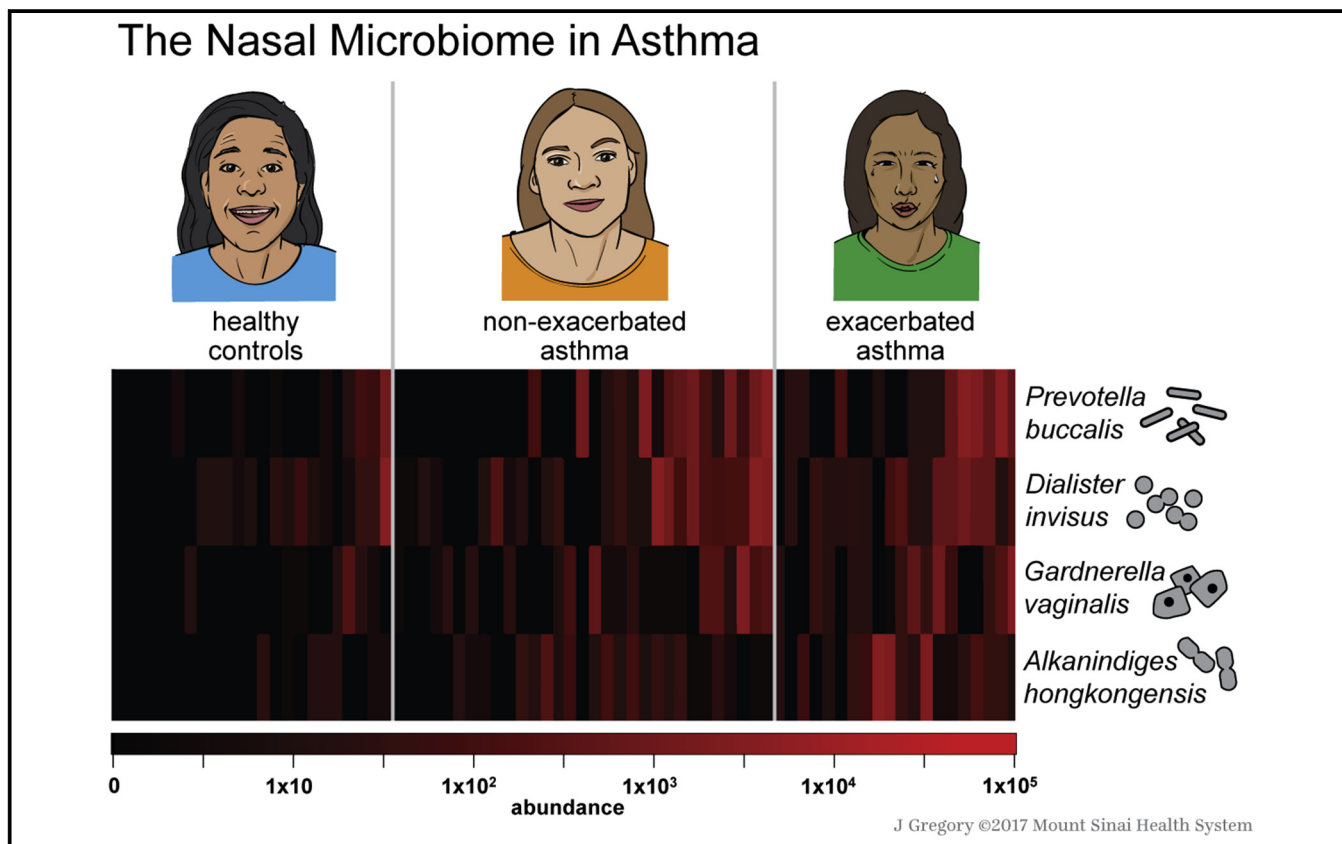


The nasal microbiome in asthma



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GRAPHICAL ABSTRACT



Background: Nasal microbiota may influence asthma pathobiology. **Objective:** We sought to characterize the nasal microbiome of subjects with exacerbated asthma, nonexacerbated asthma, and healthy controls to identify nasal microbiota associated with asthma activity.

Methods: We performed 16S ribosomal RNA sequencing on nasal swabs obtained from 72 primarily adult subjects with exacerbated asthma (n = 20), nonexacerbated asthma (n = 31), and healthy controls (n = 21). Analyses were performed using Quantitative Insights into Microbial (QIIME); linear discriminant analysis

effect size (LEfSe); Phylogenetic Investigation of Communities by Reconstruction of Unobserved States; and Statistical Analysis of Metagenomic Profiles (PICRUST); and Statistical Analysis of Metagenomic Profiles (STAMP). Species found to be associated with asthma activity were validated using quantitative PCR. Metabolic pathways associated with differentially abundant nasal taxa were inferred through metagenomic functional prediction. **Results:** Nasal bacterial composition significantly differed among subjects with exacerbated asthma, nonexacerbated asthma, and healthy controls (permutational multivariate

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ANOVA, $P = 2.2 \times 10^{-2}$). Relative to controls, the nasal microbiota of subjects with asthma were enriched with taxa from *Bacteroidetes* (Wilcoxon-Mann-Whitney, $r = 0.33$, $P = 5.1 \times 10^{-3}$) and *Proteobacteria* ($r = 0.29$, $P = 1.4 \times 10^{-2}$). Four species were differentially abundant based on asthma status after correction for multiple comparisons: *Prevotella buccalis*, $P_{\text{adj}} = 1.0 \times 10^{-2}$; *Dialister invisus*, $P_{\text{adj}} = 9.1 \times 10^{-3}$; *Gardnerella vaginalis*, $P_{\text{adj}} = 2.8 \times 10^{-3}$; *Alkanindiges hongkongensis*, $P_{\text{adj}} = 2.6 \times 10^{-3}$. These phyla and species were also differentially abundant based on asthma activity (exacerbated asthma vs nonexacerbated asthma vs controls). Quantitative PCR confirmed species overrepresentation in asthma relative to controls for *Prevotella buccalis* (fold change = 130, $P = 2.1 \times 10^{-4}$) and *Gardnerella vaginalis* (fold change = 160, $P = 6.8 \times 10^{-4}$). Metagenomic inference revealed differential glycerolipid metabolism (Kruskal-Wallis, $P = 1.9 \times 10^{-4}$) based on asthma activity. **Conclusions:** Nasal microbiome composition differs in subjects with exacerbated asthma, nonexacerbated asthma, and healthy controls. The identified nasal taxa could be further investigated for potential mechanistic roles in asthma and as possible biomarkers of asthma activity. (J Allergy Clin Immunol 2018;142:834-43.)

