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Original Article

Analysis of Oropharyngeal Microbiota between the Patients with Bronchial Asthma and the Non-Asthmatic Persons

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Bronchial asthma can be triggered by microbial agents in the oropharynx. This study was designed to identify the differences in microbiota of oropharynx of bronchial asthmatic patients in contrast to normal controls. In order to resolve the qualitative and quantitative diversity of the 16S rRNA gene present in the oropharynx microbiota of 4 patients and 4 controls, we compared microbial communities using Sanger sequencing and 376 sequences of 16S rRNA gene were analyzed. Of the total microbial diversity detected in the oropharynx in asthmatic patients 45.6% comprised members of the Firmicutes. In contrast, Proteobacteria (44.0%) dominated the oropharyngeal microbiota in the normal control group. Members of the Bacteroidetes, Fusobacteria, Actinobacteria, TM7, Cyanobacteria and unclassified bacteria were present in both groups. In conclusion, the difference in the microbiota of the oropharynx between patients and normal individuals could trigger symptomatic attacks in bronchial asthma.

Key Words: Bronchial asthma, Microbiota, Metagenome

INTRODUCTION

Asthma is a heterogeneous syndrome of airway inflammation with an increasing incidence in populations around the world. Its heterogeneity contributes to the undetermined pathogenesis in its development and frequent exacerbation. Chronic infections cause a decline in lung function and may be a risk factor for the development and triggering of bronchial asthma. The association between respiratory infections and atopy or asthma remains to be determined and may be highly dependent on different infectious agents (1).

Respiratory infections caused by respiratory syncytial virus (RSV), rhinovirus, parainfluenzavirus, influenzavirus and metapneumovirus may be associated with childhood wheezing and exacerbation of asthma (2). Atypical bacterial pathogens such as *Chlamydophila pneumoniae* and *Mycoplasma pneumoniae* may also be associated with asthma (3~6). Most studies so far have been on childhood asthma. But there have only been a few studies explaining the association between respiratory microbes and adulthood asthma.

Human microbiota are diverse in their structure and function across individuals and different sites. The micro-

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biota in the gastrointestinal tracts have been related to inflammatory bowel diseases (7) and obesity, (8) while atherosclerosis (9) has been linked to the oral microbiota. Oral microbial environments are known to be linked to upper and lower respiratory infections (7), which can also precipitate into atopic airway diseases such as allergic rhinitis and/or bronchial asthma.

The clinical relevance of the microbiota on the oropharyngeal wall is unknown. But it is known that the changes in diversity of the oral microbiota can be induced by use of antibiotics, probiotics, diet, and also influenced by microbiota in the gut. Changes in the microbiota of GI tracts can affect mucosal immunity in the lungs, as well as the gut, by bystander suppression (10). The link between oral and airway tolerance may be related to the induction or suppression of systemic immune tolerance in a way to challenge antigens. Therefore, defining the characteristics of the oropharyngeal microbiota may lead to understanding new pathogenic roles of these microorganisms and the discovery of novel therapies for the prevention and treatment of oral complications (11).

The oral microbiota is a critical component in health and disease, even the normal healthy lung is not a sterile organ, in contrast to previous assumptions (12). In order to access such low or less dominant population culture-independent strategies must inevitably be introduced for the analysis of microbial ecology.

In the current study a metagenomics approach is used to characterize the microbial populations of the oropharyngeal larynx. This approach involves the genomic analysis of microorganisms by direct extraction and sequencing of DNA from their natural environment (13). In this study, we have developed a method based on 16S rRNA gene amplification followed by clonal Sanger sequencing for monitoring the microbial communities. A highly variable region (V1-V3) of the 16S rRNA gene is amplified using primers that target adjacent conserved regions, followed by direct sequencing of individual PCR products. Here we demonstrate the power of this method by exploring the diversity within the human oropharyngeal ecosystem.

