

Corticosteroid therapy and airflow obstruction influence the bronchial microbiome, which is distinct from that of bronchoalveolar lavage in asthmatic airways

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Background: The lung has a diverse microbiome that is modest in biomass. This microbiome differs in asthmatic patients compared with control subjects, but the effects of clinical characteristics on the microbial community composition and structure are not clear.

Objectives: We examined whether the composition and structure of the lower airway microbiome correlated with clinical characteristics of chronic persistent asthma, including airflow obstruction, use of corticosteroid medications, and presence of airway eosinophilia.

Methods: DNA was extracted from endobronchial brushings and bronchoalveolar lavage fluid collected from 39 asthmatic patients and 19 control subjects, along with negative control samples. 16S rRNA V4 amplicon sequencing was used to compare the relative abundance of bacterial genera with clinical characteristics.

Results: Differential feature selection analysis revealed significant differences in microbial diversity between brush and lavage samples from asthmatic patients and control subjects.

Lactobacillus, *Pseudomonas*, and *Rickettsia* species were significantly enriched in samples from asthmatic patients, whereas *Prevotella*, *Streptococcus*, and *Veillonella* species were enriched in brush samples from control subjects. Generalized linear models on brush samples demonstrated oral corticosteroid

use as an important factor affecting the relative abundance of the taxa that were significantly enriched in asthmatic patients. In addition, bacterial α -diversity in brush samples from asthmatic patients was correlated with FEV₁ and the proportion of lavage eosinophils.

Conclusion: The diversity and composition of the bronchial airway microbiome of asthmatic patients is distinct from that of nonasthmatic control subjects and influenced by worsening airflow obstruction and corticosteroid use. (J Allergy Clin Immunol 2015;■■■■:■■■-■■■.)

Key words: Asthma, microbiome, corticosteroids, FEV₁, bacteria, 16S ribosomal RNA

Formerly thought to be sterile,^{1,2} it is now clear that the lung is colonized by microbes from early infancy³ and exposed continuously to air, as well as nasal, oropharyngeal, and gastrointestinal tract secretions. Although normally low in biomass compared with other body sites, such as the gastrointestinal tract, the ecology of the lung microbiome is diverse and complex,^{2,4} and ecologic dynamics of the microbial community rather than just the presence of any individual species might be an important component of disease pathogenesis.

Previous investigations suggest that the lung microbiome might contribute to the pathogenesis of asthma. Both bacterial exposure and greater diversity of environmental microbial exposures in early childhood diminish the risk of subsequent asthma or allergy.^{5,6} Commensal gastrointestinal microbiota can influence the development of atopy and asthma.⁷ Infants whose upper airways were colonized with select organisms had an increased risk for asthma later in life.³ Treatment of asthmatic patients with macrolide antibiotics might provide symptomatic relief for selected patients, although recent trials dispute this.⁸⁻¹³

Recent reports suggest the microbiome in the lower airways might be different in patients with asthma. Hilty et al¹⁴ demonstrated with endobronchial brushes that the genus *Haemophilus* had a greater relative abundance and the genus *Prevotella* had a reduced relative abundance in the bronchi of adults with either asthma or chronic obstructive pulmonary disease compared with control subjects. Marri et al¹⁵ demonstrated that 3 major phyla, Firmicutes, Actinobacteria, and Proteobacteria, accounted for more than 90% of total 16S rRNA sequences in the sputum of patients with mild asthma, and again, Proteobacteria were significantly enriched compared with microbial communities in the sputum of control subjects. Goleva et al¹⁶ demonstrated that bronchoalveolar lavage (BAL) fluid of control subjects and patients with corticosteroid-resistant or corticosteroid-sensitive asthma had significantly different relative abundances of many bacterial

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Abbreviations used

BAL: Bronchoalveolar lavage
 EB: Endobronchial brushing
 FDR: False discovery rate
 GLM: Generalized linear regression model
 ICS: Inhaled corticosteroid
 OCS: Oral corticosteroid
 OTU: Operational taxonomic unit

genera. Finally, using endobronchial brush samples previously collected from the Macrolides in Asthma study, Huang et al¹⁷ demonstrated greater bacterial diversity in samples from asthmatic patients compared with those from healthy control subjects, which correlated with bronchial hyperresponsiveness.

These data suggest that the lower airway microbiome might differ in asthmatic patients. However, differences in sample collection and location within the lung, phenotypes of asthmatic patients, and differing use of corticosteroids are likely significant confounders that need to be considered to identify the microbial biomarkers of clinically relevant characteristics in asthmatic patients.

Our asthma clinical research program has been collecting lower airway samples from both carefully characterized asthmatic patients and control subjects.^{18,19} To overcome previous sampling inconsistencies, we decided to elucidate and model the variability in the microbiome of different lung regions, specifically the bronchial (endobronchial brushing [EB] samples) and small (BAL samples) airways. We show significant differences in the relative abundance of bacterial taxa between samples from asthmatic patients and those from control subjects and between EB and BAL samples, and we demonstrate that the asthmatic EB microbiome correlates with the degree of airflow obstruction. In addition, we highlight anatomic localization and corticosteroid use as important factors influencing the relative abundance patterns of differentially abundant taxa. Our data, combined with those of previous studies, help set the stage for longitudinal studies that can answer important questions about the role of the lower airway microbiome in asthmatic patients.

METHODS**Subjects**

This is a cross-sectional retrospective study. Adult asthmatic patients and control subjects were recruited from among the participants in previous asthma genetic and airway biology studies that were originally conducted from 2011 to 2013. Approval for the retrospective use of samples generated from these subjects was obtained from the Institutional Review Board at the University of Chicago. All subjects provided written informed consent at the time of recruitment. Additional information, including details of negative brush and reagent control sample collection, is provided in the [Methods](#) section in this article's Online Repository at www.jacionline.org.

Bronchoscopy was done by using standard methods and conscious sedation. Because of the nature of this study with retrospective identification of subjects, no oral or nasal control samples were available for microbial analysis. Sample collection at bronchoscopy is described in detail in the [Methods](#) section in this article's Online Repository.