Key words: Nasal, microbiome, 16S rRNA sequencing, asthma, upper respiratory tract, upper airway

Resident microbial communities have been increasingly implicated in health and disease.¹ Respiratory microbiota have been associated with asthma development and exacerbations,^{2,3} and differences in the bronchial microbiome are observed in asthmatic versus control subjects.⁴⁻¹³ Although the bronchial microbiome has been frequently studied in asthma, the nasal microbiome in asthma has received far less attention.^{4,14-17}

A better understanding of the nasal microbiome of asthma is important for several reasons. First, nasopharyngeal bacterial species have been associated with the development of childhood wheeze,¹⁷ suggesting a potential role for nasal microbiota in asthma pathogenesis. Second, the nasal passages are directly connected to the lungs. A shared biology to the upper and lower airways (ie, the united airway) is suggested by epidemiologic data, clinical practice, and molecular findings.¹⁸⁻²⁰ For such shared biology, assessment of nasal microbiota is far less invasive than bronchoscopic profiling of the bronchial microbiome. Nasal microbial markers of asthma for detection and disease monitoring could be more feasible to assess in population studies of asthma. That said, bacterial communities vary across the human body,²¹ so dedicated studies of the nasal microbiome will likely identify nasal microbiota associated with asthma that are distinct from those known from bronchial studies.

In this study, we applied 16S ribosomal RNA (rRNA) sequencing to profile the nasal microbiome of subjects with exacerbated asthma, nonexacerbated asthma, and healthy controls, yielding data for one of the largest studies of the nasal microbiome and asthma activity to date. Our analyses identified bacterial taxa that marked each study group, with quantitative PCR (qPCR) corroborating our major findings.

METHODS

Subject recruitment and sample collection

Participants were recruited from the Mount Sinai Health System's emergency department and clinics in New York, NY, over a 12-month period.

Abbreviations used

LEfSe: Linear discriminant analysis effect size
OTU: Operational taxonomic unit
PERMANOVA: Permutational multivariate ANOVA
qPCR: Quantitative PCR
rRNA: Ribosomal RNA

The study was approved by the Mount Sinai Institutional Review Board, and all subjects provided written informed consent. Exacerbated asthma was defined as self-reported and physician-diagnosed current asthma presenting with a chief complaint of shortness of breath to the emergency department with an encounter diagnosis of asthma exacerbation. Nonexacerbated asthma was defined as self-reported and physician-diagnosed current asthma presenting for routine, nonurgent, asthma follow-up care. Healthy controls had no self-report or physician diagnosis of past or current asthma. Individuals with nonasthma pulmonary diagnoses were excluded from all groups.

The study physician administered to each subject a detailed questionnaire and asthma control test. The study physician coached each participant through 3 peak flow measurements and obtained nasal samples in duplicate from the anterior nares using a sterile cotton swab.

DNA isolation and 16S rRNA sequencing

Given the low biomass nature of nasal samples, we instituted precautions for potential false positives from contaminants by including 3 blank swab controls with our clinical collections. DNA was isolated from experimental samples and blank swabs using the QIAamp DNA MicroKit (Qiagen, Hilden, Germany). The V3-V4 region of the 16S rRNA gene was amplified using previously described primer sequences.²² Amplicon cleaning, indexing, and sequencing were performed according to the Illumina MiSeq 16S Metagenomic Sequencing Library Preparation Protocol (Illumina, San Diego, Calif). Samples were multiplexed using a dual-index approach with the Nextera XT Index kit version 2 (Illumina). The final library was sequenced at 2 × 250 bp on the Illumina MiSeq platform. Bacterial burden was quantified with qPCR for the 16S rRNA gene.²³ Quality control on the raw reads was performed using Quantitative Insights into Microbial Ecology (QIIME 1.9.1, <http://qiime.org>) as previously described.²⁴ There were 10,910,593 sequences in total after demultiplexing and filtering for read quality, with a median of 150,615 sequences per experimental sample.

Microbiome analyses

We used QIIME 1.9.1 to analyze the microbiome sequence data. Operational taxonomic units (OTUs), defined as taxonomic units based on DNA sequences that share high identity²⁵ were constructed using a >97% similarity threshold to the 16S rRNA representative sequence provided in the Greengenes database version 13.5.0 (Greengenes Consortium, <http://greengenes.lbl.gov>). To remove potential signal from contaminants, we removed from analysis taxa with >10% relative abundance in the blank swab samples and OTUs with <2 total occurrences among the samples. In total 10,266,559 reads remained, which clustered into 6,182 OTUs.

Alpha diversity (the richness of a sample in terms of the diversity of OTUs observed in it) was estimated using Faith phylogenetic diversity.²⁶ Beta diversity (distance between samples based on differences in OTUs present in each sample) was measured using weighted UniFrac.²⁷ Association between microbiome composition and study group was tested using permutational multivariate ANOVA (PERMANOVA), a nonparametric test similar to ANOVA. Significance of PERMANOVA tests was determined using 2000 permutations with adjustment for multiple testing using the Benjamini-Hochberg method. Linear discriminant analysis effect size (LEfSe),²⁸ a method for biomarker discovery, was used to determine genera that best characterize each study group. LEfSe scores measure the consistency of differences in relative abundance between taxa in the groups analyzed (control vs nonexacerbated asthma vs exacerbated asthma), with a higher

