Gut microbiome signature and nasal lavage inflammatory markers in young people with asthma

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Background: Asthma is a complex disease and a severe global public health problem resulting from interactions between genetic background and environmental exposures. It has been suggested that gut microbiota may be related to asthma development; however, such relationships needs further investigation.

Objective: This study aimed to characterize the gut microbiota as well as the nasal lavage cytokine profile of asthmatic and nonasthmatic individuals.

Methods: Stool and nasal lavage samples were collected from 29 children and adolescents with type 2 asthma and 28 children without asthma in Brazil. Amplicon sequencing of the stool bacterial V4 region of the 16S rRNA gene was performed using Illumina MiSeq. Microbiota analysis was performed by QIIME 2 and PICRUSt2. Type 2 asthma phenotype was characterized by high sputum eosinophil counts and positive skin prick tests for house dust mite, cockroach, and/or cat or dog dander. The nasal immune marker profile was assessed using a customized multiplex panel.

Results: Stool microbiota differed significantly between asthmatic and nonasthmatic participants ($P = .001$). *Bacteroides* was more abundant in participants with asthma ($P < .05$), while *Prevotella* was more abundant in nonasthmatic individuals ($P < .05$). In people with asthma, the relative abundance of *Bacteroides* correlated with IL-4 concentration in nasal lavage samples. Inference of microbiota functional capacity identified differential fatty acid biosynthesis in asthmatic compared to nonasthmatic subjects.

Conclusion: The stool microbiota differed between asthmatic and nonasthmatic young people in Brazil. Asthma was associated with higher *Bacteroides* levels, which correlated with nasal IL-4 concentration. (J Allergy Clin Immunol Global 2024;3:100242.)

Key words: *Asthma, microbiome, stool, gut microbiota*

Asthma is a serious global public health problem that affects 339 million people.[1] Its prevalence, severity, and mortality vary across the world.[2] However, the disease has multiple clinical phenotypes and pathophysiologic characteristics[3] and is a result of complex interactions between genetic factors, immunologic status, and environmental exposure.[4]

Type 2 (T2) asthma is the most studied asthma endotype, characterized by inflammation with eosinophils, including the expression of cytokines such as IL-4, IL-5, and IL-13.[5] These cytokines may be secreted by T_H2 CD4 lymphocytes or T2 innate lymphoid cells.[5-7] T2-high asthma also involves increased activation of dendritic cells and B cells, mediated by an IgE-dependent mechanism.[8] These biomarkers are hypothesized to contribute to the establishment and persistence of T2 asthma.

Evidence suggests that early-life environmental exposures can shape the composition of the gut microbiome, which can modulate the development of immune function, play a role in disease causation, and provide protection through mechanisms such as attenuation of allergic sensitization.[9] The immune system of the gut mucosa represents the major immune component in vertebrates, working in close collaboration with the intestinal microbiome, with which it interacts to achieve intestinal homeostasis.[10] The epithelium controls the local immunologic activities of IgA, defensins, and lysozymes, which are also regulated by the production of IL-25, IL-33, and thymic stromal lymphopoietin, which in turn stimulate T2 inflammation, which is classically known to support the development of asthma.[11]

Microbial dysbiosis has been associated with lung disorders and respiratory infections[12] and can be caused by many factors related to lifestyle and environmental exposure.[13] Studies have suggested the role of gut microbiota on asthma development is

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Abbreviations used
KEGG: Kyoto Encyclopedia of Genes and Genomes (genome.jp/kegg)
SCFA: Short-chain fatty acid
SPT: Skin prick test
T2: Type 2

related to an imbalance in microbiota composition,[12] and others have shown that the composition of gut microbes differs considerably between asthmatic and nonasthmatic subjects;[14] in addition, asthma severity is linked to changing gut microbiome composition.[15]

The gut–lung relationship has been studied predominately in European and North American people with asthma. The characteristics of the gut microbiota and its role in T2 asthma in low- and middle-income country settings is poorly understood. The ability to understand pathophysiologic mechanisms related to T2 asthma, as well as geographical variation, is crucial for the development and appropriate evaluation of novel therapies.