Sample processing

DNA was extracted by using methods detailed in the Methods section in this article's Online Repository. Primers specific for the V4 region (515-

806 bp) of the 16S rRNA encoding gene were used to generate amplicons. Samples with sufficiently high DNA loading after amplification were sequenced in a paired-end 150 bp run by using the Illumina MiSeq (Illumina, San Diego, Calif) at the National Laboratory for High-Throughput Genome Analysis Core at Argonne National Laboratory (Argonne, Ill). Paired-end reads were quality trimmed and processed for operational taxonomic unit (OTU) picking by using the UPARSE²⁰ pipeline set at 97% sequence identity cutoff. Taxonomic status was assigned to high-quality OTUs (<1% incorrect bases) by using "parallel assign taxonomy" script from QIIME software.²¹ Multiple sequence alignment and phylogenetic reconstruction were performed with PyNast and FastTree.²¹ The Phyloseq package²² was used for ordination and α - and β -diversity analysis.

Statistical analysis

By using the Phyloseq package,²² the OTU matrix was processed to remove OTUs containing less than 5 reads to reduce the PCR and sequencing-based bias; then the OTU table was rarified to the minimum numbers of reads present in the smallest library.

For ANOVA and *P* value correction, we performed *post hoc* test using the Tukey-Kramer test with effect size of "eta squared" and corrected for multiple tests by using the method of Benjamini and Hochberg. MetagenomeSeq²³ was used to identify the differentially abundant taxa across groups. Clinical data are expressed as means $\pm$ SEMs. Two-group analysis was performed with Welch *t* tests, followed by the Storey false discovery rate (FDR) correction. We then used a generalized linear regression model (GLM) to examine the contribution of patients' demographic data on the abundance patterns of the differentially abundant bacterial genera.

RESULTS

In this study we used specimens collected from 3 asthmatic patients and 19 control subjects for lung microbiome evaluation. Study subjects' characteristics are summarized in [Table I](#). As expected, asthmatic patients had a lower FEV₁ compared with control subjects (*P* = .001), a higher concentration of exhaled nitric oxide (*P* = .05), and a larger percentage of eosinophils in BAL fluid (*P* = .001). There were no identified differences based on sex, ancestry, or the proportion of subjects with a serum IgE level of greater than 100 U/mL, although asthmatic patients were significantly older than the control population (44.2 vs 34.3 years old, *P* < .004; [Table I](#)).

Airway microbial community in EB versus BAL samples

We sequenced bacterial 16S rRNA of EB and BAL samples from 39 asthmatic patients and 19 control subjects, 5 negative brush samples, and 7 negative reagent samples. A total of 1.1 million 16S rRNA V4 amplicon sequence reads (average sample depth, 14,918) were generated, which, after quality control (<0.1% incorrect bases), were clustered into 2,134 OTUs. By using a strict quality control, OTUs (*n* = 403) with variance of greater than 0.000001 were kept for further analysis.

By using the Greengenes database, 38 phyla were identified in our data set; of these, 6 (Firmicutes, Proteobacteria, Bacteroides, Fusobacteria, Acidobacteria, and Actinobacteria) accounted for 85.9% of all sequences, unclassified sequences accounted for 9.1% of sequences, and the remaining phyla accounted for 1.3% of sequences. Similarly, although 303 genera were identified, the top 25 represented 64.7% of sequences ([Fig 1](#)).

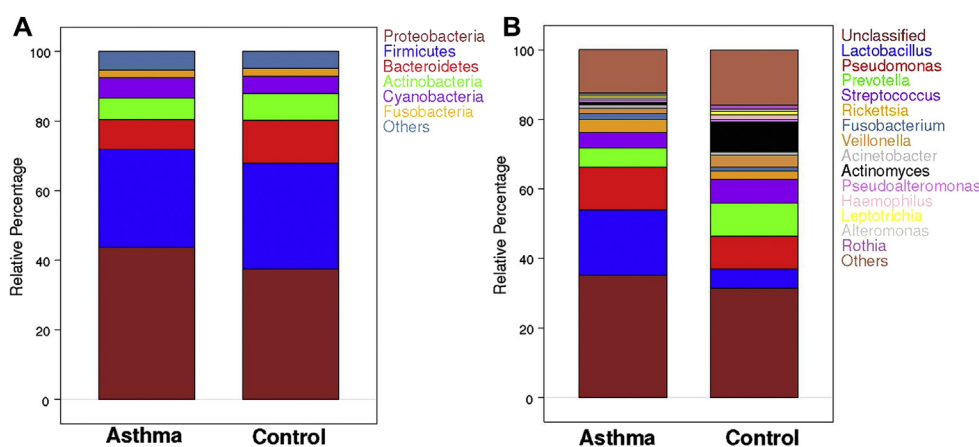
Microbial community α -diversity was significantly different between EB and BAL samples within both specimen groups at the

TABLE I. Demographic and clinical characteristics of study subjects

	Asthmatic patients (n = 39)	Control subjects (n = 19)	P value
Age (y), mean $\pm$ SE	44.2 $\pm$ 1.8	34.3 $\pm$ 3.0	.004
Sex (male/female)	11/28	8/11	—
Race (European American/African America/other)	21/17/1	7/10/2	—
ICS use (yes/no)	29/10	0/19	.001
OCS use (yes/no)	17/22	0/19	.001
Serum IgE (IU/mL), mean $\pm$ SE	242 $\pm$ 61	200 $\pm$ 109	—
Serum IgE >100 IU/mL (yes/no)	19/20	5/14	—
ENO (ppb), mean $\pm$ SE	36.8 $\pm$ 7.6*	21.9 $\pm$ 3.2	—
BAL fluid eosinophils (proportion), mean $\pm$ SE	4.2 $\pm$ 0.4	0.6 $\pm$ 0.3	.001
BAL fluid neutrophils (proportion), mean $\pm$ SE	5.4 $\pm$ 0.3	5.1 $\pm$ 0.5	—
FEV ₁ (% predicted), mean $\pm$ SE	77.2 $\pm$ 3.1	94.4 $\pm$ 2.6	.001
$\geq$ 80% of predicted value, no. (%)	16 (41)	18 (95)	
60% to 80% of predicted value, no. (%)	16 (41)	1 (5)	
$\leq$ 60% of predicted value, no. (%)	7 (18)	0 (0)	

ENO, Exhaled nitric oxide.

*This data point has 1 missing value.

