and details of the acute illness. Emergency department and hospital chart reviews provided further clinical data, such as vital signs, physical examination, medical management, and disposition. All data were reviewed at the EMNet Coordinating Center, and site investigators were queried about missing data and discrepancies identified by manual data checks.

Nasopharyngeal aspirates were collected by trained site investigators using the standardized protocol used in a previous cohort study of children with bronchiolitis.^{E4,E5} All sites used the same collection equipment (Medline Industries, Mundelein, Ill) and collected the samples within 24 hours of hospitalization. The nasopharyngeal sample was added to transport medium, immediately placed on ice, and then stored at -80°C . Frozen samples were shipped in batches on dry ice to Baylor College of Medicine, where they were tested for (1) 17 respiratory viruses (eg, respiratory syncytial virus and rhinovirus) by using real-time PCR assays,^{E4-E6} and (2) microbiota by using 16S rRNA gene sequencing.

16S rRNA gene sequencing

16S rRNA gene sequencing methods were adapted from the methods developed for the NIH-Human Microbiome Project.^{E7,E8} Because 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, Calif),^{E9} 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, San Diego, Calif) using the 2×250 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.^{E10,E11}

Sequencing read pairs were demultiplexed on the basis of unique molecular barcodes, and reads were merged using USEARCH v7.0.1090,^{E12} 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.^{E13} In addition, 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 resequenced to ensure that most 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.^{E14} OTUs were determined by mapping the centroids to the SILVA database^{E15} containing only the 16S V4 region to determine taxonomies. A custom script constructed a rarefied OTU table (rarefaction was performed at only 1 sequence depth) from the output files generated in the previous 2 steps for downstream analyses of alpha diversity (eg, Shannon index) and beta diversity (eg, weighted UniFrac).^{E16,E17} Shannon diversity index is a quantitative measure that takes into account not only richness but also the proportion of each bacteria (evenness) within the local community. The weighted UniFrac algorithm calculates the distance between microbial communities on the basis of phylogenetic relatedness of lineages and relative abundance in each sample.

Compositional analysis and clustering

16S rRNA gene sequencing of the nasopharyngeal samples from the enrolled infants ($n = 1,016$) obtained 17,399,260 high-quality merged sequences, of which 16,685,637 (95.9%) were mapped to the database. Of 1,016 infant samples, 1,005 (98.9%) had sufficient sequence depth (rarefaction cutoff, 2,128 reads per sample) and were analyzed in the present study.

As previously described,^{E2} by using the partitioning around medoids method^{E18} with weighted UniFrac distance, we identified 4 distinct microbiota profiles: (1) *Haemophilus*-dominant profile (19.2%), (2) *Moraxella*-dominant profile (21.9%), (3) *Streptococcus*-dominant profile (28.2%), and (4) mixed profile (30.7%) (Fig E1). The number of clusters was determined using the average silhouette score.^{E19}

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. Nontemplate controls (extraction chemistries) were included in the microbial DNA extraction process and the resulting material was subsequently used for PCR amplification. In addition, at the step of amplification, another set of nontemplate controls (PCR-mix) was included to evaluate the potential introduction of contamination at this step. Similarly, a positive control composed 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 nontemplate controls or the absence of amplicons in the positive control. In the present study, no amplicons were observed in the nontemplate controls and a negligible amount of raw reads was recovered after sequencing.

LL-37 measurement

The sole human cathelicidin is human cationic antimicrobial peptide of 18 kDa (hCAP-18), which is encoded by the *CAMP* gene and regulated by multiple factors (eg, vitamin D, Toll-like receptor agonists).^{E20} LL-37, the main active form of human cathelicidin, is generated proteolytically from hCAP-18.^{E21} Serum LL-37 concentration was quantified by using a commercially available ELISA (Hycult Biotech, Uden, Netherlands). In particular, the lower limit of detection of the LL-37 assay is 1 ng/mL, with an intraassay coefficient of variation of less than 12%. The antigens recognized by this ELISA are within the peptide LL-37. The assay was performed according to the manufacturer's instructions and the sample was diluted 1:20 before testing.