In this study, we aimed to characterize the gut microbiota from T2 asthmatic and nonasthmatic subjects from Brazil and investigate the association with asthma status and cytokine production in nasal lavage samples.

METHODS

Study population

Fifty-seven Brazilian adolescents and young adults were selected for the present study on the bases of asthma diagnosis, presence of eosinophils in respiratory secretions, and skin prick test (SPT) for common aeroallergens. Twenty-nine T2 asthma cases (asthma group) occured in subjects with an asthma diagnosis, presence of eosinophils in sputum of ≥2.5%, and positive SPT result for at least one allergen. The nonasthma group comprised 28 control subjects without an asthma diagnosis. The inclusion and exclusion criteria are described in Table I. The project was approved by the Brazilian National Research Ethics Council (approval 47840415.3.0000.5030).

Recruitment and sample collection of the selected individuals were carried out at the reference outpatient clinic for severe asthma in the city of Salvador, Brazil, the ProAR (Programa para o Controle da Asma na Bahia).[16] The recruitment was done by applying questionnaires based on the International Study of Asthma and Allergies in Childhood (ISAAC) phase 2 (asthma management) and phase 3 (environmental risk factors) modules, with additional validated questions about the clinical severity of asthma.[17] Written informed consent was obtained from each participant or child's legal guardian.

Sputum induction

To characterize the eosinophilic asthma phenotype, sputum induction was conducted according to a protocol previously used by our team.[18,19] Individuals were pretreated with 400 mg of inhaled salbutamol by inhalation; on average, 100 μL of induced sputum was collected. A differential count of 200 nonsquamous cells was performed using an optical microscope. An eosinophilic sputum inflammatory phenotype was defined as having eosinophils of ≥2.5%.[19]

Skin prick test

To define atopic asthma, all patients with asthma and controls underwent SPTs against house dust mites (*Dermatophagoides pteronyssinus* and *Blomia tropicalis*), cockroaches (*Blattella germanica* and *Periplaneta americana*), and cat and dog dander. Histamine and saline were used as positive and negative controls, respectively. The diameter of the wheals was measured after 15 minutes. Results were considered positive if the mean of the largest perpendicular diameter was at least 3 mm larger than the negative control.

Nasal lavage and cytokine measurement

Nasal lavage was performed using nasal atomizers (MAD Nasal) by instilling 5 mL of sterile saline solution (0.9% NaCl) into each nostril, with the individual's head tilted 30 degrees backward.[20] After 10 seconds, a sample of at least 7 mL (considering 2 nostrils) was collected and stored in a sterile conical tube and frozen at −80°C until use. Cytokines were measured in the supernatants of the nasal lavage samples, as follows.

For the analysis of inflammatory and anti-inflammatory cytokines, a customized multiplex panel (ProcartaPlex Custom Assay Kit, 9 PPX-1246 Invitrogen Plex) for the Luminex MAGPIX (Life Technologies) instrument was used. The panel allowed us to measure the levels of various cytokines, including inflammatory IL-4 (11.47/47,000 pg/mL), IL-5 (5.37/22,000 pg/mL), IL-8 (2.18/8,950 pg/mL), IL-13 (2.46/10,100 pg/mL), and IL-17A (1.86/7,650 pg/mL), as well as the anti-inflammatory cytokine IL-10 (1.50/6,150 pg/mL), in supernatants of nasal lavage.

Stool sample collection

To perform sequencing of the gut microbiome, stool samples were collected during participant visits using a commercial sterile collector. Stool DNA was extracted and purified according to the protocol of the Human Microbiome Project,[21] which uses the PowerSoil kit protocol (Qiagen).

16S rRNA amplicon sequencing

The 16S rRNA gene amplicon library preparation and sequencing were conducted following the Earth Microbiome Project protocol.[22] Briefly, PCR amplification of the extracted DNA was performed for 30 cycles targeting the V4 hypervariable 16S region using universal primers 515F (GTGCCAGCM GCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTA AT), with a Golay barcode on the forward primer. The sequencing was performed using the Illumina MiSeq Reagent Kit v2 (500 cycles) on an Illumina MiSeq platform in the Genomics and Microarray Core of the University of Colorado Anschutz Medical Campus. A total of 57 paired-end read samples were generated by the end of the sequencing runs. Raw files and associated metadata are available at the Sequence Read Archive (SRA) under accession number PRJNA950484 (www.ncbi.nlm.nih.gov/sra/PRJNA950484).