**FIG 1.** Relative abundance (as a percentage) of bacteria at the phylum (A) and genera (B) levels identified in each sampling group.

genus level with the Simpson index (diversity), Chao index (richness), and Shannon index (evenness), where EB samples were significantly more diverse than BAL samples (Fig 2, A). Microbial community structure variance (β -diversity) was greater between sampling locations (EB vs BAL samples) compared with within each location (see Fig E1 in this article's Online Repository at www.jacionline.org). Fig 2, B, depicts the β -diversity of EB and BAL samples for both asthmatic patients and control subjects combined. Two-group analysis (Welch *t* test with the Storey FDR correction) demonstrated a significant difference between the microbial community β -diversity in the EB and BAL samples for both the asthmatic group ($P < .005$) and the control group ($P < .005$; Fig 2, B). Significant differences in the relative abundance of several genera were observed between EB and BAL samples. *Lactobacillus* and *Pseudomonas* species were significantly less abundant, whereas *Prevotella* and *Streptococcus* species were significantly more abundant in EB samples from asthmatic patients compared with BAL samples from asthmatic patients (Fig 2, C, and Table II). Similar trends were observed when comparing EB samples from control subjects and BAL samples from control subjects (Fig 2, D, and Table III), suggesting that this lung biogeography is absolute.

In EB samples 2-group analysis revealed that *Pseudoalteromonas* species was significantly more abundant in asthmatic patients compared with control subjects, whereas *Actinomyces* and *Prevotella* species were significantly less abundant in samples from asthmatic patients (Fig 3, A, and Table IV). In BAL samples *Rickettsia*, *Staphylococcus*, *Marinobacter*, and *Novosphingobium* species were enriched, whereas *Sphingobium* species levels were significantly less in asthmatic patients compared with control subjects (Fig 3, B, and Table V).

Clinical influences on relative abundance of specific genera

Moving forward, we chose to focus solely on EB samples because they had the most robust difference compared with negative control samples (ie, brush and reagent). To explore the relationship between clinical characteristics and the lung microbiome, we used simple linear regression to compare the microbiome community and clinical variables in EB samples. A significant decrease in the relative abundance of Firmicutes, Bacteroidetes, and Actinobacteria was demonstrated for EB

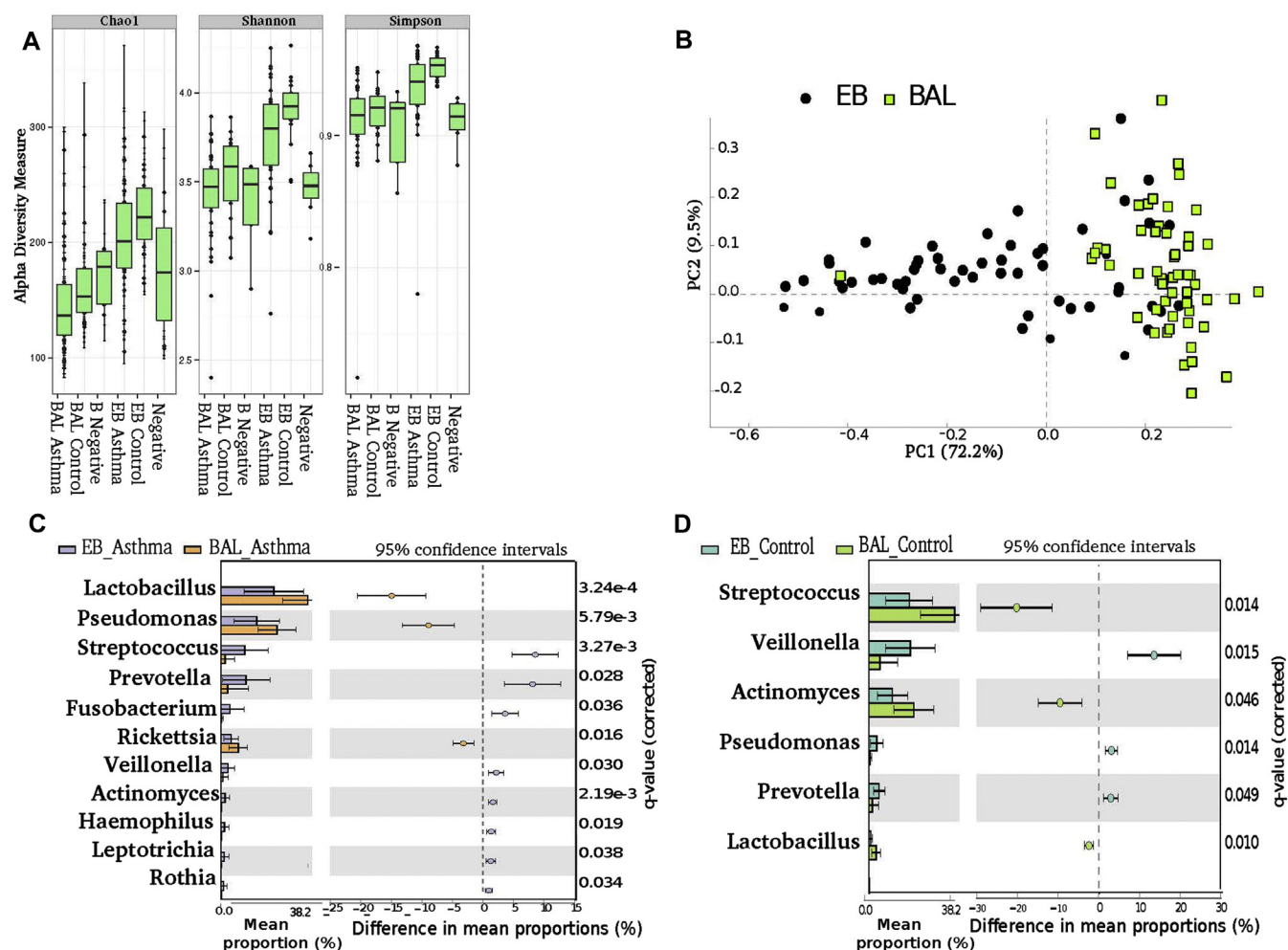


FIG 2. Analysis of microbial communities by sampling location. **A**, α -Diversity of all samples according to location. **B**, Ordination plot of principle component analysis of β -diversity of EB, BAL, and negative control samples. **C** and **D**, Significantly differentially abundant genera in EB samples from asthmatic patients versus BAL samples from asthmatic patients (Fig 2, C) and EB samples from control subjects versus BAL samples from control subjects (Fig 2, D).

