RESEARCH NOTE

Sphingolipid metabolism potential in fecal microbiome and bronchiolitis in infants: a case–control study

Kohei Hasegawa1*, Christopher J. Stewart2, Jonathan M. Mansbach3, Rachel W. Linnemann4, Nadim J. Ajami2, Joseph F. Petrosino2 and Carlos A. Camargo1

Abstract

Objective: Emerging evidence demonstrated that the structure of fecal microbiome is associated with the likelihood of bronchiolitis in infants. However, no study has examined functional profiles of fecal microbiome in infants with bronchiolitis. In this context, we conducted a case–control study. As a part of multicenter prospective study, we collected stool samples from 40 infants hospitalized with bronchiolitis (cases). We concurrently enrolled 115 age-matched healthy controls.

Results: First, by applying 16S rRNA gene sequencing to these 155 fecal samples, we identified the taxonomic profiles of fecal microbiome. Next, based on the taxonomy data, we inferred the functional capabilities of fecal microbiome and tested for differences in the functional capabilities between cases and controls. Overall, the median age was 3 months and 45% were female. Among 274 metabolic pathways surveyed, there were significant differences between bronchiolitis cases and healthy controls for 37 pathways, including lipid metabolic pathways (false discovery rate [FDR] <0.05). Particularly, the fecal microbiome of bronchiolitis cases had consistently higher abundances of gene function related to the sphingolipid metabolic pathways compared to that of controls (FDR <0.05). These pathways were more abundant in infants with Bacteroides-dominant microbiome profile compared to the others (FDR <0.001). On the basis of the predicted metagenome in this case–control study, we found significant differences in the functional potential of fecal microbiome between infants with bronchiolitis and healthy controls. Although causal inferences remain premature, our data suggest a potential link between the bacteria-derived metabolites, modulations of host immune response, and development of bronchiolitis.

Keywords: Microbiome, Infants, Bronchiolitis, Bacteroides, Sphingolipids

Introduction

Bronchiolitis is a common acute respiratory infection and the leading cause of hospitalizations in US infants [1, 2]. Although bronchiolitis has been considered virus-induced inflammation of small airways [3], recent studies demonstrate that the pathobiology involves complex interrelations among respiratory viruses, host immune response, and human microbiome [4–10]. Emerging evidence also indicates the existence of “gut-lung axis” in which the gut microbiome conditions immunologic responses in the lungs to environmental challenges (e.g., viral infection) [11]. Indeed, we have previously demonstrated, in a case–control study of infants hospitalized for bronchiolitis and healthy controls [11], that the taxonomy profiles of the fecal microbiome were associated with the likelihood of bronchiolitis—e.g., infants with the Bacteroides-dominant profile were more likely to have bronchiolitis. Although previous studies suggest that the gut microbiome-derived metabolites (e.g., sphingolipids) may play an important role in the host immune development [12, 13], the functional profiles of fecal microbiome...
in infants were not examined in the earlier study. To address this knowledge gap, we determined the predicted function of fecal microbiome in infants with bronchiolitis and healthy infants.

**Main text**

**Methods**

This study was a secondary analysis of the data from a case–control study of infants hospitalized for bronchiolitis and healthy controls. The study design, setting, participants, and methods of data collection have been reported previously [11]. In brief, as a part of a multicenter prospective cohort study, called the 35th Multi-center Airway Research Collaboration (MARC-35) [4–7, 9], we enrolled 40 infants (aged <12 months) hospitalized for an attending physician diagnosis of bronchiolitis from November 2013 through April 2014. Bronchiolitis was diagnosed according to the American Academy of Pediatrics guidelines [14]. Exclusion criteria were a transfer to a participating hospital >48 h after the original hospitalization, delayed consent (>24 h after hospitalization), gestational age \( \leq 32 \) weeks, and known comorbidities (cardiopulmonary disease, immunodeficiency, immunosuppression). In addition, during the same period, we also enrolled 115 healthy infants as the controls (age-matched within 1.5 months of cases) [11, 15–17]. We excluded infants with current fever, respiratory illness, or gastrointestinal illness, antibiotic treatment in the preceding 7 days, gestational age \( \leq 32 \) weeks, or known comorbidities. Taken together, a total of 155 infants were eligible for the current analysis. From these infants, by using a standardized protocol [11, 15, 17], investigators conducted a structured interview and medical record review, and collected fecal specimens at the time of hospitalization (cases) or at home before the clinic visit (controls). The fecal samples were immediately stored at \(-80^\circ\text{C}\). The institutional review board at each of the participating hospitals approved the study. Written informed consent was obtained from the parent or guardian.

16S rRNA gene sequencing was performed based on the methods adapted from the NIH Human Microbiome Project. Briefly, bacterial genomic DNA was extracted using MO BIO PowerMag DNA Isolation Kit (Mo Bio Lab; Carlsbad, CA). The 16S rDNA V4 regions were amplified by PCR and sequenced in the MiSeq platform (Illumina; SanDiego, CA) using \( 2 \times 250 \) bp paired-end protocol. Sequencing read pairs were demultiplexed based on the unique molecular barcodes, and reads were merged using USEARCH v7.0.1090, allowing no mismatches and a minimum overlap of 50 bases. We trimmed the merged reads 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. We also applied a quality filter to the resulting merged reads, discarded the reads containing >0.05 expected errors. We constructed rarefaction curves of bacterial operational taxonomic units (OTUs) using sequence data for each sample to ensure coverage of the bacterial diversity present (Fig. 1). 16S rRNA gene sequences were clustered into OTUs at a similarity cutoff value of 97% using the UPARSE algorithm; OTUs were mapped to the SILVA Database to determine taxonomies. Abundances were recovered by mapping the demultiplexed reads to the UPARSE OTUs.