TABLE I. Baseline characteristics of the participating subjects

	All (n = 72)	Healthy control (n = 21)	Nonexacerbated asthma (n = 31)	Exacerbated asthma (n = 20)	P value*
Female	48 (66.7)	12 (57.1)	22 (71.0)	14 (70.0)	.59
Age (y)	35.8 ± 16.0	26.9 ± 8.4	44.4 ± 18.3	31.8 ± 11.1	9.2 × 10 ⁻⁵
Race					.0021
Caucasian	14 (19.4)	10 (47.6)	3 (9.7)	1 (5.0)	
African American	20 (27.8)	2 (9.5)	11 (35.5)	7 (35.0)	
Latino	25 (34.7)	3 (14.3)	12 (38.7)	10 (50.0)	
Asian	4 (5.6)	3 (14.3)	1 (3.2)	0 (0.0)	
Other	9 (12.5)	3 (14.3)	4 (12.9)	2 (10.0)	
Hospitalization for asthma, past year	29 (40.3)	0 (0.0)	14 (45.2)	15 (75.0)	4.8 × 10 ⁻⁷
ED visit for asthma, past year	46 (63.9)	0 (0.0)	26 (83.9)	20 (100.0)	2.0 × 10 ⁻¹⁴
Last wheeze, cough, or chest tightness					5.6 × 10 ⁻¹⁰
Currently	20 (27.8)	0 (0.0)	2 (6.5)	18 (90.0)	
≤ 2 wk	14 (19.4)	0 (0.0)	14 (45.2)	0 (0.0)	
≤ 3 mo	8 (11.1)	1 (4.8)	7 (22.6)	0 (0.0)	
>3 mo	11 (15.3)	1 (4.8)	8 (25.8)	2 (10.0)	
Medication use, past 24 h					
Short-acting β-agonist	38 (52.8)	0 (0.0)	19 (61.3)	19 (95.0)	4.7 × 10 ⁻¹¹
Inhaled corticosteroid	30 (41.7)	0 (0.0)	22 (71.0)	8 (40.0)	2.2 × 10 ⁻⁷
Long-acting β-agonist	16 (22.2)	0 (0.0)	10 (32.3)	6 (30.0)	5.6 × 10 ⁻³
Leukotriene receptor antagonist	17 (23.6)	0 (0.0)	14 (45.2)	3 (15.0)	1.8 × 10 ⁻⁴
≥1 controller medication†	32 (44.4)	0 (0.0)	23 (74.2)	9 (45.0)	3.4 × 10 ⁻⁸
Systemic steroids	9 (12.5)	0 (0.0)	3 (9.7)	6 (30.0)	.013
Nasal steroid	14 (19.4)	1 (4.8)	11 (35.4)	2 (10.0)	.014
Antihistamines	26 (35.6)	2 (9.5)	19 (61.3)	5 (25.0)	2.3 × 10 ⁻⁴
ACT score	17.7 ± 6.2	24.7 ± 1.0	15.0 ± 4.8	14.2 ± 5.4	1.4 × 10 ⁻⁵
Peak flow, % predicted	81.8 ± 22.4	98.4 ± 11.9	79.1 ± 22.1	72.6 ± 19.6	.0041
Allergic rhinitis symptoms, past year	43 (59.7)	7 (33.3)	24 (77.4)	12 (60.0)	.0069
Last upper respiratory infection					.011
Currently	13 (18.1)	1 (4.8)	2 (6.5)	10 (50.0)	
≤2 wk	12 (16.7)	4 (19.0)	6 (19.4)	2 (10.0)	
≤3 mo	18 (25.0)	5 (23.8)	10 (32.3)	3 (15.0)	
>3 mo	27 (37.5)	10 (47.6)	12 (38.7)	5 (25.0)	
Antibiotics, past 2 mo	16 (22.2)	2 (9.5)	8 (25.8)	6 (30.0)	.22

Values are n (%) or mean ± SD.

ACT, Asthma control test; ED, emergency department.

*Fisher exact test, except ANOVA for age and Kruskal-Wallis test for ACT score and peak flow.

†Inhaled corticosteroid and/or long-acting β-agonist and/or leukotriene receptor antagonist.

score indicating higher consistency. We considered taxa with linear discriminant analysis score >2 and $P < .05$ to be significant. To identify species represented by the genera revealed by LEfSe, we first identified the OTUs associated with those genera, filtered low abundance OTUs (<50 copies), performed Kruskal-Wallis tests on each remaining OTU, and used Basic Local Alignment Search Tool (BLAST) (<https://blast.ncbi.nlm.nih.gov>) to align the sequences of these OTUs against the Greengenes database, retaining species with identity match of >97%.

qPCR validation

To validate species identified by our analyses, we used qPCR to measure their abundances in nasal samples from asthmatic and control subjects. We included universal primers (Microbial DNA qPCR Kit for Pan Bacteria 3, QIAGEN) to measure total bacterial load in addition to qPCR with species-specific primers for *Prevotella buccalis*, *Gardnerella vaginalis*, and *Dialister invisus* (Microbial DNA qPCR Assay kit specific for each, QIAGEN). We used 1.5 ng of nasal DNA from a subset of the controls (n = 3) and asthmatic subjects (nonexacerbated n = 3 plus exacerbated n = 1) as the template for qPCR reactions. Samples were chosen based on availability of DNA, as we sought to focus experimental validation on samples with high residual yield after 16S rRNA sequencing template needs. The qPCR reactions were prepared according to the manufacturer's instructions (Qiagen). Samples were loaded into a 384-well plate and read using the ABI 7900HT Real-Time PCR system (Applied Biosystems, Foster City, Calif). Thermocycling conditions were 95°C for 10 minutes, 40 cycles of

95°C for 15 seconds, and 60°C for 2 minutes. For fold change analyses, unpaired *t* tests were performed to calculate *P* values.

Prediction of metagenome functional content

We used Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) 1.0.0²⁹ to predict metagenome function from the 16S rRNA data. Bray-Curtis distances were used to determine similarity of samples based on metagenomic composition. We used Statistical Analysis of Metagenomic Profiles (STAMP)³⁰ to analyze the predicted metagenome and identify pathways associated with asthma activity. The Benjamini-Hochberg procedure was used to control the false discovery rate due to multiple testing.

RESULTS

Study population

Nasal samples were obtained from 72 participants, whose baseline characteristics are shown in Table I. Consistent with racial demographics of asthma in the New York area,^{31,32} subjects with asthma included more African Americans and Latinos than Caucasians. The participants were primarily adults, with median age 32 years, interquartile range 23 to 47 years, range 10 to 73 years. Subjects with asthma generally had mild to moderate

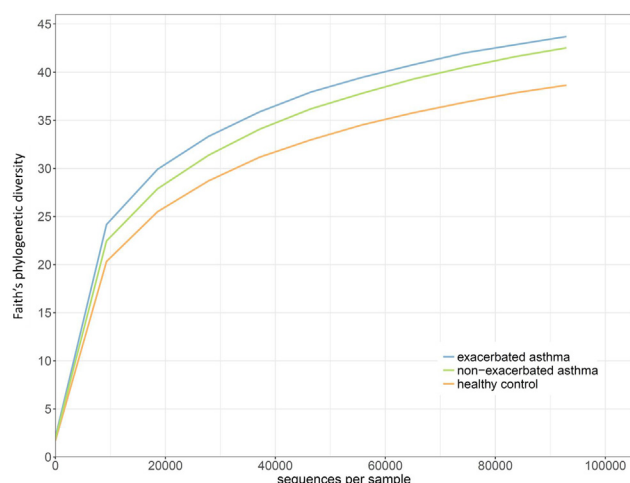


FIG 1. Trend toward increased nasal bacterial alpha diversity with greater asthma activity. Rarefaction plot showing the total branch length using Faith phylogenetic diversity (PD) is plotted against fraction of retained reads for healthy controls ($n = 21$) and subjects with nonexacerbated asthma ($n = 31$) and exacerbated asthma ($n = 20$). Differences were not statistically significant at $P \leq .05$.

persistent asthma, and those with exacerbated asthma had the highest prevalence rates of hospitalizations and emergency department visits for asthma in the past year. Control subjects had no health care needs for asthma, while those with nonexacerbated asthma had intermediate rates of hospitalization and emergency department visits for asthma. Short-acting β agonist and inhaled corticosteroids were the most commonly used medications in the past 24 hours among those with asthma. Relative to controls and as expected, asthma control test score and peak flow measures were lower in those with nonexacerbated asthma, and lowest in subjects with exacerbated asthma. Subjects with asthma were more likely to have had allergic rhinitis symptoms and recent upper respiratory infection.