TABLE II. Significantly differentiated relative abundance proportions of genera of EB samples from asthmatic patients and BAL samples from asthmatic patients

Genus	BAL samples	EB samples
<i>Lactobacillus</i>	38.21 $\pm$ 10.88	23.26 $\pm$ 13.12*
<i>Pseudomonas</i>	24.64 $\pm$ 8.29	15.73 $\pm$ 9.95*
<i>Streptococcus</i>	1.81 $\pm$ 4.35	10.40 $\pm$ 10.48*
<i>Prevotella</i>	2.95 $\pm$ 9.25	11.09 $\pm$ 10.46*
<i>Fusobacterium</i>	0.29 $\pm$ 0.85	3.93 $\pm$ 6.36*
<i>Rickettsia</i>	7.67 $\pm$ 4.01	4.45 $\pm$ 3.30*
<i>Veillonella</i>	0.87 $\pm$ 4.35	3.07 $\pm$ 3.03*
<i>Actinomyces</i>	0.15 $\pm$ 0.34	1.80 $\pm$ 1.98*
<i>Haemophilus</i>	0.16 $\pm$ 0.40	1.53 $\pm$ 2.05*
<i>Leptotrichia</i>	0.08 $\pm$ 0.42	1.37 $\pm$ 2.24*
<i>Rothia</i>	3.14 $\pm$ 2.14	2.28 $\pm$ 1.43*

Proportions are shown as means $\pm$ SDs.

* $P < .01$.

samples in subjects with the lowest FEV₁ (Table V). Also, at the genus level, *Streptococcus*, *Prevotella*, *Gemella*, and *Veillonella* levels were significantly reduced in EB samples from the patients

TABLE III. Significantly differentiated relative abundance proportions of genera of EB samples from control subjects and BAL samples from control subjects

Genus	BAL samples	EB samples
<i>Streptococcus</i>	38.23 $\pm$ 15.09	18.05 $\pm$ 10.43*
<i>Veillonella</i>	4.93 $\pm$ 8.04	18.56 $\pm$ 10.96*
<i>Actinomyces</i>	20.05 $\pm$ 8.97	10.51 $\pm$ 6.79*
<i>Pseudomonas</i>	0.39 $\pm$ 0.74	3.51 $\pm$ 2.84*
<i>Prevotella</i>	1.59 $\pm$ 2.67	4.54 $\pm$ 2.53*
<i>Lactobacillus</i>	3.26 $\pm$ 2.00	0.85 $\pm$ 0.68*

Proportions are shown as means $\pm$ SDs.

* $P < .01$.

with the lowest FEV₁ levels (Table V). Finally, the β -diversity between samples in the lowest FEV₁ group was significantly greater than for the highest FEV₁ group. This suggests that FEV₁ levels influence the diversity and structure of the EB-associated microbiome.

Many of the asthmatic patients in this study used an inhaled corticosteroid (ICS) on a daily basis, and 48% were using oral

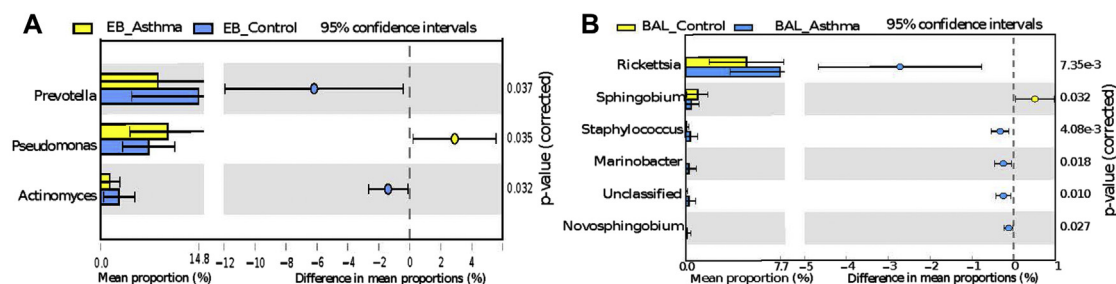


FIG 3. Differentially abundant genera across asthmatic patients and control subjects. **A**, Significantly differentiated genera between EB samples from asthmatic patients and EB samples from control subjects. **B**, Significantly differentiated genera between BAL samples from asthmatic patients and BAL samples from control subjects.

TABLE IV. Significantly differentiated relative abundance proportions of genera of EB samples from control subjects and EB samples from asthmatic patients

Genus	EB samples from asthmatic patients	EB samples from control subjects
<i>Prevotella</i>	8.67 ± 9.18	14.84 ± 10.13*
<i>Pseudomonas</i>	10.16 ± 5.76	7.25 ± 3.97*
<i>Actinomyces</i>	1.34 ± 1.57	2.76 ± 2.37*

Proportions are shown as means ± SDs.

* $P < .01$.

TABLE V. Significantly differentiated relative abundance proportions of genera of BAL samples from control subjects and BAL samples from asthmatic patients

Genus	BAL samples from control subjects	BAL samples from asthmatic patients
<i>Rickettsia</i>	4.94 ± 3.04	7.66 ± 4.01*
<i>Sphingobium</i>	0.99 ± 0.86	0.47 ± 0.63*
<i>Staphylococcus</i>	0.07 ± 0.18	0.40 ± 0.60*
<i>Marinobacter</i>	0.01 ± 0.06	0.26 ± 0.61*
Unclassified	0.03 ± 0.11	0.28 ± 0.54*
<i>Novosphingobium</i>	0.00 ± 0.00	0.12 ± 0.32*

Proportions are shown as means ± SDs.

* $P < .01$.

corticosteroids (OCSs) at the time of bronchoscopy. Subjects were stratified according to no corticosteroid use, use of ICSs only, or use of both ICSs and OCSs. No subjects used OCSs without ICSs. In the EB samples there was a decrease in the relative abundance of both Bacteroidetes and Fusobacteria and an increase in Proteobacteria based on OCS use (Table VI). At the genus level, GLM predicted OCSs as being a significant influence in the relative abundance proportions of *Pseudomonas*, *Rickettsia*, *Prevotella*, *Lactobacillus*, and *Streptococcus* species (Fig 4). There was a decreased relative abundance of *Prevotella* species and an increase of *Pseudomonas* species based on increasing corticosteroid use (Table VII). These data suggested that OCS use was associated with significant changes in the relative abundance of the EB microbial community. Additionally, GLM revealed a significant relationship between relative abundance proportion of *Rickettsia* species and exhaled nitric oxide and BAL fluid eosinophils (Fig 4).