To infer the functional capabilities of the fecal microbiome based on the OTU (taxonomy) data, we used a bioinformatic approach, Tax4Fun [18]. This approach links the 16S rRNA gene sequences with the functional annotation of sequenced bacterial genomes by identifying a nearest neighbor based on a minimal 16S rRNA gene sequence similarity. Next, the predicted metagenomes were categorized by function at the Kyoto Encyclopedia of Genes and Genomes (KEGG) ortholog and pathway levels [19]. We tested for significant differences in the functional category abundances between cases and controls using Welch’s unequal variances t test. Resulting P values were adjusted for multiple hypothesis testing by converting to false discovery rate q values using the Benjamini–Hochberg procedure, with q values of <0.05 considered statistically significant. To validate the findings, we performed random permutation testing with 1000 permutations for each of the pathways of interest, which corresponds to the situation when the abundance of pathways is randomly assigned to cases and controls.
contained in dataset. Once the dataset was permuted, we tested for the differences in abundances between cases and controls. We repeated the randomization 1000 times and recorded the squared error of the models averaged for every repetition. Additionally, to further examine the differences in the pathways of interest, we constructed multivariable linear regression models adjusting for potential confounders (age, sex, race/ethnicity, maternal antibiotic use during pregnancy, history of prematurity, mode of delivery, feeding status, and lifetime history of antibiotic and corticosteroid use), based on a priori knowledge [5, 6, 9]. Furthermore, to determine the relationship between the abundance of bacteria genus and metabolic pathways of interest, we examined their correlations with the use of scatterplots fitting locally weighted scatterplot smoothed (LOWESS) curve as well as Spearman’s correlation. The analyses used R version 3.3 with phyloseq package [20] and STAMP version 2.1 [21].

**Results**

At the four participating hospitals, a total of 40 infants hospitalized for bronchiolitis (cases) and 115 age-matched healthy infants (controls) were enrolled (Table 1). Overall, the median age was 3 months (IQR, 2–5 months) and 55% were male. All 155 fecal specimens had sufficient depth to obtain high degree of sequence

| Table 1  Patient characteristics of 40 cases (infants with bronchiolitis) and 115 controls (healthy infants) at enrollment |
|-----------------------------------------------|
| Characteristics                              | Infants with bronchiolitis n = 40 | Healthy control infants n = 115 | P value* |
| Demographics                                 |                                  |                               |
| Age (mo), median (IQR)                       | 3.2 (1.6–4.9)                    | 3.8 (2.0–4.9)                 | 0.52     |
| Male sex                                     | 22 (55)                          | 64 (56)                       | 0.99     |
| Race/ethnicity                               |                                  |                               |
| Non-hispanic white                           | 23 (58)                          | 61 (53)                       | 0.04     |
| Non-hispanic black                           | 6 (15)                           | 11 (10)                       |          |
| Hispanic                                     | 10 (25)                          | 19 (17)                       |          |
| Other                                        | 1 (3)                            | 24 (21)                       |          |
| Prenatal history                             |                                  |                               |
| Parental history of asthma                   | 16 (40)                          | 21 (18)                       | 0.01     |
| Maternal smoking during pregnancy            | 8 (20)                           | 3 (3)                         |          |
| Maternal antibiotic use during pregnancy     | 11 (28)                          | 13 (11)                       | 0.02     |
| Maternal antibiotic use during labor         | 12 (30)                          | 35 (30)                       | 0.82     |
| Past medical history and home environmental characteristics |                                  |                               |
| Mode of birth, C-section                     | 9 (23)                           | 43 (37)                       | 0.13     |
| Prematurity (32–37 weeks)                    | 12 (30)                          | 11 (10)                       | 0.004    |
| Previous breathing problems before enrollment† | 8 (20)                           | 0 (0)                         | 0.001    |
| History of eczema                            | 8 (20)                           | 17 (15)                       | 0.56     |
| Ever attended daycare                        | 9 (23)                           | 14 (12)                       | 0.16     |
| Smoking exposure at home                     | 8 (20)                           | 4 (3)                         | 0.002    |
| Mostly breastfed for the first 3 months of age | 16 (40)                          | 89 (77)                       | 0.009    |
| Systemic antibiotic use before enrollmentab  | 8 (20)                           | 13 (11)                       | 0.24     |
| Systemic corticosteroid use before enrollment | 9 (23)                           | 0 (0)                         | 0.001    |
| Clinical course                              |                                  |                               |
| Systemic antibiotic use during pre-hospitalization visit | 8 (20)        | –                             |          |
| Systemic corticosteroid use during pre-hospitalization visit | 3 (8)        | –                             |          |
| Hospital length-of-stay (day), median (IQR)  | 3 (2–4)                          | –                             |          |
| Admission to intensive care unit             | 8 (20)                           | –                             |          |
| Use of mechanical ventilation†               | 5 (13)                           | –                             |          |

Data are no. (%) of infants unless otherwise indicated. Percentages may not equal 100 because of missingness or rounding

IQR interquartile range

* Chi square, Fisher exact, or Wilcoxon-Mann–Whitney tests, as appropriate

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

ab Lifetime use of systemic antibiotic use before the enrollment. Infants with systemic antibiotic treatment in the preceding 7 days were not enrolled to the control group
coverage (rarefaction cutoff, 1470 reads/specimen; Fig. 1). The fecal microbiome were dominated by four genera: *Escherichia* (22%), *Bifidobacterium* (19%), *Enterobacter* (15%), and *Bacteroides* (13%). The characteristics of the fecal microbiome differed between cases and controls (Table 2). For example, infants with bronchiolitis had a higher proportion of *Bacteroides*-dominant profile and lower proportion of *Enterobacter/Veillonella*-dominant profile, compared to healthy controls (P = 0.01).