Table 1. Characteristic of study participants

Sample no.	Sex	Spirometry	Respiratory manifestation	Smoking
A1	Male	*FVC/FEV1 72	Cough, wheezing,	No
A2	Female	*FVC/FEV1 86	Noctonal dyspnea	No
A3	Female	*FVC/FEV1 63	Wheezing, DOE	No
A4	Female	*FVC/FEV1 62	Dyspnea, wheezing	No
N1	Female	−	No symptom	No
N2	Male	−	No symptom	No
N3	Male	−	No symptom	No
N4	Male	−	No symptom	No

*FVC, Forced vital capacity; FEV, Forced expiratory volume.

MATERIALS AND METHODS

Study participants and sample preparation

We selected 4 controls from Chung-Ang University College of Medicine (CAU) and 4 patients with bronchial asthma in Chung-Ang University Hospital (Table 1). Oropharyngeal swabs were collected from the participants under protocols approved by the Chung-Ang University College of Medicine IRB (Protocol #2010-02-01) using 3 M quick swabs (3 M Microbiology Products, St. Paul, MN, USA). The samples were transported immediately to the lab for identification and subsequent metagenomic analysis. Those with bronchial asthma had mild intermittent disease severity and were treated as naive. Each swab sample was aseptically placed in a microfuge tube, centrifuged for 10 min., and the supernatant was removed. To detect possible contamination, negative controls were prepared and then subjected to the same procedures.

DNA extraction from oropharyngeal swab samples

The first step in the analysis was extraction of DNA from the bacterial pellet obtained from oropharyngeal swab samples followed by PCR analysis. Samples were extracted individually using the cetyltrimethylammonium bromide

method (14). Purified DNA was dissolved in sterile water with 40 μg/ml of RNase A and quantified using an Infinite 200 NanoQuant (Tecan, Männedorf, Switzerland) at a wavelength of 260 nm.

PCR for cloning

PCR amplification of the 16S rRNA gene was carried out following established procedures (15). The 1,550 base pairs of the 16S rRNA gene are a structural part of the 30S ribosomal small subunit (SSU) and consist of eight highly conserved regions (U1-U8) and nine variable regions (V1-V9) across the bacterial domain (16). The V1-V3 region of the 16S rRNA gene was amplified with primers 8F (5'-CTGCTGCCTYCCGTA-3') and 530R (5'-GTATTAC-CGCGGCTGCTG-3'), respectively. PCRs were performed in final reaction mixtures of 25 μl containing 5~25 ng genomic DNA, 0.4 mM of each primer, 0.2 mM dNTPs (Takara Bio, Shiga, Japan), 1.5 mM $MgCl_2$ (Invitrogen, Carlsbad, CA, USA), 2.0 U Hot start *Taq* polymerase (Takara) and 1.0 μl reaction buffer (Invitrogen). The PCR amplification was carried out in GeneAmp PCR system 9700 (Applied Biosystem) with the following conditions: initial denaturation for 5 min at 94℃ followed by 30 cycles of 30 s at 94℃, 30 s at 55℃ and 1 min at 72℃ with a final extension for 10 min at 72℃ and cooling to 4℃. The PCR products were resolved on 1.2% Seakem LE agarose gel (FMC Bioproducts, Rockland, ME, U.S.A.) and visualized after ethidium bromide staining. The resultant PCR products were ligated into the pCR2.1-TOPO vector system (Invitrogen) and transformed into One Shot TOP10 *E. coli* strains (Invitrogen). The transformants were plated onto Luria-Bertani (LB) medium (Difco, Detroit, MI., USA) containing ampicillin/IPTG/X-Gal and white colonies were screened for the target fragment. Colony PCR with the M13F (5'-GTAAAACGACGGCCAGT-3') and M13R (5'-GTAAAACGA CGGCCAGT-3') was used to confirm the presence of the gene of interest and the resultant fragments were analysed by agarose gel electrophoresis.