Bioinformatic analysis

Bioinformatic analyses and sample quality checks were performed using QIIME 2 (v2021.4) software.[23] Using the q2-demux plugin, raw sequence data were demultiplexed and quality filtered before being denoised with DADA2 through q2-dada2.[24] To construct

TABLE I. Inclusion and exclusion criteria

Criteria	Patient	Control
Inclusion		
	• Asthma symptoms and/or receipt of asthma medications in last 12 months.	• No past or current history of asthma.
	• Must not have received cromolyn, fast-acting β-agonists, or ipratropium bromide 6 hours before test.	
	• Must not have received theophylline 12 hours before test.	
	• Must not have received long-acting β-agonists 24 hours before test.	
	• No receipt of antihistamines 48 hours before test.	
Exclusion		
	• Acute exacerbation of asthma.	• Receipt of antihistamines 48 hours before test.
	• FEV_1 < 75% (of reference value).	• Any infection detected in last 4 weeks.
	• Any infection detected in last 4 weeks.	• Any chronic illness (including asthma).
	• Other chronic diseases (in addition to asthma).	• Pregnancy.
	• Pregnancy.	

FEV_1, Forced expiratory volume in 1 second.

phylogenetic trees, a fragment insertion tree was created using the q2-fragment-insertion plugin.[25] The q2-feature-classifier[26] classify-sklearn naive Bayes taxonomy classifier was used to classify amplicon sequence variants against the Greengenes 13_8_99% OTU reference sequences set trimmed to 250 bp of the V4 hypervariable region (corresponding to the 515F-806R primers).[27]

To test if the microbial community structure and diversity were different between the groups, the α diversity (ie, observed features and Shannon index[28]) and β diversity (ie, principal coordinate analysis using Bray-Curtis distances) were calculated by R v4.1.0 statistical software (R Project; www.r-project.org) via the 'phyloseq' R package after samples were rarefied to 26,300 sequences per sample. To test if the potential function of the microbiota was different between groups, we used PICRUSt2[29] to predict pathway abundances of the Kyoto Encyclopedia of Genes and Genomes (KEGG; genome.jp/kegg) features.

Statistical analysis

Statistical analysis was conducted by R and STAMP v2.1.3.[30] Demographic data and clinical characteristics are expressed as means ± SDs for numerical variables, the differences between groups were evaluated by 1-way ANOVA, and the chi-square test was used for categorical variables. The Wilcoxon rank-sum test was used to analyze the α diversity, and the analysis of similarities (aka ANOSIM) was evaluated for β diversity between-group comparisons. Adjustments for multiple comparisons were made using the false discovery rate method. Only results meeting these criteria were deemed statistically significant. $P < .05$ was considered statistically significant. To identify which features contributed to the differences between groups, the similarity percentage analysis (aka SIMPER) was used. The correlation analysis between microbial composition and cytokines was performed by the Spearman correlation test.

TABLE II. Characteristics of study population by asthma status

Characteristic	Asthma status: Asthma (n = 29)	Asthma status: No asthma (n = 28)
Sex		
Female	18 (62.1)	19 (67.8)
Male	11 (37.9)	9 (32.2)
Ethnicity		
White	2 (6.9)	3 (11.1)
Black	26 (8962)	24 (85.7)
Asian	1 (3.4)	0
Age (years)	17.21 ± 2.66	19.36 ± 1.70
BMI (kg/m^2)	22.62 ± 4.19	22.91 ± 6.72
Height (m)	1.65 ± 0.08	1.62 ± 0.33
Weight (kg)	62.80 ± 15.82	65.81 ± 21.64
SPT positive for at least one allergen tested	29 (100)	18 (69.2)

Data are presented as nos. (%) or means ± SDs (continuous).
BMI, Body mass index.