Between the infants with bronchiolitis and healthy controls, we compared the functional potential of fecal microbiome inferred from the 16S rRNA gene sequencing data. Of 6402 KEGG orthologs (orthologous genes) surveyed, the abundances of 319 genes were significantly different (q < 0.05; Table 3). The functional differences involved genes with diverse metabolic functions—e.g., carbohydrate, amino acid, and lipid metabolism. To make the data presentation and interpretation more meaningful, the genes were further consolidated into 274 KEGG pathways. Among these, there were significant differences between bronchiolitis cases and healthy controls for 37 pathways, including lipid metabolic pathways (q < 0.05; Table 4; Fig. 2). Particularly, the fecal microbiome of bronchiolitis cases had consistently higher abundances of gene function related to the sphingolipid metabolic pathways compared to that of controls (all q < 0.05)—i.e., sphingolipid (ko00600) and glycosphingolipid (ko00603, ko00604) metabolic pathways (Fig. 3). For each of these 3 pathways, the permutation test was significant (all random permutation P < 0.05), supporting the validity of the observed between-group differences. In the multivariable models adjusting for 9 patient-level factors (age, sex, race/ethnicity, maternal antibiotic use during pregnancy, history of prematurity, mode of delivery, feeding status, and lifetime history of antibiotic and corticosteroid use), the difference in the abundances of 3 sphingolipid metabolic pathways remained significant (all P < 0.05). Additionally, these pathways were more abundant in infants with *Bacteroides*-dominant microbiome profile compared to the other microbiome profiles (all q < 0.001; Fig. 4). Likewise, there was a positive correlation between the abundance of *Bacteroides* genus and each of the 3 sphingolipid metabolic pathways (all P < 0.001; Fig. 5; Table 4).

### Discussion

By predicting the functional potential of the fecal microbiome from 40 infants with bronchiolitis and 115 healthy age-matched controls enrolled in a case–control study, we found significant differences in the abundance of genes related to multiple metabolic pathways. Of these, the gene function related to sphingolipid metabolic

---

**Table 2 Richness, alpha-diversity, and relative abundance of fecal microbiome in infants with bronchiolitis and healthy controls**

| Richness, median (IQR) | Infants with bronchiolitis n = 40 | Healthy control infants n = 115 | P value |
|------------------------|-----------------------------------|---------------------------------|---------|
| Number of genera       | 17 (13–23)                        | 13 (10–18)                      | 0.004   |
| Alpha-diversity, median (IQR) shannon index | 2.21 (1.68–2.65) | 1.93 (1.44–2.49) | 0.27    |
| Relative abundance of 10 most abundant genera, mean (standard deviation) | | | |
| *Escherichia*           | 0.21 (0.24)                      | 0.23 (0.26)                    | 0.91*   |
| *Bifidobacterium*       | 0.16 (0.20)                      | 0.20 (0.21)                    | 0.49*   |
| *Enterobacter*          | 0.10 (0.21)                      | 0.17 (0.24)                    | 0.27*   |
| *Bacteroides*           | 0.20 (0.23)                      | 0.10 (0.19)                    | 0.10*   |
| *Veillonella*           | 0.03 (0.09)                      | 0.06 (0.12)                    | 0.31*   |
| Lachnospiraceae incertae sedis | 0.06 (0.10) | 0.04 (0.10) | 0.49*   |
| Streptococcus           | 0.02 (0.09)                      | 0.03 (0.05)                    | 0.91*   |
| Clostridium sensu strictos 1 | 0.01 (0.01) | 0.03 (0.06) | 0.16*   |
| Enterococcus            | 0.01 (0.03)                      | 0.02 (0.04)                    | 0.48*   |
| Akkermansia             | 0.02 (0.09)                      | 0.02 (0.08)                    | 0.91*   |
| Microbiome profile, n (%) | | | 0.01 |
| *Bacteroides*-dominant profile | 19 (48) | 24 (21) |
| *Bifidobacterium*-dominant profile | 6 (15) | 26 (23) |
| *Escherichia*-dominant profile | 10 (25) | 36 (31) |
| *Enterobacter/Veillonella*-dominant profile | 5 (12) | 29 (25) |

*IQR* interquartile range

* Benjamini–Hochberg corrected false discovery rate (q value) accounting for multiple comparisons
Table 3 Predicted KEGG orthologs with significant differences in relative abundance between infants with bronchiolitis and healthy controls