16S rRNA gene sequence analysis

Each clone was sequenced in duplicate with the M13 forward and/or reverse primers using the BigDye terminator cycle sequencing kit and an ABI PRISM 3100 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). Sequences were edited and aligned with Clustal X (17). The nucleotide sequence homologies of the amplified 16S rRNA gene V1-V3 regions were determined using the Nucleotide BLAST program available on the BLAST network service of the National Center for Biotechnology Information (NCBI) and EzTaxon Server version 2.1 (http://147.47.212.35:8080). Accurate identification of organisms by comparative analysis of 16S rRNA gene sequences is strongly dependent on the quality of the database used. The curated Ribosomal Database Project (RDP-II, http://rdp.cme.msu.edu) provides 1,921,179 16S rRNA gene sequences in an aligned and annotated format and has achieved major improvements in the detection of sequence anomalies (18). Notably, among all of the online tools provided by the RDP-II web site, the RDP classifier tool has demonstrated effective taxonomic classification of short 16S rRNA gene sequences. A tree was built using the neighbor-joining method in Clustal_X (17) with 1000 bootstrap replicates. UniFrac was used to evaluate the relatedness of samples (19, 20). The Clustal_X tree served as the input tree for UniFrac (http://bmf.colorado.edu/unifrac). Weighted and normalized Principal Component Analysis (PCA) and Jackknife Environment Clusters were performed to evaluate similarity among samples, where each sample represents an environment.

Statistical analysis

Factor analysis was applied in order to extract meaningful variables from the total observed set of variables. Principal components were identified with over 70% of the cumulative variance. The component matrix of the chosen principal components was calculated and variables whose factor loadings were below 0.6 deleted. A Generalized Linear Model (GLM) using the final set of chosen variables was constructed. The following equation (a) represents the GLM,

$$g(\mu) = \alpha + \beta\chi \qquad \text{(a)}$$

where, t is the total number of Occurrences, $\chi = \begin{cases} 1, \text{Normal } group \\ 0, \text{Dandruff } group \end{cases}$ and $g(\mu) = \log(\mu/t)$ in the pooled group of samples from asthma patients and healthy controls. The parameter beta is tested at a 5% significance level by (a).

RESULTS

Identification of genera in the asthma patient and control groups

The diversity of the oropharyngeal swab sample microbiota was characterized by sequencing the V1-V3 region of the 16S rRNA gene, amplified from genomic DNA samples of 4 asthma patients and 4 controls. About 50 clones were sequenced from each sample. Based on the analysis of 376 16S rRNA gene clones, the diversity of the microbiota from the 2 groups of bronchial asthmatics and non-asthmatic controls was showing a total of 39 different bacterial genera representing six different bacterial phyla.

A total of 39 different bacterial genera were present in the non-asthmatic control samples. The majority of sequences in the metagenome library from the control group had *Neisseria* spp. (23.3%), *Prevotella* spp. (7.8%) and *Streptococcus* spp. (9.3%) identified by GenBank, Eztaxon and the RDP database analysis tool. By contrast, a total of 21 different bacterial taxa were present in the samples from the group with bronchial asthma. The majority of sequences in the bronchial asthma library were identified as *Streptococcus* spp. (29.5%), *Prevotella* spp. (22.4%), *Neisseria* spp. (6.0%), and *Veillonella* spp. (13.7%).

To examine the diversity of the microbial community present in oropharyngeal swab samples, assembled sequences were assigned to bacterial genera based on the taxonomy of their closest relatives as judged by BLAST n analysis. While the two data sets show broad taxonomic similarity, there is however a notable variation in each sample at species level.

It should be noted however, that this analysis is coverage-limited. To correlate the variation of bacteria between the swab samples, the BLAST n hits were classified by genera. Fig. 1 shows that the bronchial asthma sample is dominated by *Streptococcus* spp., *Prevotella* spp., *Neisseria* spp., and *Veillonella* spp., infecting the oropharynx. Estimated diversity in the affected patients is 72.6%, whereas in the control group it is 46.6%. Relatively there was a higher number of bacterial genera represented in normal controls than in the bronchial asthma samples, which included *Granulicatella* spp. 3.6%, *Campylobacter* spp. 2.6%, *Capnocytophaga* spp. 2.6%, *Xylanibacter* spp. 2.6%, *Sphingomonas* spp. 1.6%, *Phocoenobacter* spp. 1.0%, *Schlegelella* spp. 1.0%, *Atopobium* spp. 0.5%, *Catonella* spp. 0.5%, *Dialister* spp.