RESULTS

Study population characteristics

Clinical parameters and demographic characteristics are shown in Table II. There were 18 female and 11 male participants with asthma, with a mean ± SD age of 17.21 ± 2.66 years. In comparison, nonasthmatic individuals comprised 19 female and 9 male subjects with a mean ± SD age of 19.36 ± 1.70 years. All subjects had a normal body mass index (18.5-24.9 kg/m^2), and most individuals had self-declared Black skin color.

Stool microbiota differs between those with and without asthma

Sequencing depth ranged from 26,318 to 108,737 reads per sample (mean = 50,931) (see Table E1 in the Online Repository

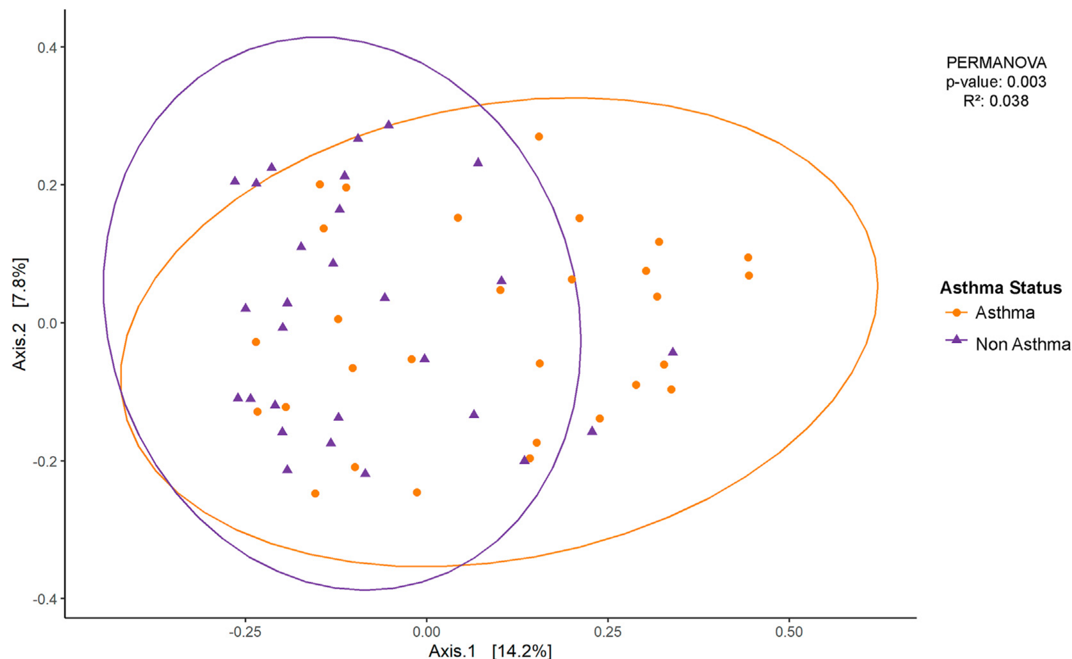

FIG 1. Differences between asthmatic and nonasthmatic groups by Bray-Curtis dissimilarity indices using principal coordinate analysis. Each dot represents 1 sample; corresponding group can be found in legend. There was significant difference in β diversity between both groups. Statistics were calculated by pairwise PERMANOVA.

at www.jaci-global.org); and the number of unique amplicon sequence variants was 2,436 after denoising with DADA2. After rarefying to 26,300 sequences per sample (see Fig E1 in the Online Repository), overall composition, taxonomic richness (number of unique amplicon sequence variants), and diversity (Shannon-Wiener index) were calculated. There was a divergence in the gut microbiota between asthmatic and nonasthmatic subjects (P = .01, R^2 = 0.038, PERMANOVA) (Fig 1).

Although not statistically significant, those with asthma had a lower richness (P = .144; see Tables E2 and E3 in the Online Repository at www.jaci-global.org) and diversity (P = .145, Table E3) of microbiota compared to nonasthmatic subjects (Fig 2).

A total of 15 phyla, 25 classes, 39 orders, 75 families, 165 genera, and 235 species were detected across all samples. The predominant phyla were largely consistent in both groups, but different relative abundances were observed (see Fig E2 in the Online Repository at www.jaci-global.org). When comparing the relative abundance of the top 10 (>2%) most common genera between asthmatic and nonasthmatic subjects, some differences were observed, with a higher abundance of *Bacteroides* in the asthma group and a higher abundance of *Prevotella* in the nonasthma group (Fig 3).