TABLE VI. Relative abundance (proportion) of phyla and genera in EB samples from asthmatic patients stratified by FEV₁

FEV ₁ (% predicted)	>80%	60% to 80%	<60%
Phyla			
Firmicutes	33.6 ± 1.4	32.3 ± 2.2	24.0 ± 1.3*
Proteobacteria	30.7 ± 3.5	37.4 ± 3.3	43.8 ± 6.8
Bacteroidetes	15.1 ± 2.3	11.4 ± 2.1	6.7 ± 2.7*
Fusobacteria	5.0 ± 1.3	3.1 ± 1.0	5.6 ± 5.3
Actinobacteria	4.7 ± 0.7	3.7 ± 0.5	2.5 ± 0.4†
Genera			
<i>Lactobacillus</i>	11.9 ± 1.7	16.3 ± 1.8	18.6 ± 2.9*
<i>Streptococcus</i>	10.1 ± 1.5	8.2 ± 2.6	2.2 ± 1.3*
<i>Prevotella</i>	9.7 ± 2.1	7.4 ± 2.2	1.9 ± 1.5†
<i>Pseudomonas</i>	9.1 ± 1.4	9.8 ± 1.4	12.8 ± 2.8
<i>Veillonella</i>	3.2 ± 0.6	2.0 ± 0.6	0.3 ± 0.2*
<i>Gemella</i>	1.7 ± 0.3	1.1 ± 0.4	0.1 ± 0.0*

* $P < .05$ and † $P < .01$ versus high FEV₁ by using Metastats.

Clinical influences on endobronchial microbiome diversity

We used mixed-model regression to determine what clinical factors were associated with changes in diversity in EB asthmatic samples. Inverse Simpson and Shannon indices in EB samples correlated with an FEV₁ in the lowest quartile, OCS use, European ancestry, and BAL eosinophil proportions in the highest quartile. In this model 1 subject was excluded as an outlier after testing by using jackknife residual and Cook distance; doing so corrected skew significantly without affecting fit. A best-fit model for each index is shown in Fig 5. Neutrophil proportion in the BAL fluid was not a significant predictor of diversity or evenness.

DISCUSSION

In this study we compared the microbial communities in the central airways and peripheral lung in both asthmatic patients and control subjects. We examined the relationship of corticosteroid use and degree of airflow obstruction, 2 key clinical parameters of asthma, on these communities. We demonstrate that both the diversity and relative abundance in the microbiome in peripheral airways (EB) differs significantly from that of the samples taken from central airways (BAL) and negative brush and reagent controls in both asthmatic patients and nonasthmatic subjects.

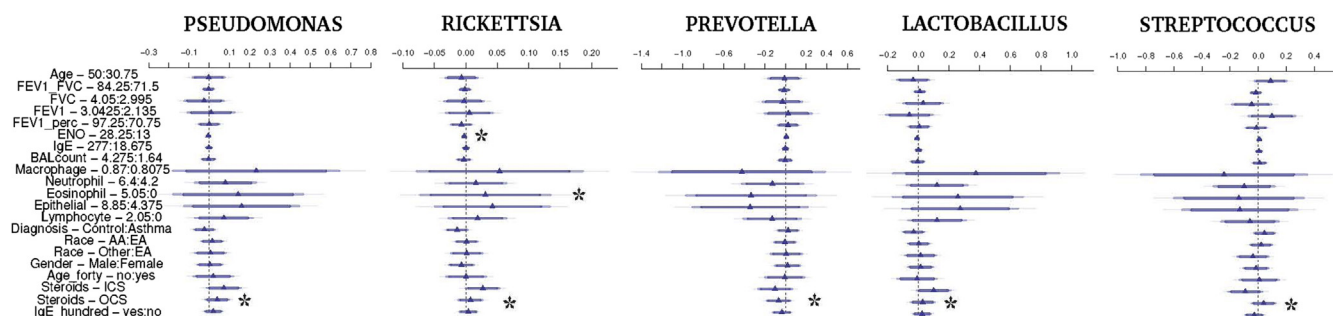


FIG 4. GLM fitting analysis across significantly important taxa (genera as predicted by MetagenomeSeq) across samples from asthmatic patients. For each genus, the GLM model was constructed, validated, and analyzed by using ANOVA. * $P < .05$. ENO, Exhaled nitric oxide; FVC, forced vital capacity.

TABLE VII. Relative abundance (proportion) of phyla and genera in EB samples from asthmatic patients stratified by corticosteroid use

Corticosteroid use:	No corticosteroids	ICSs only	ICSs and OCSs
Phyla			
Proteobacteria	28.4 ± 2.6	39.1 ± 2.7*	37.4 ± 3.5*
Bacteroidetes	17.6 ± 1.8	12.0 ± 1.6*	10.1 ± 2.1†
Fusobacteria	6.0 ± 1.3	3.0 ± 1.1*	2.2 ± 0.9*
Genera			
<i>Prevotella</i>	12.1 ± 1.7	6.2 ± 1.3*	6.6 ± 2.1*
<i>Pseudomonas</i>	7.2 ± 0.9	10.4 ± 1.8	11.0 ± 1.3*
<i>Veillonella</i>	3.1 ± 0.4	1.4 ± 0.4*	2.4 ± 0.7

* $P < .05$ and † $P < .01$ versus no corticosteroid use by using Metastats.

The BAL specimens showed slight differences between the negative control samples. In EB samples of asthmatic patients, there are significant differences in diversity and relative abundance based on both corticosteroid use and FEV₁. These data demonstrate that the lower airway microbiome in asthmatic patients differs by location, disease severity, and corticosteroid use.

We demonstrated a greater number of bacterial taxa present in our samples than noted in previous studies. In contrast to the study by Huang et al,¹⁷ we report a significant number of taxa, identified sequences, and observed unique species in control subjects without demonstrable airways disease. We note that approximately two thirds of asthmatic patients in the Huang et al study had not used corticosteroids within 2 years of study. Whether this difference accounts for the observed differences is not clear. Goleva et al¹⁶ demonstrated equal bacterial numbers in the BAL fluid of both healthy subjects and asthmatic patients that was approximately one half-log lower than that observed in our study with similar coverage, richness, and diversity.