| KEGG orthologs                                                                 | Mean abundance in cases (%) | Mean abundance in controls (%) | Raw P value | FDR corrected q value |
|--------------------------------------------------------------------------------|------------------------------|-------------------------------|-------------|-----------------------|
| K00179; indolepyruvate ferredoxin oxidoreductase, alpha subunit [EC:1.2.7.8]    | 0.02                         | 0.009                         | <0.001      | 0.026                 |
| K00180; indolepyruvate ferredoxin oxidoreductase, beta subunit [EC:1.2.7.8]     | 0.007                        | 0.003                         | <0.001      | 0.028                 |
| K02489; two-component system, cell cycle sensor kinase and response regulator [EC:2.7.13.3] | 0.004                        | 0.002                         | 0.001       | 0.038                 |
| K03319; divalent anion:Na⁺ symporter, DASS family                             | 0.019                        | 0.044                         | <0.001      | 0.028                 |
| K08082; two-component system, LytT family, sensor histidine kinase AlgZ [EC:2.7.13.3] | 0.011                        | 0.005                         | 0.001       | 0.041                 |
| K08196; MFS transporter, AAHS family, cis, cis-muconate transporter            | 0.001                        | 0.002                         | 0.002       | 0.044                 |
| K10715; two-component system, sensor histidine kinase RpfC [EC:2.7.13.3]        | 0.009                        | 0.005                         | 0.001       | 0.036                 |
| K10916; two-component system, CAI-1 autoinducer sensor kinase/phosphatase CqsS [EC:2.7.13.3 3.1.3.-] | 0.001                        | 0.000                         | 0.001       | 0.031                 |
| K11382; MFS transporter, OPA family, phosphoglycerate transporter protein      | 0.006                        | 0.015                         | 0.000       | 0.028                 |
| K11383; two-component system, NtrC family, sensor histidine kinase KinB [EC:2.7.13.3] | 0.001                        | 0.000                         | 0.001       | 0.034                 |
| K11520; two-component system, OmpR family, manganese sensing sensor histidine kinase [EC:2.7.13.3] | 0.001                        | 0.000                         | 0.000       | 0.028                 |
| K11527; two-component system, unclassified family, sensor histidine kinase and response regulator [EC:2.7.13.3] | 0.023                        | 0.011                         | 0.001       | 0.038                 |
| K15850; two-component system, autoinducer 1 sensor kinase/phosphatase LuxN [EC:2.7.13.3 3.1.3.-] | 0.001                        | 0.000                         | <0.001      | 0.026                 |
| K15913; UDP-4-amino-4,6-dideoxy-N-acetyl-d-glucosamine 4-acetyltransferase [EC:2.3.1.-] | 0.000                        | 0.000                         | 0.002       | 0.049                 |
| K16014; ATP-binding cassette, subfamily C, bacterial CytCD                      | 0.005                        | 0.010                         | 0.002       | 0.042                 |
| K00176; 2-oxoglutarate ferredoxin oxidoreductase subunit delta [EC:1.2.7.3]     | 0.001                        | 0.001                         | 0.001       | 0.035                 |
| K00177; 2-oxoglutarate ferredoxin oxidoreductase subunit gamma [EC:1.2.7.3]     | 0.010                        | 0.005                         | 0.001       | 0.041                 |
| K00200; formylmethanofuran dehydrogenase subunit A [EC:1.2.99.5]               | 0.001                        | 0.000                         | 0.001       | 0.038                 |
| K00316; spermidine dehydrogenase [EC:1.5.99.6]                                 | 0.000                        | 0.000                         | 0.002       | 0.046                 |
| K00406; cytochrome c oxidase cbb3-type subunit III                              | 0.002                        | 0.004                         | 0.001       | 0.030                 |
| K00436; hydrogen dehydrogenase [EC:1.12.1.2]                                   | 0.002                        | 0.001                         | <0.001      | 0.023                 |
| K00824; 2-oxaloacetyltransaminase [EC:2.6.1.21]                                 | 0.004                        | 0.008                         | 0.002       | 0.045                 |
| K00832; aromatic-amino-acid transaminase [EC:2.6.1.57]                          | 0.010                        | 0.020                         | <0.001      | 0.026                 |
| K00856; adenosine kinase [EC:2.7.1.20]                                          | 0.002                        | 0.001                         | 0.001       | 0.034                 |
| K00908; Ca2⁺/calmodulin-dependent protein kinase [EC:2.7.11.17]                 | 0.001                        | 0.000                         | <0.001      | 0.023                 |
| K01235; alpha-glucuronidase [EC:3.2.1.139]                                      | 0.017                        | 0.008                         | 0.002       | 0.041                 |
| K01601; ribulose-bisphosphate carboxylase large chain [EC:4.1.1.39]             | 0.003                        | 0.010                         | 0.002       | 0.042                 |
| K01841; phosphohemolpyruvate phosphomutase [EC:5.4.2.9]                         | 0.008                        | 0.004                         | 0.002       | 0.049                 |
| K01906; 6-carboxyhexanoate-CoA ligase [EC:6.2.1.14]                              | 0.003                        | 0.007                         | 0.001       | 0.037                 |
| K01912; phenylacetate-CoA ligase [EC:6.2.1.30]                                  | 0.037                        | 0.018                         | 0.001       | 0.041                 |
| K02121; V-type H⁺-transporting ATPase subunit E [EC:3.6.3.14]                    | 0.010                        | 0.005                         | <0.001      | 0.024                 |
| K02655; type IV pilus assembly protein PilE                                     | 0.002                        | 0.005                         | <0.001      | 0.023                 |
| K03330; glutamyl-tRNA (Gln) amidotransferase subunit E [EC:6.3.5.7]             | 0.001                        | 0.003                         | 0.001       | 0.042                 |
| K03404; magnesium chelatase subunit D [EC:6.6.1.1]                               | 0.007                        | 0.019                         | 0.002       | 0.046                 |
| K03756; putrescine:ornithine antiporter                                         | 0.007                        | 0.016                         | <0.001      | 0.024                 |
| K04561; nitric oxide reductase subunit B [EC:1.7.2.5]                            | 0.006                        | 0.017                         | <0.001      | 0.026                 |
| K05586; bidirectional [NiFe] hydrogenase diaphorase subunit [EC:1.6.5.3]        | 0.001                        | 0.000                         | 0.001       | 0.032                 |
| K05588; bidirectional [NiFe] hydrogenase diaphorase subunit [EC:1.6.5.3]        | 0.001                        | 0.000                         | 0.002       | 0.043                 |
| K05589; cell division protein FtsB                                              | 0.002                        | 0.003                         | <0.001      | 0.024                 |
| K05989; alpha-l-rhamnosidase [EC:3.2.1.40]                                      | 0.056                        | 0.028                         | 0.002       | 0.045                 |
| K06138; pyrroloquinoline quinone biosynthesis protein D                          | 0.001                        | 0.000                         | 0.001       | 0.034                 |
| K07326; hemolysin activation/secretion protein                                 | 0.001                        | 0.003                         | <0.001      | 0.026                 |
pathways was consistently more abundant in the fecal microbiome of bronchiolitis cases compared to that of healthy controls. The current study extends the previously identified association of Bacteroides-dominated fecal microbiome profile with higher likelihoods of bronchiolitis by demonstrating the functional potential of the gut microbiome in infants.