On the one hand, we found that the asthma group had an increased abundance of the genera *Bacteroides* (P = .007), *Parabacteroides* (P = .01), and *Phascolarctobacterium* compared to the nonasthma group. On the other hand, *Prevotella* had an increased abundance in the nonasthma group compared to the asthma group (Fig 4).

Patients with asthma had decreased IL-10 production in nasal lavage samples

A total of 24 asthmatic and 20 nonasthmatic patients had detectable levels of proinflammatory (IL-5, IL-8, and IL-17A) and anti-inflammatory (IL-10, IL-4, and IL-13) cytokines in nasal lavage fluid. Among the cytokines evaluated, the levels of IL-10 in nasal lavage samples were decreased compared to nonasthmatic subjects (P < .04) (Fig 5, *A*). No significant differences were found for IL-13, IL-17A, IL-4, IL-5, and IL-8 (Fig 5, *B-F,* respectively).

Bacteroides abundance is correlated with nasal IL-4 levels in asthma

Spearman correlation coefficients were estimated between the relative abundance of the significantly different genera (ie, *Bacteroides, Parabacteroides, Prevotella, Phascolarctobacterium,* and *Dialister*) and asthma and nonasthma groups with cytokine levels (Fig 6). There was a positive correlation between the presence of *Bacteroides* and IL-4 production (P = .04, r = 0.12) in people with asthma.

IL-10 is correlated with other cytokines in studied population

IL-10 exhibited correlations with the other cytokines within the study population. Fig 7 illustrates the correlations among the cytokines in both nonasthmatic individuals and those with asthma. There was a positive correlation between IL-10 and IL-13, IL-17A, IL-4, and IL-5.

Predicted metabolic and biosynthetic pathways are different between asthmatic and nonasthmatic subjects

The KEGG Pathway database is a collection of human produced pathway maps that represent the understanding of