Nasal bacterial alpha diversity and asthma activity

Rarefaction curves calculated based on Faith phylogenetic diversity for healthy control, nonexacerbated asthma, and exacerbated asthma subjects showed a positive trend between nasal bacterial alpha diversity and asthma activity (Fig 1). These differences in phylogenetic diversity were not statistically significant, however.

Distinct nasal microbiome composition associated with asthma activity

Healthy controls, subjects with nonexacerbated asthma, and subjects with exacerbated asthma demonstrated distinct nasal microbiome compositions (PERMANOVA, $P = .022$, pseudo- $F = 1.9$). At the phylum level, *Bacteroidetes* and *Proteobacteria* were enriched in subjects with exacerbated and nonexacerbated asthma relative to healthy controls (Wilcoxon-Mann-Whitney, $r = 0.33$, $P = 5.1 \times 10^{-3}$ and $r = 0.29$; $P = 1.4 \times 10^{-2}$, respectively) (Fig 2). These phyla were also differentially abundant in 3-way comparisons based on asthma activity (ie, exacerbated asthma vs nonexacerbated asthma vs healthy control): *Bacteroidetes* Kruskal-Wallis, $P = 9.7 \times 10^{-3}$ and

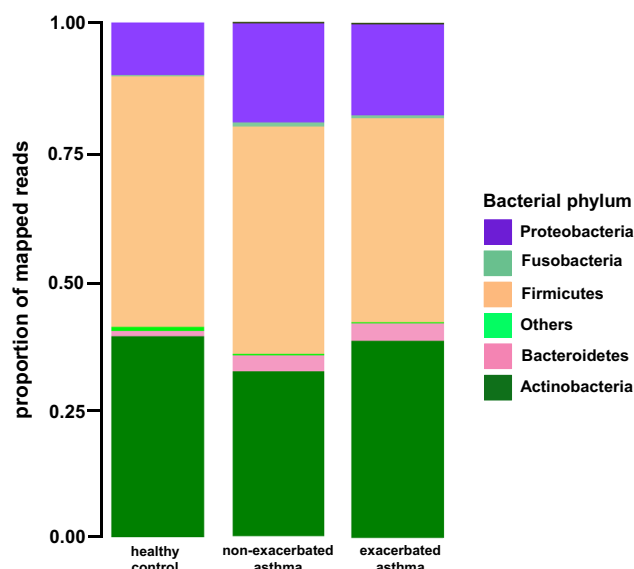


FIG 2. Distinct bacterial composition in subjects with exacerbated asthma, nonexacerbated asthma, and healthy controls. At the phylum level, *Bacteroidetes* and *Proteobacteria* were significantly enriched in subjects with nonexacerbated and exacerbated asthma relative to controls ($P = 5.1 \times 10^{-3}$ and $P = 1.4 \times 10^{-2}$, respectively). These phyla were also differentially abundant in 3-way comparisons based on asthma activity. Phyla with $<0.1\%$ abundance are aggregated as "Others."

Proteobacteria, $P = 5.0 \times 10^{-2}$ (see Fig E1 in this article's Online Repository at www.jacionline.org).

To more specifically identify bacterial genera associated with asthma activity, we used LEfSe to compare bacterial abundances at the genus level. Four bacterial genera were differentially abundant between the study groups with linear discriminant analysis score >2 and $P < .05$ (Fig 3). *Prevotella* (phylum *Bacteroidetes*), *Alkanindiges* (phylum *Proteobacteria*), and *Gardnerella* (phylum *Actinobacteria*) were enriched in subjects with exacerbated asthma, while *Dialister* (phylum *Firmicutes*) was significantly more abundant in subjects with nonexacerbated asthma. No taxa were enriched in healthy controls relative to nonexacerbated or exacerbated asthmatic subjects.

To further hone in on nasal bacterial species associated with asthma activity, we identified OTUs associated with the genera identified by LEfSe, and filtered samples with <50 copies, leaving 10 OTUs for species-level analysis. We identified 4 species that were differentially abundant based on asthma status (ie, exacerbated asthma plus nonexacerbated asthma vs healthy control subjects) after correction for multiple testing at $P_{\text{adj}} \leq .05$: *Prevotella buccalis* ($P_{\text{adj}} = 1.0 \times 10^{-2}$), *Dialister invisus* ($P_{\text{adj}} = 9.1 \times 10^{-3}$), *Gardnerella vaginalis* ($P_{\text{adj}} = 2.8 \times 10^{-3}$), and *Alkanindiges hongkongensis* ($P_{\text{adj}} = 2.6 \times 10^{-3}$). These species were also differentially abundant in 3-way comparisons based on asthma activity (ie, exacerbated asthma vs nonexacerbated asthma vs healthy control subjects): *Prevotella buccalis* ($P_{\text{adj}} = 5.3 \times 10^{-2}$), *Dialister invisus* ($P_{\text{adj}} = 2.6 \times 10^{-2}$), *Gardnerella vaginalis* ($P_{\text{adj}} = 4.6 \times 10^{-3}$), and *Alkanindiges hongkongensis* ($P_{\text{adj}} = 6.2 \times 10^{-4}$). The abundances of these species in the samples grouped by asthma activity are shown as a heat map in Fig 4.