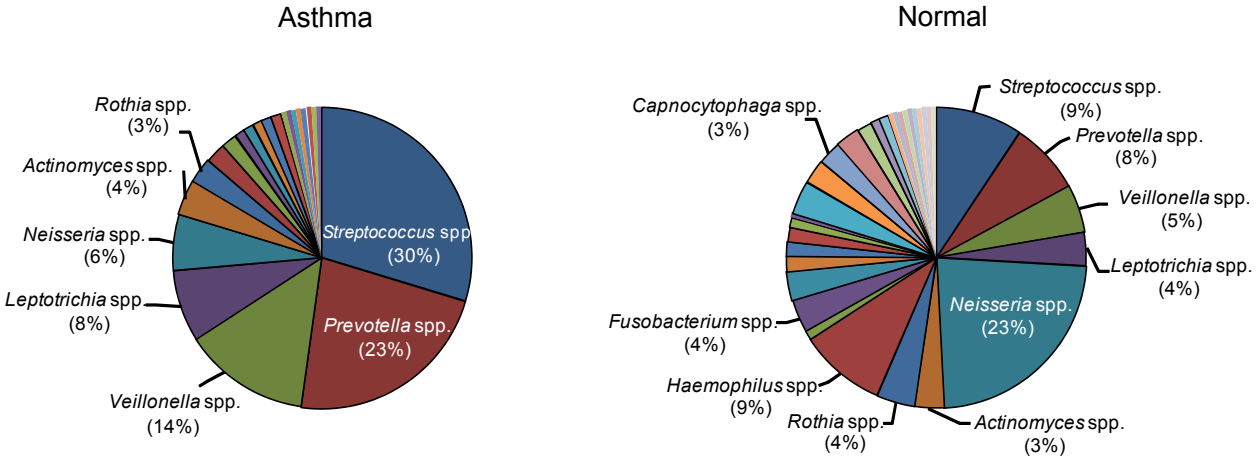

Figure 1. Comparison of the microbial diversity in the oropharynx bronchial asthma and non-asthmatic persons.

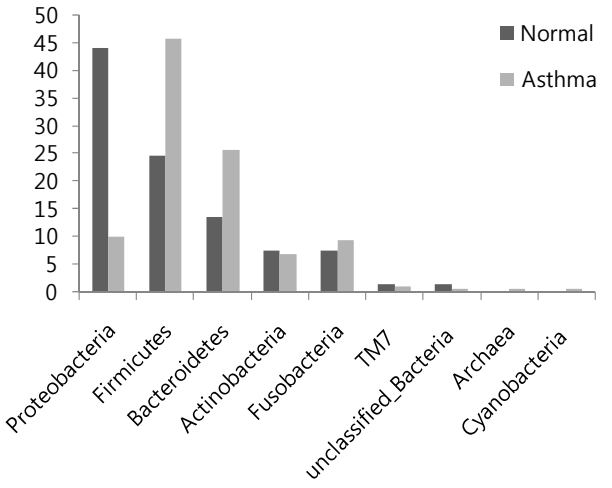

Figure 2. Relative abundance of the main phyla identified in bronchial asthma and non-asthmatic persons. The comparison of the proportion of each phylum among the oropharyngeal microbiota of the asthmatic versus non-asthmatic persons.

0.5%, *Enterobacter* spp. 0.5%, *Gemella* spp. 0.5%, *Kaistella* spp. 0.5%, *Microvirgula* spp. 0.5%, *Novosphingobium* spp. 0.5%, *Oscillibacter* spp. 0.5%, and *Parvimonas* spp. 0.5%. The presence of several genera in the bronchial asthma oropharynx samples secluded them from the non-asthmatic control samples. For instance, *Bergeriella* spp., *Megasphaera* spp., *Paraprevotella* spp., *Phycicola* spp., *Pilibacter* spp. and *Streptobacillus* spp. were detected in the bronchial asthma oropharynx samples and were absent in the non-asthmatic controls.