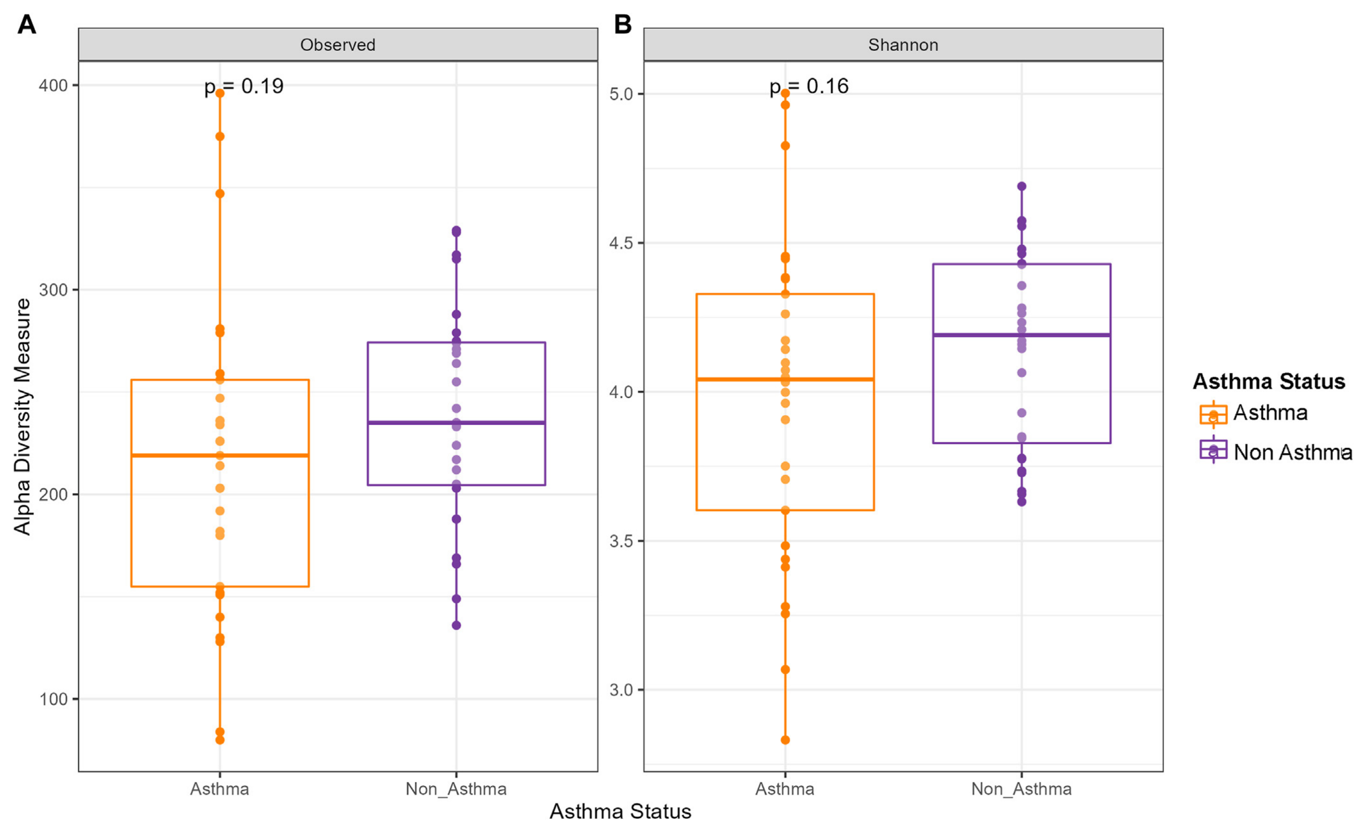

FIG 2. Comparison of gut microbiota α diversity between groups, including **(A)** species richness (represented by observed species) and **(B)** evenness (represented by Shannon). Asthmatic group had less bacterial diversity, richness, and evenness, although not statistically significant, compared to nonasthmatic group (Student *t* test $P > .05$, Table E3).