Regarding potential confounders, there were no significant differences in nasal microbiome composition based on population

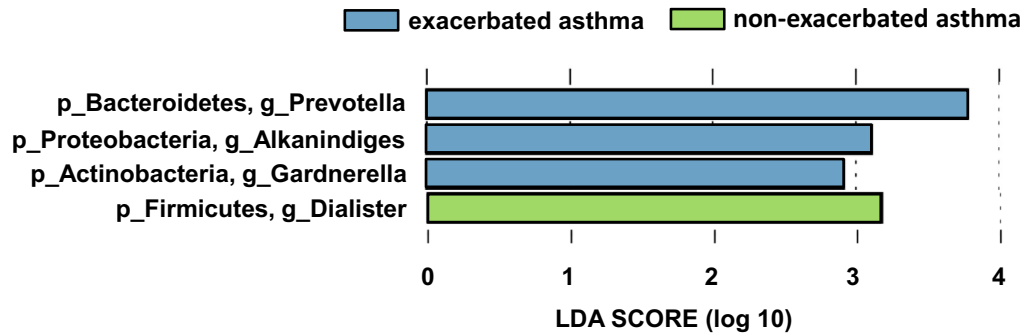


FIG 3. Linear discriminant analysis (LDA) demonstrated distinct bacterial genera enriched in exacerbated and nonexacerbated asthma. Genera (g) with $P < .05$ and LDA score > 2 were considered significant and are shown here with notation for their corresponding phylum (p).

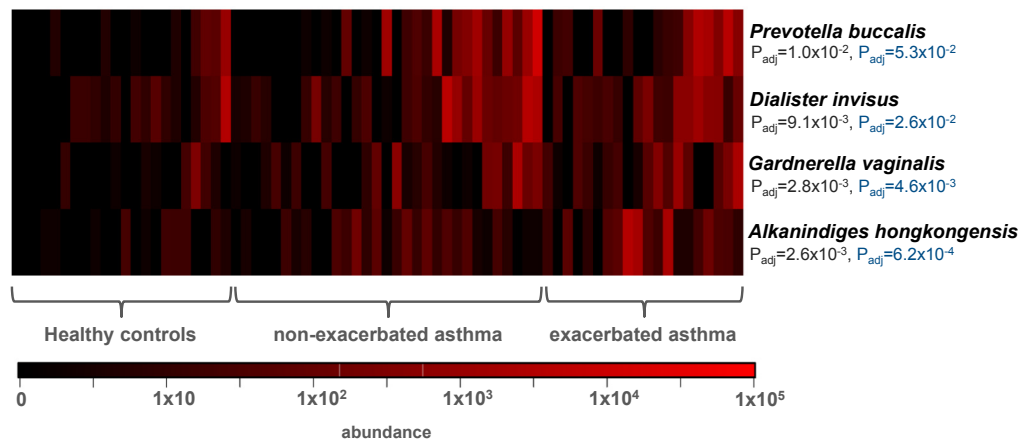


FIG 4. Heat map showing abundance of species significantly associated with asthma status and asthma activity. P_{adj} for 2-way comparison based on asthma status (ie, exacerbated asthma and nonexacerbated asthma vs healthy control) using the Wilcoxon-Mann-Whitney test with adjustment for multiple testing via the Benjamini-Hochberg method if first shown in black font. P_{adj} for 3-way comparison based on asthma activity (ie, exacerbated asthma vs nonexacerbated asthma vs healthy control) using the Kruskal-Wallis test with adjustment for multiple testing using the Benjamini-Hochberg method is then shown in blue font. Each column of the heat map represents an individual.

characteristics such as age (PERMANOVA, $P = .13$), sex ($P = .64$), allergic rhinitis status ($P = 1.00$), last upper respiratory infection ($P = .48$), recent antibiotic use ($P = .44$), antihistamine use ($P = .99$), nasal steroid use ($P = .32$), inhaled steroid use ($P = .19$) or systemic steroid use ($P = .93$), supporting that these characteristics were not confounders of the associations between nasal microbiome composition and asthma. Although race was associated with nasal microbiome composition (PERMANOVA, $P = .0005$), OTU abundances of *Prevotella buccalis*, *Dialister invisus*, *Gardnerella vaginalis*, and *Alkanindiges hongkongensis* in particular were not associated with race, providing reassurance that the reported associations for these 4 species and asthma were not confounded by race.

Although use of nasal steroids, inhaled steroids, and systemic steroids were not associated with nasal microbiome composition, we additionally constructed multivariate logistic regression models for asthma and OTU abundances adjusted for each type of steroid use as exploratory analyses. A study of bronchial microbiota before and after inhaled steroid treatment suggested that inhaled corticosteroids could influence selected taxa, although the sample size for this was limited and the upper airway was not profiled.³³ We therefore thought that a directed

exploration of steroid use in this study of the nasal microbiome of asthma might be insightful. In models adjusted for nasal steroids, nasal abundances of *Prevotella buccalis*, *Alkanindiges hongkongensis*, *Gardnerella vaginalis*, and *Dialister invisus* all remained significantly associated with asthma (see Table E1 in this article's Online Repository at www.jacionline.org). Significant associations with asthma were also found for these 4 species in multivariate models adjusted for systemic steroids. Three of the 4 species (*Alkanindiges hongkongensis*, *Gardnerella vaginalis*, and *Dialister invisus*) remained significantly associated with asthma in models adjusted for inhaled corticosteroids (Table E1).

qPCR validation of nasal species differentially abundant in asthma

To validate the species we identified as differentially abundant in asthma from our 16S rRNA-based microbiome analyses, we used qPCR to quantify the presence of *Gardnerella vaginalis*, *Prevotella buccalis*, and *Dialister invisus* in a subset of our cohort; there were no qPCR kits available for *Alkanindiges hongkongensis*, a recently identified species. Fold changes of

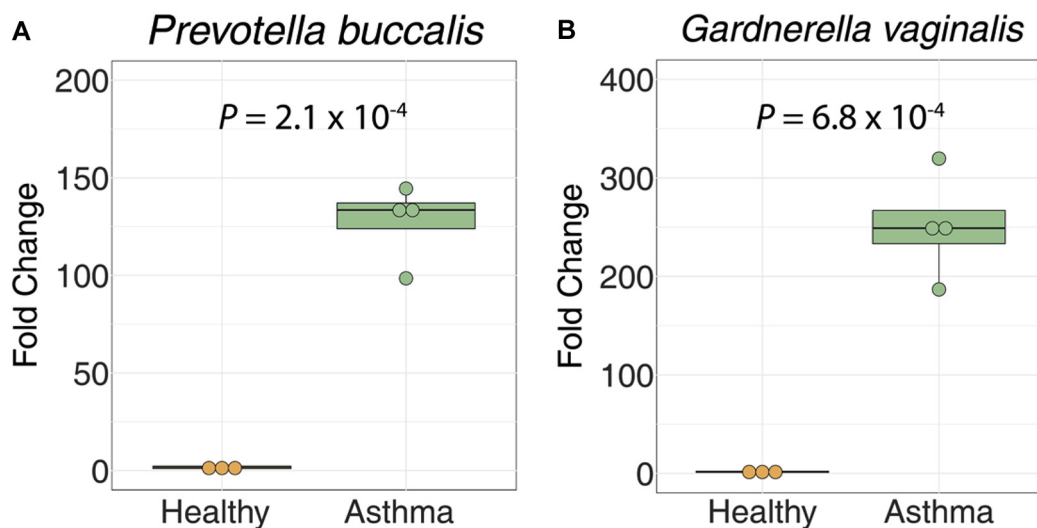


FIG 5. qPCR validation of *Prevotella buccalis* (**A**) and *Gardnerella vaginalis* (**B**) overrepresentation in subjects with asthma relative to controls. Pan bacteria, a measure of total bacterial load, was used as a “reference gene” to normalize each sample. *t* test was used to assess difference in fold change between asthmatic and healthy samples.