Sphingolipids are a class of complex lipids containing a backbone of sphingoid bases. These lipids have long been known as structural components of human cell membranes and as a component of surfactant, but have more recently emerged as signaling molecules that modulate the host immune response and contribute to the pathogenesis of respiratory diseases, such as bronchiolitis, pneumonia, and asthma [7, 22]. While sphingolipids production is ubiquitous in eukaryotes, it is also produced by several bacteria genera such as Bacteroides, Prevotella, and Porphyromonas [12]. Recently, experimental models reported that Bacteroides-derived sphingolipids (e.g., α-galactosylceramide) play an important role in host immunomodulation similar to lipopolysaccharide (LPS), another family of bacteria-derived glycolipid. For example, Wieland Brown et al. demonstrated that Bacteroides-derived α-galactosylceramide binds to

| KEGG orthologs                                      | Mean abundance in cases (%) | Mean abundance in controls (%) | Raw P value | FDR corrected q value |
|-----------------------------------------------------|------------------------------|--------------------------------|-------------|----------------------|
| K07536; 2-ketocyclohexanecarboxyl-CoA hydrolase [EC:3.1.2.-] | 0.001                        | 0.002                          | 0.001       | 0.041                |
| K09002; hypothetical protein                        | 0.006                        | 0.018                          | 0.001       | 0.041                |
| K09020; ureidoacrylate peracid hydrolase [EC:3.5.1.110] | 0.002                        | 0.003                          | 0.001       | 0.031                |
| K09162; hypothetical protein                        | 0.003                        | 0.008                          | 0.001       | 0.034                |
| K09459; phosphonopyruvate decarboxylase [EC:4.1.1.82] | 0.005                        | 0.003                          | 0.003       | 0.050                |
| K09477; citrate:succinate antiporter                 | 0.008                        | 0.016                          | 0.001       | 0.034                |
| K09758; aspartate-4-decarboxylase [EC:4.1.1.12]       | 0.015                        | 0.007                          | <0.001      | 0.026                |
| K09800; hypothetical protein                        | 0.025                        | 0.053                          | <0.001      | 0.026                |
| K09824; hypothetical protein                        | 0.007                        | 0.014                          | <0.001      | 0.026                |
| K10960; geranylgeranyl reductase [EC:1.3.1.83]        | 0.001                        | 0.000                          | <0.001      | 0.027                |
| K10974; cytosine permease                           | 0.007                        | 0.016                          | <0.001      | 0.026                |
| K11016; hemolysin                                   | 0.001                        | 0.002                          | 0.001       | 0.029                |
| K11106; d-tartrate/succinate antiporter              | 0.009                        | 0.020                          | <0.001      | 0.026                |
| K11607; manganese/iron transport system ATP-binding protein | 0.004                        | 0.009                          | <0.001      | 0.022                |
| K11707; manganese/zinc/iron transport system substrate-binding protein | 0.003                        | 0.007                          | 0.001       | 0.041                |
| K11708; manganese/zinc/iron transport system permease protein | 0.003                        | 0.007                          | 0.002       | 0.042                |
| K11709; manganese/zinc/iron transport system permease protein | 0.004                        | 0.008                          | 0.001       | 0.034                |
| K11719; lipopolysaccharide export system protein LptC | 0.003                        | 0.007                          | <0.001      | 0.023                |
| K11931; biofilm PGA synthesis lipoprotein PgaB [EC:3.-.-] | 0.008                        | 0.016                          | <0.001      | 0.028                |
| K12341; adhesin YadA                                 | 0.005                        | 0.012                          | 0.001       | 0.041                |
| K12681; pertactin                                    | 0.001                        | 0.002                          | 0.002       | 0.041                |
| K12982; heptosyltransferase I [EC:2.4.-]             | 0.001                        | 0.002                          | <0.001      | 0.028                |
| K13256; protein PsiE                                 | 0.003                        | 0.006                          | <0.001      | 0.029                |
| K13498; indole-3-glycerol phosphate synthase/phosphoribosylanthranilate isomerase [EC:4.1.1.48 5.3.1.24] | 0.008                        | 0.017                          | <0.001      | 0.022                |
| K13818; molybdopterin-guanine dinucleotide biosynthesis protein | 0.001                        | 0.003                          | 0.001       | 0.029                |
| K14448; (2S)-methylsuccinyl-CoA dehydrogenase         | 0.002                        | 0.001                          | 0.001       | 0.032                |
| K14564; nucleolar protein 56                         | 0.001                        | 0.000                          | 0.002       | 0.043                |
| K14665; amidohydrolase [EC:3.5.1.-]                  | 0.002                        | 0.004                          | 0.001       | 0.031                |
| K15125; filamentous hemagglutinin                     | 0.032                        | 0.092                          | 0.000       | 0.024                |
| K15669; α-glycero-α-manno-heptose 1-phosphate guanylyltransferase [EC:2.7.7.71] | 0.002                        | 0.001                          | 0.001       | 0.031                |
| K15905; nitrite oxidoreductase alpha subunit          | 0.001                        | 0.003                          | 0.001       | 0.041                |
| K16201; dipeptide transport system permease protein  | 0.001                        | 0.003                          | 0.002       | 0.045                |