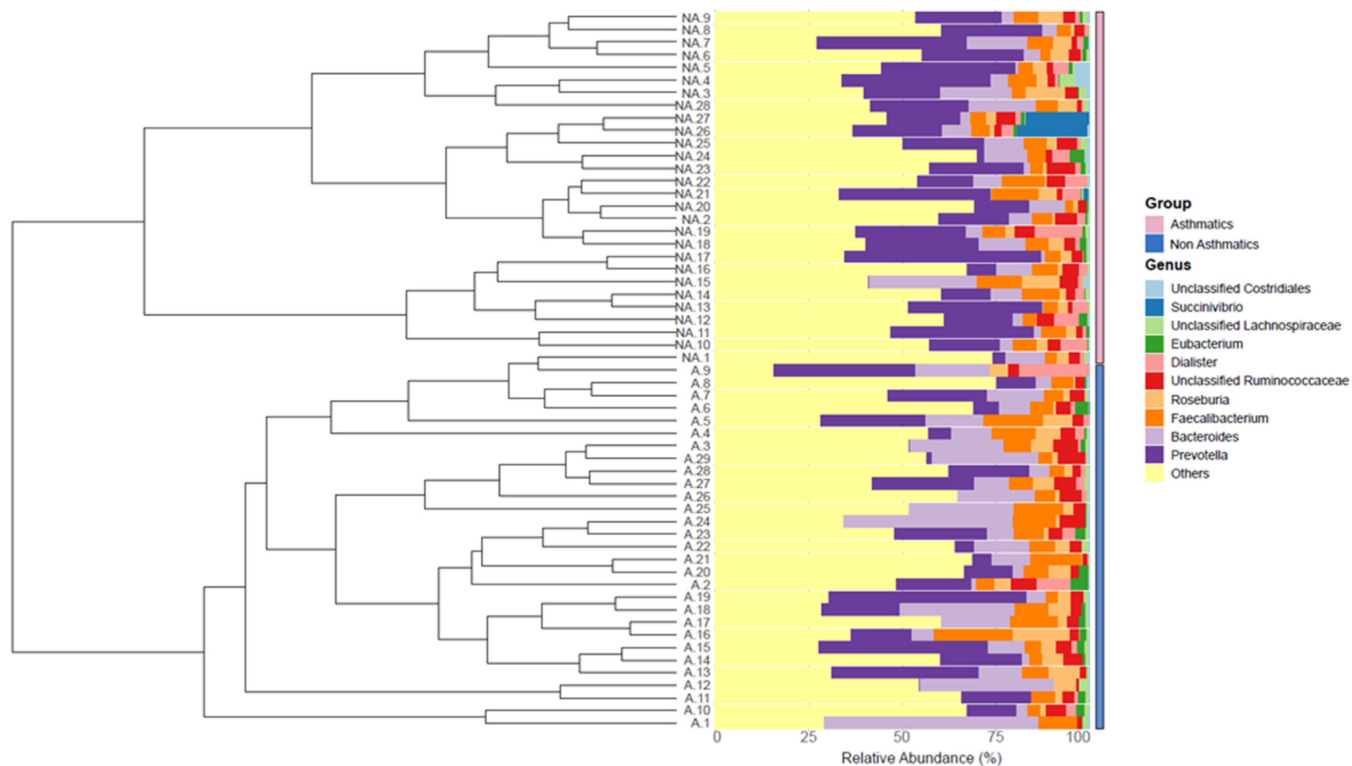

FIG 3. Relative abundance of 10 most abundant genera in asthmatic and nonasthmatic groups. Genera are shown in different colors; height of bars represents relative abundance.

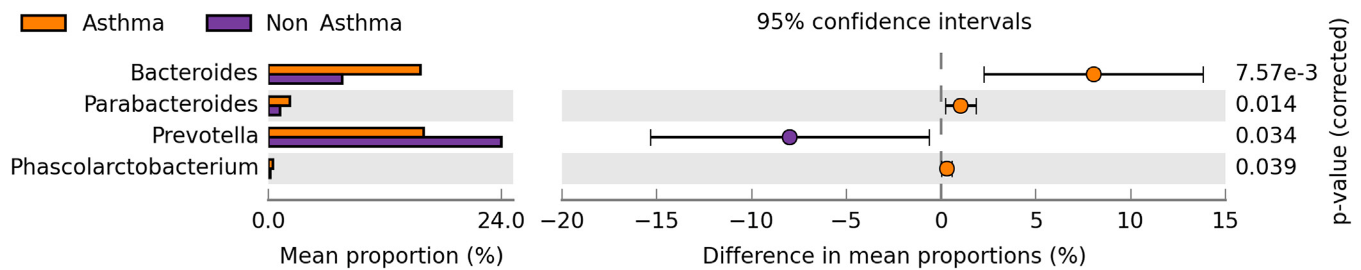

FIG 4. Extended bar plot for significantly different genera between groups showing mean proportion and difference in mean proportion. Asthmatic individuals are shown in *orange* and nonasthmatic individuals in *purple.* Error bars indicate SDs; corrected *P* values are indicated at right.