There are differences in our study versus previously published work relating to the effect of corticosteroids on the microbiome. Goleva et al¹⁶ compared asthmatic patients who were (“sensitive”) or were not (“resistant”) responsive to a course of treatment with OCSs for 7 days with healthy subjects. Their study demonstrated a reduction in commensal genera, such as *Prevotella* and *Veillonella*, and an increase in both Actinobacteria and Proteobacteria in the asthmatic patients. We demonstrated significant differences based on corticosteroid treatment, particularly the combination of ICSs and OCSs, in α - and β -diversity, with an increased abundance of Proteobacteria and the genus *Pseudomonas*, and decreased abundance of Bacteroidetes, Fusobacteria, and *Prevotella* species. However, we did not specifically test

corticosteroid responsiveness, and the Goleva et al study did not segregate data based on current or recent corticosteroid use. Although our studies might not be directly comparable, both suggest that corticosteroid therapy in asthmatic patients is associated with changes in relative abundance in the lower airway microbiome.

We also demonstrated changes in the lower airway microbiome in asthmatic patients based on FEV₁ as a marker of airflow obstruction. Subjects with an FEV₁ of less than 60% of predicted value had lower α -diversity, higher β -diversity, and a lower relative abundance of the phyla Firmicutes, Bacteroidetes, and Actinobacteria and of the potential pathogenic genus *Streptococcus* and the commensal genera *Veillonella* and *Prevotella* compared with asthmatic patients with an FEV₁ of greater than 80% of predicted value. There was a corresponding increase in the phylum Proteobacteria and of the genus *Pseudomonas*, but these did not reach statistical significance. Huang et al¹⁷ demonstrated that increased diversity in EB community composition correlated with greater bronchial hyperresponsiveness. Their data and ours taken together suggest that airway reactivity and airflow obstruction, both cardinal features of chronic asthma, are associated with changes in microbial burden, diversity, and membership.

Previous studies of the airway microbiome in asthmatic patients have examined the presence of microbial 16S-rRNA in sputum,¹⁵ BAL fluid,^{4,14,16} and/or EB samples.^{14,17} In these studies 5 major phyla, Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria, and Fusobacterium, account for more than 90% of identified sequences, a finding we also observed in both EB and BAL samples. Marri et al¹⁵ suggested, based on sputum analysis, that the microbiome of patients with mild asthma is similar to that of patients with more severe disease and demonstrate a greater abundance of Proteobacteria and lower abundance of both Firmicutes and Actinobacteria in sputum from asthmatic patients. Our data from lower airway samples suggest otherwise: for both EB and BAL samples, the 5 major phyla are comparable between asthmatic patients and control subjects, although both are different than that found in the negative control subjects. To date, no comprehensive study has been done to examine the relation of sputum or the oral microbiome to the lower airway microbiome in asthmatic patients.

The presence of bacteria in the lower airways raises the possibility that either specific microbes or the combined effects of the microbial community could contribute to airway inflammation in asthmatic patients through modulation of the innate immune system. In support of the latter, some asthmatic patients

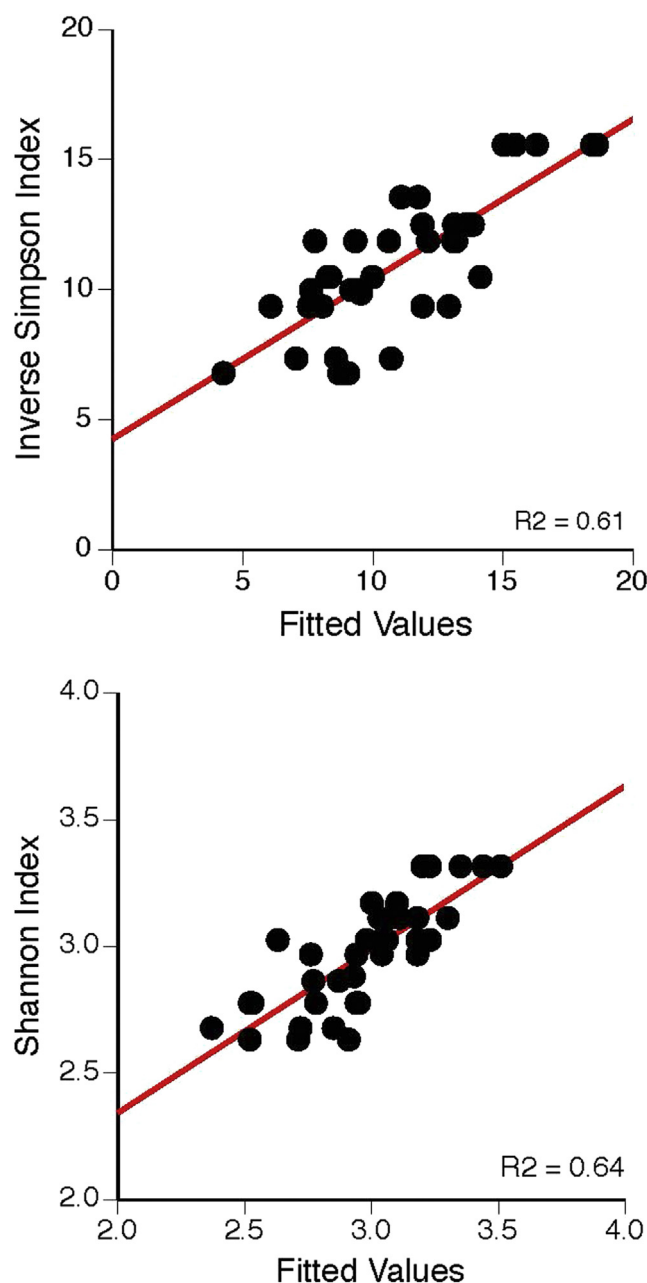


FIG 5. Mixed-model regression demonstrating a relationship between Shannon (*top panel*) and inverse Simpson (*bottom panel*) measurements of diversity and FEV₁, corticosteroid use, white ancestry, and BAL fluid eosinophils for EB samples from asthmatic patients only.

have a significant proportion of neutrophils in bronchial wall biopsy specimens or BAL fluid^{24,25} or in sputum.²⁶⁻²⁸ However, we did not find a correlation between the neutrophil proportion in BAL fluid and changes in either diversity or relative abundance in EB or BAL sample microbes from asthmatic patients at any level.