Of 6402 KEGG orthologs surveyed, the relative abundances of 319 genes were significantly different (FDR, q < 0.05) between infants with bronchiolitis and healthy controls. Of these, 74 orthologs with a ratio of abundance >2.0 are displayed.
Table 4 Predicted KEGG pathways with significant differences in relative abundance between infants with bronchiolitis and healthy controls

| KEGG pathway                          | Difference in relative abundance | Correlation with Bacteroides abundance |
|---------------------------------------|----------------------------------|----------------------------------------|
|                                       | Mean abundance in cases (%)      | Mean abundance in controls (%)         | Raw P value | FDR corrected q value | Spearman’s rho | P value |
| ko00051; fructose and mannose metabolism | 1.691                           | 1.447                                  | 0.001       | 0.043                | 0.55           | <0.001 |
| ko00052; galactose metabolism         | 1.473                           | 1.298                                  | 0.004       | 0.035                | 0.66           | <0.001 |
| ko00140; steroid hormone biosynthesis | 0.098                           | 0.063                                  | 0.007       | 0.049                | 0.66           | <0.001 |
| ko00190; oxidative phosphorylation    | 1.332                           | 1.215                                  | 0.005       | 0.041                | 0.41           | <0.001 |
| ko00311; penicillin and cephalosporin biosynthesis | 0.068                         | 0.060                                  | 0.001       | 0.041                | 0.44           | <0.001 |
| ko00450; selenocompound metabolism    | 0.626                           | 0.703                                  | 0.001       | 0.046                | −0.61          | <0.001 |
| ko00460; cyanoamino acid metabolism   | 0.280                           | 0.222                                  | 0.004       | 0.037                | 0.82           | <0.001 |
| ko00472; d-arginine and d-ornithine metabolism | 0.001                          | 0.002                                  | 0.004       | 0.037                | −0.52          | <0.001 |
| ko00480; glutathione metabolism       | 0.678                           | 0.764                                  | 0.001       | 0.035                | −0.49          | <0.001 |
| ko00511; other glycan degradation     | 1.198                           | 0.910                                  | 0.002       | 0.035                | 0.72           | <0.001 |
| ko00520; amino sugar and nucleotide sugar metabolism | 2.751                         | 2.450                                  | 0.002       | 0.037                | 0.77           | <0.001 |
| ko00523; polyketide sugar unit biosynthesis | 0.196                         | 0.163                                  | 0.002       | 0.035                | 0.80           | <0.001 |
| ko00531; glycosaminoglycan degradation | 0.256                         | 0.152                                  | 0.005       | 0.044                | 0.73           | <0.001 |
| ko00532; glycosaminoglycan biosynthesis | 0.035                         | 0.026                                  | 0.001       | 0.048                | 0.72           | <0.001 |
| ko00591; linoleic acid metabolism     | 0.120                           | 0.113                                  | 0.006       | 0.047                | 0.52           | <0.001 |
| ko00600; sphingolipid metabolism     | 0.489                           | 0.351                                  | 0.003       | 0.034                | 0.77           | <0.001 |
| ko00603; glycosphingolipid biosynthesis | 0.134                         | 0.097                                  | 0.003       | 0.031                | 0.73           | <0.001 |
| ko00604; glycosphingolipid biosynthesis | 0.061                         | 0.039                                  | 0.006       | 0.048                | 0.74           | <0.001 |
| ko00642; Ethylbenzene degradation     | 0.084                           | 0.075                                  | 0.002       | 0.034                | 0.25           | 0.001 |
| ko00660; C5-branched dibasic acid metabolism | 0.171                         | 0.191                                  | <0.001      | 0.028                | −0.50          | <0.001 |
| ko00940; phenylpropanoid biosynthesis | 0.213                           | 0.163                                  | 0.006       | 0.048                | 0.81           | <0.001 |
| ko00944; flavone and flavonol biosynthesis | 0.015                           | 0.009                                  | 0.002       | 0.036                | 0.77           | <0.001 |
| ko03015; mRNA surveillance pathway    | 0.003                           | 0.001                                  | 0.003       | 0.032                | 0.80           | <0.001 |
| ko04141; protein processing in endoplasmic reticulum | 0.063                           | 0.049                                  | 0.006       | 0.049                | 0.69           | <0.001 |
| ko04142; lysosome                     | 0.310                           | 0.194                                  | 0.006       | 0.047                | 0.76           | <0.001 |
| ko04210; apoptosis                    | 0.043                           | 0.025                                  | 0.002       | 0.030                | 0.69           | <0.001 |
| ko04612; antigen processing and presentation | 0.014                           | 0.010                                  | 0.002       | 0.031                | 0.58           | <0.001 |
| ko04621; NOD-like receptor signaling pathway | 0.056                           | 0.042                                  | 0.001       | 0.040                | 0.74           | <0.001 |
| ko04721; synaptic vesicle cycle       | 0.001                           | 0.000                                  | 0.002       | 0.033                | 0.58           | <0.001 |
| ko04725; cholinergic synapse          | 0.002                           | 0.005                                  | 0.003       | 0.031                | −0.12          | 0.14 |
| ko04914; progesterone-mediated oocyte maturation | 0.014                           | 0.010                                  | 0.002       | 0.033                | 0.58           | <0.001 |
| ko04930; type II diabetes mellitus    | 0.026                           | 0.028                                  | 0.001       | 0.036                | −0.47          | <0.001 |
| ko04962; vasopressin-regulated water reabsorption | 0.001                           | 0.000                                  | 0.002       | 0.036                | 0.58           | <0.001 |
| ko05110; vibrio cholerae infection    | 0.001                           | 0.004                                  | 0.006       | 0.046                | −0.41          | <0.001 |
| ko05133; pertussis                    | 0.356                           | 0.609                                  | 0.002       | 0.029                | −0.24          | 0.03 |
| ko05211; renal cell carcinoma         | 0.013                           | 0.019                                  | <0.001      | 0.028                | −0.58          | <0.001 |
| ko05215; prostate cancer              | 0.016                           | 0.011                                  | 0.002       | 0.037                | 0.63           | <0.001 |