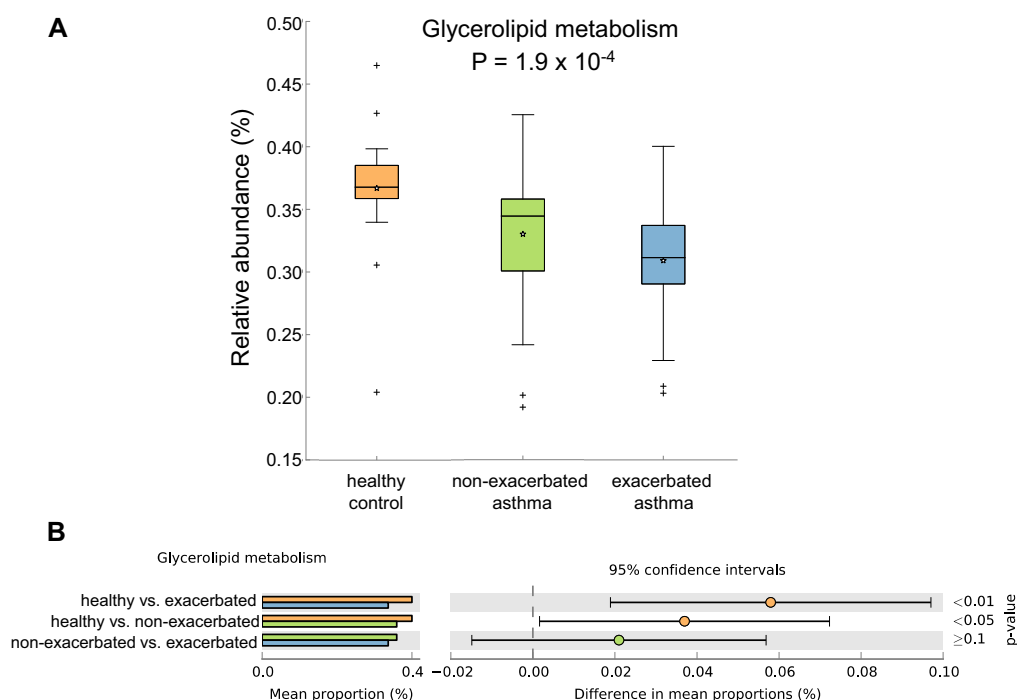


FIG 6. Glycerolipid metabolism associated with asthma activity. **A**, Metagenomic inference showed that glycerolipid metabolism was significantly different between subjects with exacerbated asthma, nonexacerbated asthma, and healthy controls. **B**, Pairwise comparison showed that this pathway was significantly decreased in subjects with exacerbated asthma versus healthy controls, as well as in subjects with nonexacerbated asthma versus healthy controls.

qPCR abundances in asthmatic versus healthy subjects for *Prevotella buccalis* and *Gardnerella vaginalis* are shown in Fig 5. We measured a 130-fold increase for *Prevotella buccalis* (*t* test, $P = 2.1 \times 10^{-4}$) (Fig 5, A) and 160-fold increase for *Gardnerella vaginalis* (*t* test, $P = 6.8 \times 10^{-4}$) (Fig 5, B) in nasal samples from these asthmatic versus control subjects,

demonstrating consistency with the overrepresentation of these species in asthmatic subjects based on 16S rRNA-based analyses. For *Dialister invisus*, Qiagen’s proprietary primers yielded indeterminate results on multiple attempts; sequence alignment of their targeted strain to the species sequence from our library revealed poor alignment.

TABLE II. Studies of the nasal microbiome in asthma

Reference	Subjects	Age group	Major findings
Hilty et al, 2010 ⁴	N = 24 (11 asthma, 5 COPD, 8 control subjects)	Adult	Nasal microbiota distinct from oropharynx and left upper lobe microbiota. Nasal samples in general characterized by <i>Actinobacteria</i> and <i>Firmicutes</i> .
Castro-Nallar et al, 2015 ¹⁴	N = 14 (8 asthma, 6 control subjects)	Children	<i>Moraxella catarrhalis</i> , <i>Escherichia coli</i> , and <i>Psychrobacter</i> overrepresented in asthmatic vs control subjects
Depner et al, 2017 ¹⁶	N = 68 (39 asthma, 29 control subjects)	Children	Lower alpha and beta diversity in asthmatic vs control subjects. Higher relative abundance of phylum <i>Proteobacteria</i> in children with asthma. Asthma associated with OTU in <i>Moraxella</i> in nonfarming children but not farming children. Throat samples also studied but no difference between cases and controls.
Perez-Losada et al, 2017 ¹⁵	N = 40 (40 asthma subjects)	Children	No significant differences in alpha and beta diversity observed between seasons. Interpatient variation and variation based on time.
Fazlollahi et al, (this study)	N = 72 (20 exacerbated asthma, 31 nonexacerbated asthma, 21 control subjects)	Adult (primarily)	Nasal microbial composition significantly different among subjects with exacerbated asthma, nonexacerbated asthma, and controls. Taxa from <i>Bacteroidetes</i> and <i>Proteobacteria</i> enriched in those with asthma. Four species differentially abundant after correction for multiple comparisons, with qPCR confirmation of major findings.

Reduced glycerolipid metabolism associated with asthma activity

To infer metabolic pathways associated with the nasal taxa identified as differentially abundant based on asthma activity, we used PiCRUST²⁹ and STAMP³⁰ to map microbial genes to metabolic databases to infer microbial functions differentially expressed by asthma activity (ie, metagenomic prediction). Glycerolipid metabolism was the metabolic pathway that was differentially abundant (Kruskal-Wallis, $P = 1.9 \times 10^{-4}$) across the study groups after correction for multiple testing at false discovery rate (FDR) ≤ 0.10 (Fig 6, A). Relative to healthy controls, glycerolipid metabolism was reduced in subjects with nonexacerbated asthma and further reduced in subjects with exacerbated asthma (Fig 6, B).