Outcome measure

The outcome of interest was *intensive care use*, 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.^{E4,E22}

Statistical analyses

First, we dichotomized the serum LL-37 levels to classify patients into 2 groups: low LL-37 (≤ 46 ng/mL) and high LL-37 (> 46 ng/mL). We compared the patients' characteristics, clinical presentation, virology, nasopharyngeal

microbiota profiles, and laboratory data, by LL-37 status, using the chi-square test or the Wilcoxon-Mann-Whitney test as appropriate. Next, we tested for an interaction between LL-37 status and microbiota profiles with regard to risks of intensive care use by fitting a random-effects model adjusting for 12 patient-level variables (ie, age, sex, race/ethnicity, gestational age, history of breathing problems, daycare attendance, siblings at home, breast-feeding, lifetime history of antibiotic use, history of corticosteroid use, use of antibiotics during the prehospitalization visit, and respiratory viruses detected by PCR). The model also accounted for patient clustering at the hospital level. We chose these potential confounders on the basis of clinical plausibility and *a priori* knowledge.^{E4,E5,E22-E25} Because the model indicated a significant LL-37 $\times$ Microbiota interaction ($P_{\text{interaction}} = .02$), we repeated the analysis with stratification by LL-37 status. Analyses used R version 3.2 (R Foundation, Vienna, Austria). All *P* values were 2-tailed, with *P* < .05 considered statistically significant.

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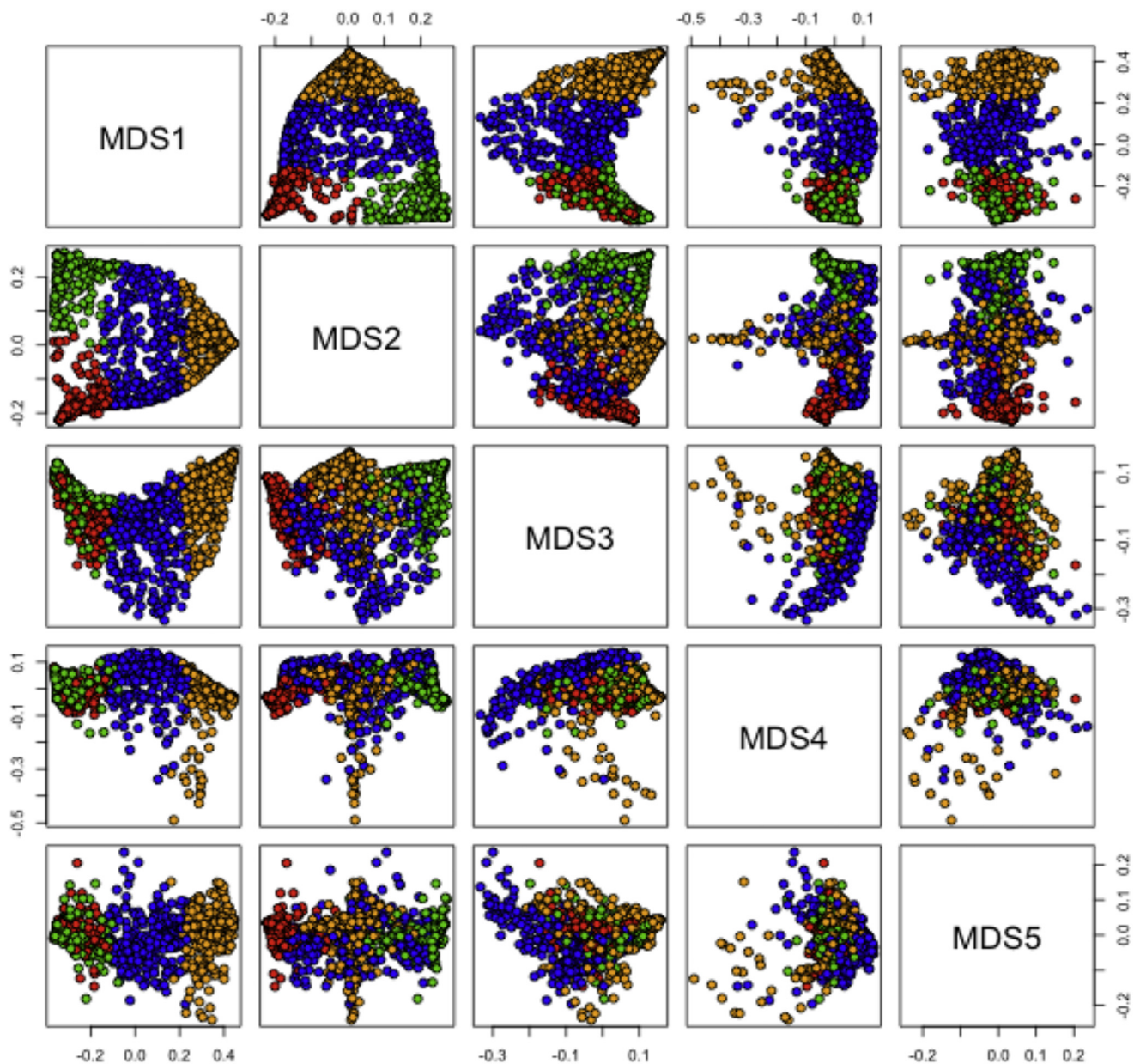