*Italicics results are the pathways of interest (sphingolipid metabolic pathways)*
CD1d and activates mouse and human invariant natural killer T (iNKT) cells both in vitro and in vivo [12]. In contrast, An et al., using neonatal mouse models, found that treatment with a different Bacteroides-derived glycophospholipids (GSL-Bf717) reduces the number of colonic iNKT cells and subsequent colonic inflammation [13]. Although reverse causation—e.g., bronchiolitis per se or treatments for bronchiolitis result in perturbation of the fecal microbiome—is also possible, these prior studies, coupled with our findings, collectively suggest that Bacteroides-dominant microbiome in the gut, through their sphingolipid production, may contribute to inappropriate immune responses and bronchiolitis pathogenesis in infants. Our data should encourage future investigations into the mechanisms linking the individual gut microbiome-derived metabolites to the host immune response in the gut and respiratory tract (the gut-lung axis).
In sum, on the basis of the predicted metagenome in this case–control study, we found significant differences in the functional potential of fecal microbiome between infants with bronchiolitis and healthy controls. Particularly, the fecal microbiome in infants with bronchiolitis had consistently higher abundances of gene function related to the sphingolipid metabolic pathways. Although causal inferences remain premature, our data may suggest a potential link between the bacteria-derived metabolites, modulations of host immune response, and development of bronchiolitis. Our findings should facilitate further metagenomic, metatranscriptomic, and metabolomic (including \emph{Bacteroides}–derived galactosylceramide [13]) investigations into the role of gut microbiome in the bronchiolitis pathogenesis. Our data also encourage researchers to integrate these “omics” approaches with mechanistic evaluations in experimental models in order to develop new preventive and therapeutic strategies (e.g., microbiome modification) for infants with bronchiolitis.

**Limitations**

Our study has several potential limitations. First, the location of fecal sample collection differed between cases and controls. However, in both populations, the fecal samples were refrigerated immediately after collection.
and the literature reported that refrigeration is associated with no significant alteration in fecal microbiota composition [23]. Second, the functional potential of fecal microbiome was inferred from the 16S rRNA gene sequencing data rather than measured by metabolomics or metatranscriptomics, or from metagenomic sequencing. However, a study has shown a strong correlation between the predicted metagenome and metagenome sequencing data ($r > 0.85$) in the NIH Human Microbiome Project samples (including fecal samples) [18]. Third, the concentration of metabolites was not measured in the fecal samples. This is an important area
for examination in our future work. Lastly, the study design precluded us from examining the succession of fecal microbiome and its relation to the development of respiratory disease in early childhood. To address this question, the study populations are currently being followed longitudinally to age 6 years, with fecal sample collections at multiple time-points.

**Abbreviations**

iNKT: invariant natural killer T; KEGG: Kyoto Encyclopedia of Genes and Genomes; MARC: Multicenter Airway Research Collaboration; OTU: operational taxonomic unit.

**Authors’ contributions**

KH carried out the statistical analysis, drafted the initial manuscript, and approved the final manuscript as submitted. CJS carried out the initial analyses, reviewed and revised the manuscript, and approved the final manuscript.
as submitted. RWL conceptualized and designed the study, enrolled the subjects, reviewed and revised the manuscript, and approved the final manuscript as submitted. JMM and CAC conceptualized and designed the study, obtained the funding, reviewed and revised the manuscript, and approved the final manuscript as submitted. NJA and JFP generated the microbiome data, carried out the initial statistical analysis, reviewed and revised the manuscript. All authors read and approved the final manuscript.

Author details
1 Department of Emergency Medicine, Massachusetts General Hospital, Harvard Medical School, 50 Staniford Street, Boston, MA 02115, USA. 2 Department of Molecular Virology and Microbiology, Alkek Center for Metagenomics and Microbiome Research, Baylor College of Medicine, Houston, TX, USA. 3 Department of Medicine, Boston Children’s Hospital, Boston, MA, USA. 4 Department of Pediatrics, Emory University School of Medicine, Atlanta, GA, USA.

Acknowledgements
The authors thank Pedro A. Piedra, MD (Baylor College of Medicine), Ashley F. Sullivan, MS MPH (Massachusetts General Hospital), the site investigators at Massachusetts General Hospital, Alfred I. duPont Hospital for Children, Boston Children’s Hospital, and Kosair Children’s Hospital, and all of the study families for their contributions to the study.

Competing interests
Dr. Mansbach has provided bronchiolitis-related consultation for Regeneneron. Drs. Ajami and Petrosino own shares at Diversigen Inc., a microbiome research company. The authors declare that they have no competing interests.

Availability of data and materials
The datasets generated and analyzed during the current study are not publicly available due to the data sharing agreement (based on the informed consent) but may be available from the corresponding author on reasonable request.

Consent for publication
Not applicable.

Ethics approval and consent to participate
The institutional review board at each of the participating hospitals approved the study. Written informed consent was obtained from the parent or guardian.