Heterogeneity in asthma is well described, and there are multiple proposed asthma phenotypes based on age, the presence of concurrent allergies, increase in serum IgE levels, cellularity of BAL fluid (eosinophil or neutrophil enriched), response to corticosteroid therapy, and obesity.^{29,30} Our study examines 2 distinct groups of asthmatic patients: those with

relatively mild airflow obstruction whose symptoms were well controlled by either ICS therapy or no corticosteroid therapy at all and those with more severe airflow obstruction and a need for OCS therapy. Asthmatic patients with the greatest degree of obstruction and those using OCS therapy had differences in the relative abundance of potentially pathogenic taxa compared with asthmatic patients with milder disease. Furthermore, eosinophil proportion, but not neutrophil proportion, in BAL fluid correlated with microbial diversity in the EB microbiome in asthmatic patients. Whether these differences persist over time or change with therapy or airflow obstruction is not clear.

Our study has several limitations. We note that changes in microbial communities do not establish causality. Although our study is about equal to or greater in size than previous studies,¹⁴⁻¹⁷ it is still small and does not include patients with the full spectrum of asthma phenotypes. It is not clear how remote use of antibiotics, corticosteroids, and other therapies for asthma influence the airway microbiome. Other features of the airway, such as the composition or rheology of mucus in the sol layer, the presence and activity of macrophages, the presence of inflammatory mediators, and the secretion of local host defense factors, might also influence the composition of the microbial communities and would not be detected in our study.

One important consideration is that the majority of asthmatic patients in the group with the greatest airway obstruction, as measured based on FEV₁, are patients treated with OCSs. This partial concordance might account for similarities within the correlation of our data. In our asthmatic patient cohort, FEV₁ and corticosteroid use did not significantly correlate, although correlation was very close ($P = .57$). For this reason, we chose to treat the FEV₁ and corticosteroid variables separately in our data analyses.

We also note that there might be organisms that participate significantly in the lower airway microbial community that would not be considered in our analysis given the observational and analytic thresholds applied. This is a common issue in microbiome studies³¹ and awaits advances in understanding of how to assess the functional significance of sparse microbiota. Also, our study was performed at a single institution in patients drawn from a single major metropolitan center. Differences in geography, household exposures, pet ownership, and environment all can lead to differences in the microbial communities recovered² and account for some of the differences between our and previous studies.

The retrospective nature of our study precluded collection of upper airway and oral samples. Previous studies have demonstrated significant commonality between upper and lower airway microbiomes,^{4,14,32,33} and we have no reason to believe that our study would differ in this regard. Likewise, Segal et al³³ recently demonstrated in healthy subjects, using a 2-bronchoscope method in which the first instrument samples the supraglottic airway and the second the lower airways, that little carryover of organisms occurs because of the bronchoscope.

In summary, we demonstrate clear differences in the microbiome recovered from EB and BAL samples in both asthmatic patients and control subjects. Asthmatic patients differ in their EB microbiome based on corticosteroid use and degree of airflow obstruction. Understanding the role of the microbiome in airways of asthmatic patients and changes over time might lead to novel targeted therapies that improve asthma control.

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Key messages

- The microbiome of the central airways in asthmatic patients has a lower diversity and greater abundance of key bacterial pathogens, such as *Pseudomonas* species, compared with that seen in control subjects.
- Both diversity and relative abundance changes of pathogens are related to corticosteroid use and worsening airflow obstruction, as measured based on FEV₁.
- The central airway microbiome determined based on EB samples is substantially different than that of the peripheral airways determined from BAL samples.

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METHODS

Additional information concerning study subjects

Patients with mild persistent asthma or subjects without known lung disease (control subjects) were recruited from the Chicago-area community. Subjects with severe asthma were recruited from the Refractory Obstructive Lung Disease Clinic at the University of Chicago. Patients with mild persistent asthma met the criteria for step 1 or step 2 asthma, and patients with severe asthma met the criteria for step 4 through step 6 asthma, as defined by the Expert Panel Report 3 Guidelines on Asthma.^{E1} Confirmation of airway reactivity in asthmatic patients was done by using methacholine challenge when baseline FEV₁ was 60% of predicted value or greater; when this could not be done, measurement of reversibility before and after inhalation of albuterol was done per American Thoracic Society guidelines.^{E2} Control subjects were demonstrated to have a less than 16% reduction in FEV₁ after inhalation of 25 mg/mL methacholine. Subjects with a smoking history of 10 pack years or longer, who had a history of chronic obstructive pulmonary disease, or who had medical contraindications to bronchoscopy were excluded. Subjects underwent data collection, venipuncture, spirometry, and flexible video bronchoscopy at a time of clinical stability and at least 8 weeks after any known asthma exacerbation or respiratory viral illness.

Bronchoscopy was done by using standard methods and conscious sedation. After introduction of the bronchoscope into the airways, 4 EB samples were collected from the proximal portion of the right lower lobe bronchus with an epithelial cell cytology brush (Medical Engineering Laboratory, Shelby, NC). Then BAL was performed by means of instillation of 120 mL of saline into the anterior segment of the right middle lobe, followed by suction recovery. All subjects tolerated bronchoscopy without complications.

Sample processing

Negative specimens for microbiome analysis were generated from cytology brushes (n = 5) that were put through a bronchoscope that was precleared with sterile saline but were not introduced into a patient. As an additional control to identify reagent-derived contaminants, DNA was extracted from reagents used for processing of specimens on each day that batch processing was done (n = 7).

BAL fluid samples were handled at 4°C throughout initial processing. Samples were centrifuged at 1000g for 10 minutes, and then cytospin preparations of cells were made for differential counts. Supernatants were filtered through a 1.2-μm filter, pellets were collected after centrifugation at 10,000g for 10 minutes, and samples were frozen at –80°C until processed for DNA extraction. Epithelial cytology brushes (Medical Engineering Laboratory, Shelbyville, NC) used in bronchoscopy were initially stored in 1 mL of QIAzol reagent (Qiagen, Hilden, Germany) and vortexed for 3 minutes. Samples then were stored at –80°C until processed for DNA extraction.