Identification of different phyla in the asthma patient and non-asthmatic persons

Seven bacterial phyla were detected, but most sequences were assigned to three phyla: Firmicutes (45.6%), Proteobacteria (10.0%), and Bacteroidetes (25.7%) in asthmatic patients; and Proteobacteria (44.0%), Firmicutes (24.5%), and Bacteroidetes (13.5%) in the non-asthmatic controls (Fig. 2). In 4 bronchial asthma patients, a large percentage (46.7%, 41.7%, 45.7%, and 52.3%) of bacteria belonged to the Firmicutes. While in the 4 normal control samples a similar percentage (43.5%, 38.8%, 57.1%, and 40.8%) of bacteria belonged to the Proteobacteria. These results indicate a change in the bronchial asthma microbial community from Proteobacteria to Firmicutes, the latter being the major components of the microbiota. Our findings indicate that bronchial asthma may have a microbial component, which might have potential therapeutic implications.

In the Fig. 2, the seven phyla included the Firmicutes (previously referred to as the low-GC gram positives, with genera such as *Streptococcus*, *Gemella*, *Veillonella*, *Oribacterium*, *Megasphaera*, *Granulicatella*, *Parvimonas* and *Catonella*), the Bacteroidetes (e.g. *Porphyromonas*, *Prevotella*, *Capnocytophaga*, and *Cloacibacterium*), the Proteobacteria (e.g. *Neisseria*, *Haemophilus*, *Alcaligenes*, *Pseudomonas*, *Lautropia*, *Novosphingobium*, *Sphingomonas*, *Enterobacter*, *Campylobacter*, and *Moraxella*), the Fusobacteria (e.g. *Fusobacterium* and *Leptotrichia*), the Actinobacteria (previously referred to as the high-GC gram positives (e.g. *Actinomyces*, *Atopobium*, *Rothia*, and related genera), the *Cyanobacteria* (e.g., species *Streptophyta*) and the TM7 phylum, for which there are no cultivable representatives.

UniFrac and statistical analysis

Jackknife clustering of environments (Fig. 3a) showed fairly robust clustering (75% bootstrap on all nodes but one) in the bronchial asthma and non-asthmatic samples; it is similar to the PCA. Comparison of the individual samples using Fast UniFrac PCA (Fig. 3b) showed a distinct clustering by dietary treatment, when both the DNA samples were included in the analysis.

Statistical analysis showed significantly different frequencies between the bronchial asthma and non-asthmatic samples (Table 2). At the genus level, *Neisseria* spp. (p = 0.0168), *Alcaligenes* spp., *Atopobium* spp., *Campylobacter* spp., *Catonella* spp., *Dialister* spp., *Enterobacter* spp., *Fusobacterium* spp., *Gemella* spp., *Granulicatella* spp., *Haemophilus* spp., *Hallella* spp., *Microvirgula* spp., *Novosphingobium* spp., *Oscillibacter* spp., *Parvimonas* spp., *Porphyromonas* spp., *Pseudomonas* spp., *Rothia* spp., *Schlegelella* spp., *Sphingomonas* spp., *Xylanibacter* spp., and TM7 were abundant in the non-asthmatic individuals. By contrast, *Streptococcus* spp. ($p < 0.0001$) and *Anaerosporobacter* spp. were predominant in the asthmatic