DISCUSSION

In this study of the nasal microbiome in asthma, we found differences in nasal microbial composition among subjects with exacerbated asthma, nonexacerbated asthma, and healthy controls. Taxa from *Proteobacteria* and *Bacteroidetes* were enriched in subjects with exacerbated and nonexacerbated asthma relative to healthy controls. Our study identified 4 species belonging to these and other phyla that were differentially abundant based on asthma activity after correction for multiple testing, with qPCR confirming our major results. Metagenomic prediction identified glycerolipid metabolism as the metabolic pathway differentially represented by these implicated taxa.

Our study fills an important niche in airway microbiome research, which has been dominated by studies of the lower airway.⁴⁻¹³ Targeted examination of the nasal microbiome in asthma is worthwhile given potential roles for upper airway microbiota in asthma pathogenesis and disease course. For example, prior studies report associations between hypopharyngeal and nasopharyngeal bacterial species in the development of childhood wheeze.^{17,34} A culture-based study of neonates showed that hypopharyngeal colonization with *Haemophilus influenza* (phylum *Proteobacteria*), *Moraxella catarrhalis* (phylum *Proteobacteria*), and/or *Streptococcus*

pneumoniae (phylum *Firmicutes*) was associated with wheeze outcomes during the first 5 years of life.³⁴ Although the cellular impact of these bacterial colonizations were not directly examined, the investigators suggested that these associations were consistent with predominantly neutrophilic inflammation in young children with severe recurrent wheeze.³⁴ In line with this, Teo et al¹⁷ examined nasopharyngeal aspirates from an infant cohort via 16S rRNA sequencing, finding that transient abundances of *Haemophilus*, *Moraxella*, or *Streptococcus* were associated with virus-associated acute respiratory infections, and *Streptococcus* colonization was associated with atopy by age 2 years and chronic wheeze at age 5. Teo et al¹⁷ proposed that bacterial-viral interactions were the mechanisms underlying these findings. These studies on potential factors for asthma pathogenesis focused on the relationship between infant hypopharyngeal and nasopharyngeal aspirates and childhood wheeze. In contrast, we sought to characterize nasal microbiota associated with asthma activity in older subjects with established disease, as this could affect our understanding of asthma pathobiology and potential biomarkers and treatments for populations with established asthma. While the associations between gut microbiota and asthma have been studied more extensively, likely facilitated by ease of sampling access, it is very likely that airway microbiota have an equal if not greater role to play in established asthma given their immediate proximity to the site of disease.^{35,36} Multiple studies support immune-modulating interactions between nasal bacteria and viruses known to exacerbate established asthma (eg, respiratory syncytial virus, rhinovirus).³⁶⁻⁴²

We found differential enrichment of *Prevotella* (phylum *Bacteroidetes*), *Alkanindiges* (phylum *Proteobacteria*), and *Gardnerella* (phylum *Actinobacteria*) in subjects with exacerbated asthma, while *Dialister* (phylum *Firmicutes*) was significantly more abundant in subjects with nonexacerbated asthma. Given very few and limited studies of the nasal microbiome in asthma to date (Table II), it is difficult to find fair comparisons for our study. While Hilty et al⁴ sequenced 16S rRNA PCR products from nasal samples obtained from adult subjects with asthma (n = 11) and chronic obstructive pulmonary

disease ($n = 5$) and from controls ($n = 8$) as part of their study, they did not report on direct comparisons between the nasal microbiota of asthmatic versus control subjects. Their comparison of aggregated nasal samples to those from the oropharynx and left upper lobe showed that nasal specimens overall were characterized by *Actinobacteria* and *Firmicutes*.⁴ Other studies of the nasal microbiome in asthma have been of children, which may not provide optimal comparison given known changes to the microbiome between early childhood and later life.⁴³ That said, consistent with some of our findings, Depner et al¹⁶ found a higher abundance of genera from *Proteobacteria* in 39 children with asthma versus 29 controls. Perez-Losada et al¹⁵ studied nasopharyngeal microbiota in 40 children with asthma over 2 time points, finding no significant differences in alpha and beta diversity between seasons. As there were no controls nor measures of asthma activity, comparison of their findings to ours is challenging. Finally, Castro-Nallar et al¹⁴ used whole metagenomic shotgun RNA sequencing to characterize nasal brushings from a small study of 8 subjects with asthma and 6 controls, finding enrichment of *Proteobacteria* species (consistent with our findings) but underrepresentation of *Firmicutes* species in those with asthma.

While there is consistency at the phylum level between the results from our study and other examinations of nasal microbiota in asthma, we identified particular species associated with asthma activity: *Prevotella buccalis*, *Dialister invisus*, *Gardnerella vaginalis*, *Alkanindiges hongkongensis*. These species-level results are distinct from any previously reported. Not all studies report at the same taxonomic level, which is a barrier to cross-study comparisons.¹ The impact of microbiota on host is likely strain-specific.⁴⁴ However this is counterbalanced by the limited resolution of 16S rRNA sequencing to identify strains.¹ We have therefore reported our results at the most specific level (ie, species) possible for a 16S rRNA-based study. Furthermore, we used species-specific qPCR to validate our 16S rRNA-based findings of enrichment of *Prevotella buccalis* and *Gardnerella vaginalis* in subjects with asthma, thus lending confidence to our results. We note that no other study of nasal microbiome in asthma has taken this additional step of qPCR to corroborate 16S rRNA-based results.

Some information is known about the nasal bacterial species we identified as associated with asthma activity. *Prevotella* species are common bacteria at mucosal sites.⁴⁵ These gram-negative anaerobic species have been linked to several localized and systemic inflammatory diseases and represent the predominant genus in the respiratory system.⁴⁵ *Prevotella* spp abundance is associated with increased T_H17-mediated mucosal inflammation.⁴⁵ They activate Toll-like receptor 2, leading to production of IL-23 and IL-1 by antigen presentation cells and stimulation of epithelial cells to produce IL-8, IL-6, and chemokine ligand-20.⁴⁵ To our knowledge, an association between *Prevotella buccalis* in particular and asthma has not been previously reported. *Gardnerella vaginalis* is a facultative anaerobic gram-variable rod that is best known for its role in vaginosis, although it has also been detected in extravaginal sites including the lung and pleura,^{46,47} blood,⁴⁶ and skin.⁴⁶ We found its association with asthma activity in our study to be unexpected, but our corroboration of this result by qPCR supports the finding. *Dialister invisus* is a gram-negative coccobacillus first described in 2003 in endodontic infections⁴⁸ and the genitourinary tract.⁴⁹ More recently, taxa from *Dialister* have been reported in the

respiratory system, including in the lungs of patients with pneumonia⁵⁰ and sputum from cystic fibrosis patients.⁵¹ *Dialister* has also been observed in the gut microbiome of children studied for food allergen sensitization.⁵² Among the 4 biomarker species our study identified, the least is known about *Alkanindiges hongkongensis*, a recently described aerobic gram-negative coccobacillus present in the oral cavity.⁵³