All processing was done at 4°C, unless otherwise noted. BAL samples were centrifuged at 16,000g for 30 minutes at 4°C, after which the pellet was resuspended in Qiagen ATL lysis buffer. Then samples were transferred to 2-mL bead tubes containing sterile 0.1-mm glass beads (MO BIO Laboratories, Carlsbad, Calif). Samples were homogenized for 3 minutes in a BioSpec Mini-Beadbeater-16 cell disrupter (BioSpec Products, Bartlesville, Okla). After addition of proteinase K, BAL samples were incubated at 55°C for 60 minutes. Bead tubes were then centrifuged for 1 minute at 10,000g, and samples were transferred to a clean microcentrifuge tube. The remainder of sample purification and extraction was performed with the Qiagen DNeasy Blood & Tissue Kit, according to the manufacturer's protocol.

Brush samples stored in QIAzol were transferred to 2-mL bead tubes containing sterile 0.1-mm glass beads (MO BIO Laboratories). Samples were homogenized for 3 minutes in a BioSpec Mini-Beadbeater-16 cell disrupter (BioSpec Products) and then allowed to incubate at room temperature for 5 minutes. Brush samples were centrifuged at 10,000g for 1 minute and then agitated with chloroform for 15 seconds, held at room temperature for 3 minutes, and centrifuged at 12,000g for 15 minutes. After removal of the

residual aqueous phase, ethanol was added, and samples were incubated at room temperature for 2 minutes and centrifuged at 8,000g for 2 minutes to sediment DNA. The phenol/ethanol supernatant was discarded, after which DNA was washed in sodium citrate 3 times. The DNA pellet was washed in 75% ethanol for 20 minutes and centrifuged at 8,000g for 5 minutes, after which the DNA pellet was air dried and resuspended in 8 mmol/L NaOH. After resuspension, DNA was centrifuged at 14,000g for 10 minutes to remove insoluble material, and the DNA was placed in TE buffer solution. Final DNA purification with the Qiagen DNeasy Blood & Tissue Kit was done according to the kit's instructions. Then isolated DNA was dissolved in Qiagen AE buffer and stored at –80°C until analysis. DNA concentration and purity were assessed by using a NanoDrop ND-1000 Spectrophotometer.

16S rRNA amplicon library preparation and sequencing

Processing was done at the National Laboratory for High-Throughput Genome Analysis Core at Argonne National Laboratory (Argonne, Ill). All samples first underwent screening for the presence of the 16S rRNA gene within extracted total DNA by using PCR and primers specific for the 515- to 806-bp V4 region of the 16S rRNA encoding gene (338F: 5'-GTGCCAGCMGCCGCGGTAA-3'; 806R: 5'-GGACTACHVGGGTW TCTAAT-3'). Illumina 3' adapter sequences and a 12-bp barcode were added at that time. Sequencing was done with an Illumina MiSeq DNA sequencer.

Data analysis

Using the Phyloseq package,^{E3} the OTU matrix was processed to remove OTUs containing less than 5 reads to reduce the PCR and sequencing based bias; then the OTU table was rarified to the minimum numbers of reads present in the smallest library.

For ANOVA and *P* value correction, we used the following parameters: *post hoc* test, Tukey-Kramer; effect size, eta squared; and multiple test correction, Benjamini and Hochberg. MetagenomeSeq^{E4} was used to identify the differentially abundant taxons across groups. Clinical data are expressed as means ± SEMs. Two-group analysis was performed with Welch *t* tests, followed by Storey FDR correction. We then used a GLM to examine the contribution of patients' demographic data on the abundance patterns of the differentially abundant bacterial genera.

For microbial samples, paired-end reads were quality trimmed and processed for OTU picking by using the UPARSE^{E5} pipeline set at 97% sequence identity cutoff. Taxonomic status was assigned to high-quality OTUs (<1% incorrect bases) by using "parallel assign taxonomy" script from QIIME software.^{E6} Multiple sequence alignment and phylogenetic reconstruction was performed with PyNast and FastTree.^{E6} The Phyloseq package^{E3} was used for ordination and α- and β-diversity analysis. Two-group *t* tests and principal component analysis plots were generated by using STAMP software. Group diversity and taxonomy data were also calculated using the Mothur suite^{E7} where appropriate. Alignment and quality checking within Mothur was done using the SILVA ribosomal RNA sequence database, v102.^{E8} Differences in taxonomic distribution of sequences by phylum, order and genus was examined in different groups of subjects using Metastats with *q* value corrections for false discovery.^{E9}

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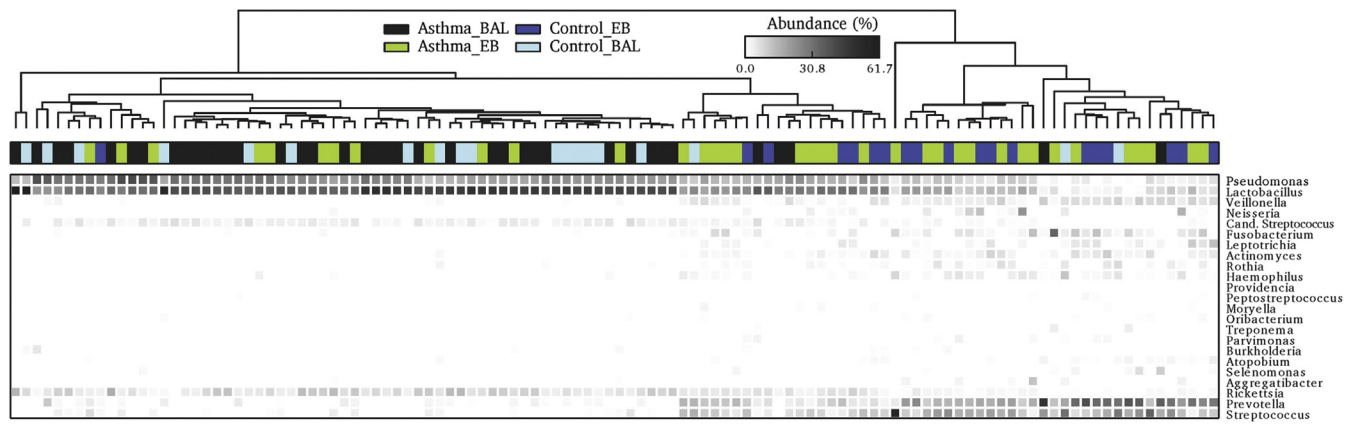


FIG E1. Clustering patterns of microbial communities in specific sampling locations based on Bray-Curtis dissimilarity measurements.