FIG E1. Metric multidimensional scaling plots based on weighted UniFrac distance. To show the differences in nasopharyngeal microbiota among 1005 infants with severe bronchiolitis, multiple metric multidimensional scaling (MDS) plots based on the weighted UniFrac distance between all subjects were generated by using the R package *cmdscale*. Each dot represents the overall bacterial community in each subject. Colors indicate 4 microbiota profiles: *Haemophilus*-dominant profile (green), *Moraxella*-dominant profile (red), *Streptococcus*-dominant profile (yellow), and mixed profile (blue). The combinations of the first 5 principal coordinates revealed that subjects cluster together according to their microbiota profile.

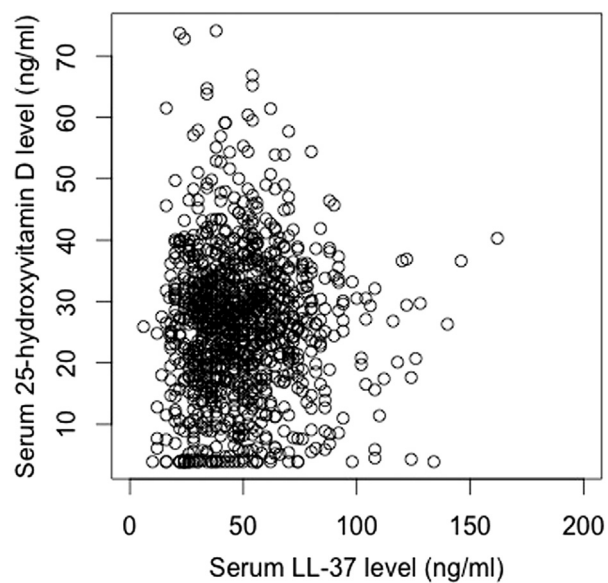


FIG E2. Scatterplot of serum LL-37 and 25(OH)D levels. There was no significant correlation between serum LL-37 and 25(OH)D levels ($r = 0.03$; $P = .33$).

TABLE E1. Principal investigators at the 17 participating sites in MARC-35

Amy D. Thompson, MD	Alfred I. duPont Hospital for Children, Wilmington, Del
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Vincent J. Wang, MD, MHA	Children's Hospital of Los Angeles, Los Angeles, Calif
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, Okla
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, DC
Richard T. Strait, MD	Cincinnati Children's Hospital and Medical Center, Cincinnati, Ohio
Ilana Waynik, MD	Connecticut Children's Medical Center, Hartford, Conn
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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, Mass
Anne K. Beasley, MD	Phoenix Children's Hospital, Phoenix, Ariz
Thida Ong, MD	Seattle Children's Hospital, Seattle, Wash
Charles G. Macias, MD, MPH	Texas Children's Hospital, Houston, Tex

TABLE E2. Richness, alpha diversity, and relative abundance by serum LL-37 level

Indices	LL-37 ≤46 ng/mL n = 515 (51%)	LL-37 >46 ng/mL n = 490 (49%)	P value
Richness			
No. of genera, median (IQR)	17 (10-24)	15 (8-24)	.06
Alpha diversity			
Shannon index, median (IQR)	0.99 (0.57-1.45)	0.89 (0.52-1.36)	.07
Relative abundance of 10 most common genera, mean ± SD			
<i>Streptococcus</i>	0.31 ± 0.29	0.31 ± 0.30	.99*
<i>Moraxella</i>	0.29 ± 0.34	0.31 ± 0.34	.99*
<i>Haemophilus</i>	0.19 ± 0.30	0.21 ± 0.31	.99*
<i>Prevotella</i>	0.02 ± 0.06	0.02 ± 0.06	.99*
<i>Staphylococcus</i>	0.02 ± 0.08	0.02 ± 0.10	.99*
<i>Neisseria</i>	0.03 ± 0.08	0.02 ± 0.06	.96*
<i>Corynebacterium</i>	0.02 ± 0.08	0.01 ± 0.04	.40*
<i>Alloprevotella</i>	0.01 ± 0.05	0.01 ± 0.04	.99*
<i>Veillonella</i>	0.01 ± 0.03	0.01 ± 0.03	.99*
<i>Gemella</i>	0.01 ± 0.03	0.01 ± 0.03	.99*

IQR, Interquartile range.

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