Funding
This study was supported by the Grants U01 AI-0887881, R01 AI-114552, R01 AI-108588, R01 AI-127507, R21 HL-129909, and UG3 OD-023253 from the National Institutes of Health (Bethesda, MD). The content of this manuscript is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Publisher’s Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Received: 16 March 2017 Accepted: 21 July 2017 Published online: 26 July 2017

References
1. Hasegawa K, Tsugawa Y, Brown DF, Mansbach JM, Camargo CA Jr. Trends in bronchiolitis hospitalizations in the United States, 2000–2009. Pediatrics. 2013;132(1):28–36.
2. Goto T, Tsugawa Y, Mansbach JM, Camargo CA Jr, Hasegawa K. Trends in infectious disease hospitalizations in US children, 2000 to 2012. Pediatr Infect Dis J. 2016;35(6):e158–63.
3. Hasegawa K, Mansbach JM, Camargo CA Jr. Infectious pathogens and bronchiolitis outcomes. Expert Review Anti Infect Ther. 2014;12(7):817–28.
4. Mansbach JM, Hasegawa K, Henke DM, Ajami NJ, Petrosino JF, Shaw CA, Piedra PA, Sullivan AF, Espindola PA, Camargo CA Jr. Respiratory syncytial virus and rhinovirus severe bronchiolitis are associated with distinct nasopharyngeal microbiota. J Allergy Clin Immunol. 2016;137(6):1909–13.
5. Hasegawa K, Mansbach JM, Ajami NJ, Espinola JA, Henke DM, Petrosino JF, Piedra PA, Shaw CA, Sullivan AF, Camargo CA Jr. Association of nasopharyngeal microbiota profiles with bronchiolitis severity in infants hospitalized for bronchiolitis. Eur Respir J. 2016;48:1329–39.
6. Hasegawa K, Mansbach JM, Ajami NJ, Petrosino JF, Freishtat RJ, Teach SJ, Piedra PA, Camargo CA Jr. Serum cathelicidin, nasopharyngeal microbiota, and disease severity in infants hospitalized with bronchiolitis. J Allergy Clin Immunol. 2017;139(4):1383–6.
7. Stewart CJ, Mansbach JM, Wong MC, Ajami NJ, Petrosino JF, Camargo CA Jr. Hasegawa K. Associations of nasopharyngeal metabolome and microbiome with severity among infants with bronchiolitis: a multi-omic analysis. Am J Respir Crit Care Med. 2017. doi:10.1164/rcrm.2017-0071OC.
8. Hasegawa K, Camargo CA Jr. Airway microbiota and acute respiratory infection in children. Exp Rev Clin Immunol. 2015;11(7):789–92.
9. Hasegawa K, Mansbach JM, Ajami NJ, Petrosino JF, Freishtat RJ, Teach SJ, Piedra PA, Camargo CA Jr. The relationship between nasopharyngeal CCL5 and microbiota on disease severity among infants with bronchiolitis. Allergy. 2017. doi:10.1111/all.13160.
10. Hasegawa K, Dumas O, Hartter TV, Camargo CA. Advancing our understanding of infant bronchiolitis through phenotyping and endotyping: clinical and molecular approaches. Exp Rev Respir Med. 2016;10(8):891–9.
11. Hasegawa K, Linnemann RW, Mansbach JM, Ajami NJ, Espinola JA, Petrosino JF, Piedra PA, Stevenson MD, Sullivan AF, Thompson AD, et al. The fecal microbiota profile and bronchiolitis in infants. Pediatrics. 2016;138(1):e20160218.
12. Wieland Brown LC, Penaranda C, Kashyap PC, Williams BB, Clardy J, Kronenberg M, Sonnenburg JL, Comstock LE, Bluestone JA, Fischbach MA. Production of alpha-galactosylceramide by a prominent member of the human gut microbiota. PLoS Biol. 2013;11(7):e1001610.
13. An D, Oh SF, Olzak T, Neves JF, Avci FY, Ertuk-Hasdemir D, Lu X, Zeissig S, Blumberg RS, Kasper DL. Sphingolipids from a symbiotic microbe regulate homeostasis of host intestinal natural killer T cells. Cell. 2014;156(1–2):123–33.
14. Ralston SL, Lieberthal AS, Meissner HC, Alversen BK, Bailey JE, Gadomski AM, Johnson DW, Light MJ, Maraqa NF, Mendonca EA, et al. Clinical practice guideline: the diagnosis, management, and prevention of bronchiolitis. Pediatrics. 2014;134(5):1474–502.
15. Hasegawa K, Linnemann RW, Mansbach JM, Ajami NJ, Espinola JA, Fiechtner LG, Petrosino JF, Camargo CA Jr. Association of household siblings with nasal and fecal microbiota in infants. Pediatr Int. 2016 (Epub ahead of print).
16. Hasegawa K, Linnemann RW, Mansbach JM, Ajami NJ, Espinola JA, Fiechtner LG, Petrosino JF, Camargo CA Jr, Taveras EM, Hasegawa K. Association of maternal gestational weight gain with the infant fecal microbiota. J Pediatr Gastroenterol Nutr. 2017. doi:10.1097/MPG.0000000000001566.
17. Robinson A, Fiechtner L, Roche B, Ajami NJ, Petrosino JF, Camargo CA Jr, Taveras EM, Hasegawa K. Association of maternal gestational weight gain with the infant fecal microbiota. J Pediatr Gastroenterol Nutr. 2017. doi:10.1097/MPG.0000000000001566.
18. Ashshauer KP, Wemheuer B, Daniel R, Meinicke P. Tax4Fun: predicting taxonomic and functional profiles. Bioinformatics. 2014;30(21):3123–4.
19. Kanehisa M, Goto S. KEGG: kyoto encyclopedia of genes and genomes. Nucleic Acids Res. 2000;28(1):27–30.
20. McMurdie PJ, Holmes S. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS ONE. 2013;8(6):e61217.
21. Parks DH, Tyson GW, Hugenholtz P, Beike RG. STAMP: statistical analysis of interactive analysis and graphics of microbiome census data. PLoS ONE. 2014;30(21):3123–4.
22. Ono JG, Worgall TS, Worgall S. Airway reactivity and sphingolipids—implications for childhood asthma. Mol Cell Pediatr. 2015;2(1):13.
23. Choo JM, Leong LE, Rogers GB. Sample storage conditions significantly influence faecal microbiome profiles. Sci Rep. 2015;5:16350.