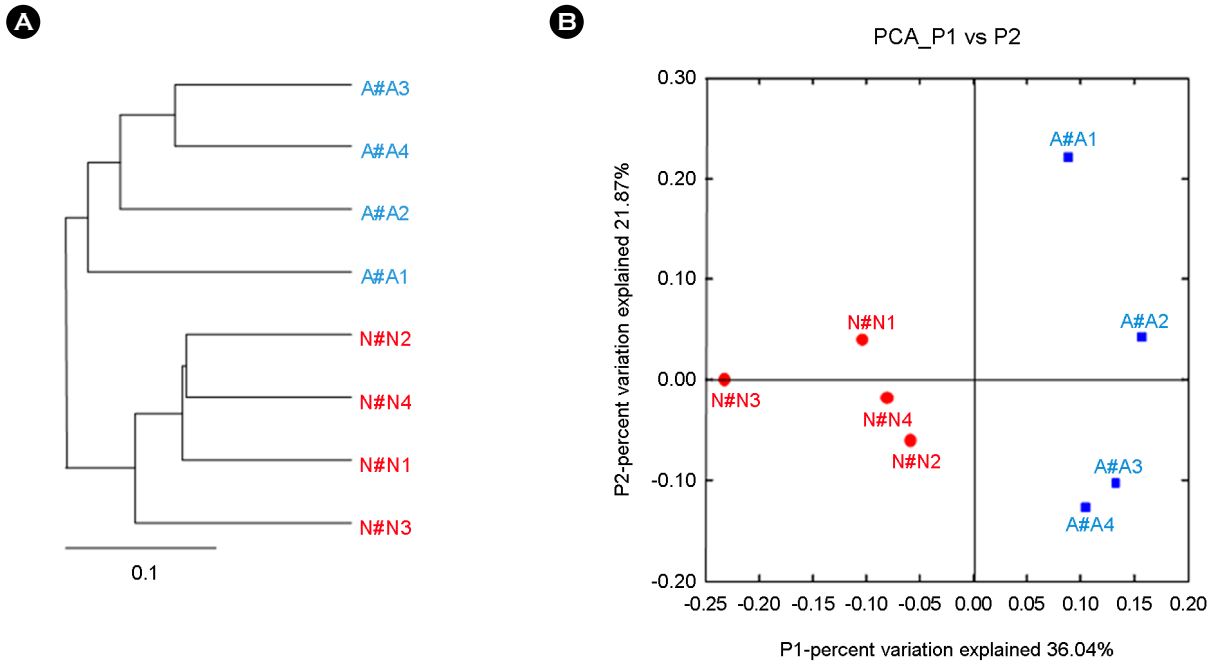

Figure 3. UniFrac analysis of V1-V3 16S rRNA gene sequences of the microbial community of oropharynx from in bronchial asthma and non-asthmatic persons. (A) A jackknifed clustering of the environments in the UniFrac dataset. The numbers next to the nodes represent the number of times that particular node was observed in a random sampling from the whole dataset. (B) Principal Component Analysis scatter plot of individuals from micorobiota in bronchial asthma and non-asthmatic persons (non-asthmatic persons, red; bronchial asthma, blue).

patients at the significant level ($p < 0.0001$).

DISCUSSION

Environmental changes are a major factor in the development of allergies, the increase in the incidence of allergic diseases over the past 20~30 years and the dichotomy in the rate of allergic disease between industrialized and developing countries. Children raised on farms may be less likely to develop asthma and allergies than others (21). The concept is that significant perturbations in gastrointestinal (GI) microbiota composition in westernized areas (due to antibiotic use, dietary changes, and other lifestyle differences) have disrupted the mechanisms of mucosal immunologic tolerance (11, 22).

Sequence analysis of the 16S rRNA genes from microbiota, isolated by oropharyngeal swab samples of diseased and controls showed that the majority of these sequences derived, belonged to phyla Firmicutes, Proteobacteria and Bacteroidetes differing in abundances. Compared to the non-asthmatic controls, the oropharynx bronchial asthma samples showed higher frequencies of the genera *Streptococcus* spp. (Phylum Firmicutes) and *Prevotella* spp. (Phylum Bacteroidetes). *Streptococcus* spp., a commensal bacterium of the oral cavity, especially *Streptococcus pneumoniae*, *Streptococcus mitis* and *Streptococcus oralis* are members of the viridians streptococci, which are associated with pneumonia and endocarditis as an opportunistic pathogen (23~26). But no clear evidence of the association of asthma with the *Streptococcus* spp. has been so far reported.