The results of our metagenomic functional prediction showed that bacterial functional pathways related to glycerolipid metabolism were significantly reduced in subjects with exacerbated and nonexacerbated asthma relative to healthy controls. As metabolism encompasses anabolism and catabolism, it is difficult to interpret the specific direction of this inferred effect on glycerolipid anabolism or catabolism, but our finding of *differential* activity varying by asthma status is consistent with recognized roles of differential lipid mediator activity in asthma and inflammation.^{54,55} Bioactive lipids such as platelet activating factor, leukotrienes, prostanoids, and sphingolipids are involved in cell signaling that leads to bronchoconstriction and inflammation (degranulation, chemotaxis, allergen) in asthmatic subjects.^{54,55} Our finding of differential glycerolipid metabolism is also consistent with study of global plasma metabolomic profiling of 20 asthmatic versus 10 control subjects, where asthmatic subjects demonstrated increases in glycerolipid metabolites, long-chain fatty acid arachidonate, and medium-chain fatty acids.⁵⁶ Kinjo et al⁵⁷ have posited that cell-membrane glycosphingolipids, which are recognized by and can activate invariant natural killer T cells, are involved in the induction of IL-4 and IL-13.

While our genus and species level findings of the nasal microbiome in asthma highlight taxa distinct from those reported for the lower airway in asthma, there are commonalities when more broadly comparing phyla represented in our nasal and others' lower airway studies of asthma. As we found greater abundance of *Alkanindiges* (phylum *Proteobacteria*) in the nasal microbiome with greater asthma activity, Hilty et al,⁴ Huang et al,⁵ and Faner et al¹³ observed higher proportions of *Proteobacteria* in bronchial samples from asthmatic subjects. Greater bacterial diversity and high *Proteobacteria* representation were also observed when comparing 16S rRNA sequenced from induced sputum samples from asthmatic versus nonasthmatic subjects.^{7,11} Zhang et al¹¹ observed increased taxa from *Firmicutes* in induced sputum from severe asthmatic versus control subjects, consistent with our observation of greater *Dialister* (phylum *Firmicutes*) in exacerbated and nonexacerbated asthmatic versus control subjects. Interestingly, although we found that *Prevotella* in the nasal passages was associated with asthma activity, a group that studied airway and upper gastrointestinal sites in healthy subjects concluded that the lower airway selectively eliminates *Prevotella* derived from the upper airways in healthy subjects.⁵⁸

We recognize the limitations of our study. First, while our study represents the largest study of the nasal microbiome in asthma to date and identifies nasal bacterial taxa associated with asthma status and asthma activity, its sample size is relatively smaller than some microbiome studies of other body sites (eg, gut) and other diseases, and determination of causality (including the possibility of reverse causation) is limited by its cross-sectional design. A longitudinal study of nasal microbiome changes over time in a larger sample of subjects with asthma could be a next step. Second, it is possible that nasal microbiota are affected by asthma medication and antibiotics use, which differed by study

group. Separating the potential effect of treatment has been a constant challenge for all human studies of asthma given the impracticality and ethics of requiring discontinuation of treatment for study participation, particularly in exacerbated asthmatic subjects. However, we found no significant associations between nasal microbiome composition and use of nasal steroids, inhaled steroids, systemic steroids, antihistamines, or antibiotics in this study. Third, we identified 4 species in particular that were differentially abundant based on asthma activity, validating *Prevotella buccalis* and *Gardnerella vaginalis* by qPCR. Unfortunately, there was poor alignment between the *Dialister invisus* strain targeted by its available qPCR kit and species sequences from our library, thus yielding indeterminate validation results for this species, and validated primers for *Alkanindiges hongkongensis* are not currently available due to its relatively recent identification. Last, we used bioinformatic approaches to infer metabolic pathways affected by the nasal bacteria differentially abundant in asthma; future work could include direct metabolite profiling to corroborate these inferences.

In summary, we found differences in nasal microbial composition among subjects with exacerbated asthma, nonexacerbated asthma, and healthy controls, with qPCR confirming our major species-specific results. Our work adds one of the largest studies to an emerging literature on the nasal microbiome in asthma. Given possible roles for upper airway microbiota in asthma pathobiology, and ease of nasal sampling for monitoring and investigation of asthma, the nasal microbiome in asthma is a compelling area of research to continue to expand.

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Key messages

- Although the lower airway microbiome has been frequently studied in asthma, the nasal microbiome in asthma has received far less attention.
- We profiled the nasal microbiome of subjects with exacerbated asthma, nonexacerbated asthma, and healthy controls, identifying bacterial taxa that marked each study group.
- Our study fills an important niche in airway microbiome research given possible roles for upper airway microbiota in asthma pathobiology and ease of nasal sampling for monitoring and investigation of asthma.

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Phylum	Healthy controls (n=21)	Non-exacerbated asthma (n=31)	Exacerbated asthma (n=20)	P-value*
Proteobacteria	10.1%	19.4%	17.5%	0.05
Fusobacteria	0.4%	0.7%	0.6%	0.56
Firmicutes	48.7%	44.2%	39.6%	0.39
Others	0.8%	0.6%	0.6%	0.14
Bacteroidetes	1.0%	3.0%	3.5%	9.7x10 ⁻³
Actinobacteria	39.0%	32.1%	38.2%	0.18

*Kruskal-wallis test

FIG E1. Phylum representation of nasal microbiota in exacerbated asthmatic, nonexacerbated asthmatic, and healthy control subjects.

TABLE E1. *P* values from multivariate analyses of OTU abundances and asthma status

	Univariate model	Model adjusted for nasal steroid use	Model adjusted for inhaled steroid use	Model adjusted for systemic steroid use
<i>Prevotella buccalis</i>	1.0×10^{-2}	2.4×10^{-2}	1.8×10^{-1}	1.2×10^{-2}
<i>Dialister invisus</i>	9.1×10^{-3}	1.3×10^{-2}	1.2×10^{-2}	2.7×10^{-2}
<i>Gardnerella vaginalis</i>	2.8×10^{-3}	4.1×10^{-3}	1.7×10^{-2}	2.8×10^{-3}
<i>Alkanindiges hongkongensis</i>	2.6×10^{-3}	8.9×10^{-4}	4.2×10^{-3}	1.2×10^{-2}

Values with $P \leq .05$ are in boldface.