Recently, the correlation of the presence of combined *Veillonella* and *Streptococcus* abundances in atherosclerotic plaque samples and in oral samples across patients has been studied (27). These two genera are known early colonizers of tooth surfaces that interact to form dental plaques: *Streptococcus* produces a preferred fermentation product for *Veillonella* (28). In addition, *Prevotella* was found in bronchial asthma patients. Our results suggest that the distribution and profile of bacterial species in the oropharynx of normal control individuals is complex, and it is important

Table 2. Statistics analysis between the bronchial asthma and non-asthmatic persons

Phylum	Genus	Average (%)		Estimate	Standard Error	Chi-square	*P*-value
		Asthma	Normal				
Firmicutes	*Streptococcus* spp.	29.5	9.3	1.5626	0.2801	31.12	<.0001
Bacteroidetes	*Prevotella* spp.	22.4	7.8	−	−	−	−
Firmicutes	*Veillonella* spp.	13.7	5.2	−	−	−	−
Fusobacteria	*Leptotrichia* spp.	7.7	3.6	−	−	−	−
Proteobacteria	*Neisseria* spp.	6	23.3	-0.8193	0.3428	5.71	0.0168
Actinobacteria	*Actinomyces* spp.	3.8	3.1	−	−	−	−
Actinobacteria	*Rothia* spp.	2.7	4.2	-0.9276	0.7105	1.7	0.1917
Proteobacteria	*Haemophilus* spp.	2.2	9.3	-1.0978	0.5758	3.63	0.0566
Firmicutes	*Oribacterium* spp.	1.6	1	−	−	−	−
Fusobacteria	*Fusobacterium* spp.	1.1	3.6	-0.5628	0.8079	0.49	0.486
Proteobacteria	*Pseudomonas* spp.	1.1	3.1	-0.4131	0.8235	0.25	0.6159
Proteobacteria	*Alcaligenes* spp.	1.1	1.6	-0.08	0.9787	0.01	0.9349
Bacteroidetes	*Hallella* spp.	1.1	1.6	0.3347	0.9153	0.13	0.7146
TM7	unclassified genus	1.1	1.6	0.2567	0.925	0.08	0.7814
Bacteroidetes	*Porphyromonas* spp.	0.6	1	0.0454	1.2281	0	0.9705
Firmicutes	*Anaerosporobacter* spp.	0.6	0.5	0.7422	1.4176	0.27	0.6006
Proteobacteria	*Bergeriella* spp.	0.6	0	−	−	−	−
Firmicutes	*Megasphaera* spp.	0.6	0	−	−	−	−
Bacteroidetes	*Paraprevotella* spp.	0.6	0	−	−	−	−
Actinobacteria	*Phycicola* spp.	0.6	0	−	−	−	−
Firmicutes	*Pilibacter* spp.	0.6	0	−	−	−	−
Fusobacteria	*Streptobacillus* spp.	0.6	0	−	−	−	−
Firmicutes	*Granulicatella* spp.	0	3.6	−	−	−	−
Proteobacteria	*Campylobacter* spp.	0	2.6	−	−	−	−
Bacteroidetes	*Capnocytophaga* spp.	0	2.6	−	−	−	−
Bacteroidetes	*Xylanibacter* spp.	0	2.6	−	−	−	−
Proteobacteria	*Sphingomonas* spp.	0	1.6	−	−	−	−
Proteobacteria	*Phocoenobacter* spp.	0	1	−	−	−	−
Proteobacteria	*Schlegelella* spp.	0	1	−	−	−	−
Actinobacteria	*Atopobium* spp.	0	0.5	−	−	−	−
Firmicutes	*Catonella* spp.	0	0.5	−	−	−	−
Firmicutes	*Dialister* spp.	0	0.5	−	−	−	−
Proteobacteria	*Enterobacter* spp.	0	0.5	−	−	−	−
Firmicutes	*Gemella* spp.	0	0.5	−	−	−	−
Bacteroidetes	*Kaistella* spp.	0	0.5	−	−	−	−
Proteobacteria	*Microvirgula* spp.	0	0.5	−	−	−	−
Proteobacteria	*Novosphingobium* spp.	0	0.5	−	−	−	−
Proteobacteria	*Oscillibacter* spp.	0	0.5	−	−	−	−
Firmicutes	*Parvimonas* spp.	0	0.5	−	−	−	−

to fully define the microbiota of the bronchial asthma before we can understand the role of microbial community in asthma disease. Further studies are needed to understand the relationships between fungal microbiota and bacterial community level to present large data set for providing a blueprint for the prevention and treatment of bronchial Asthma.

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