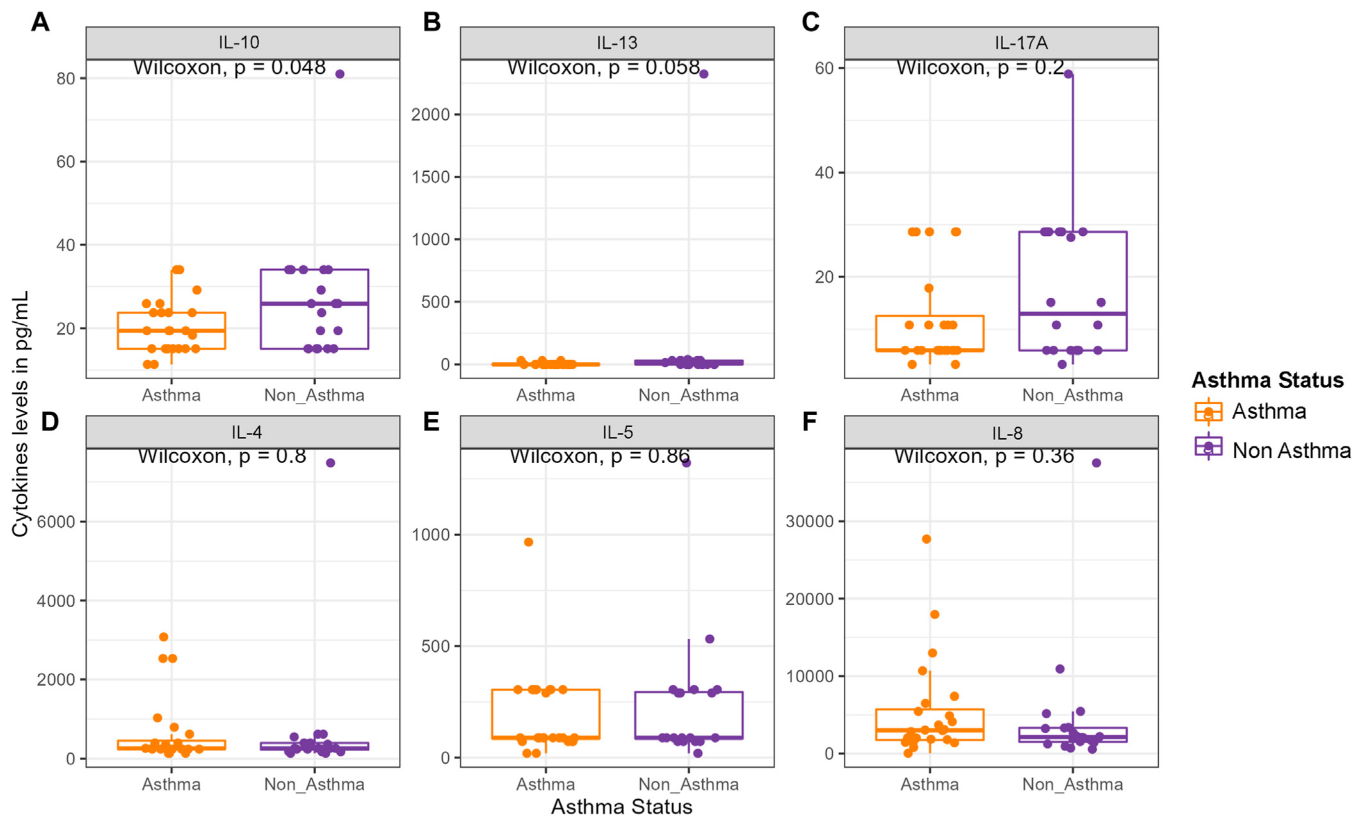

FIG 5. Cytokines IL-10 **(A)**, IL-13 **(B)**, IL-17A **(C)**, IL-4 **(D)**, IL-5 **(E)**, and IL-8 **(F)** levels of asthmatic patients quantified by Luminex in nasal lavage samples of 24 asthmatic patients and 20 nonasthmatic patients. Statistics were calculated by Mann-Whitney-Wilcoxon test.

molecular interactions. Inference of KEGG Pathway information from the taxonomic composition identified 13 metabolic pathways that differed between asthmatic and nonasthmatic subjects (Fig 8). Compared to the nonasthmatic group, fatty acid, monobactam biosynthesis, riboflavin, and purine metabolism were less abundant in the asthma group. Secondary bile acid, penicillin and cephalosporin, O-antigen nucleotide sugar, and steroid hormone biosynthesis were enriched in the asthma group. Lipoic acid, galactose, sphingolipid, glyoxylate, and dicarboxylate as well as amino sugar and nucleotide sugar metabolism were also less abundant in people with asthma. Table E3 summarizes the findings of the significant metabolic pathways and their association with asthma and allergy in other studies.

DISCUSSION

The current study used the 16S rRNA amplicon sequencing technique to assess stool bacteria composition in children and young asthmatic patients from Brazil. Our findings revealed that there were differences in the gut microbiota between groups. The β diversity indices indicated an overall significant difference between groups. This difference may be related to the distinct microbial contacts to which individuals are exposed, such as contact with greenery,[31] mode of delivery,[32] and diet.[12]

A rich diet in fermented foods increases microbial diversity and lowers several inflammation-related indicators.[33] Modification of intestinal metabolites and microorganisms is more likely to cause an impact.[34] The diet of the participants was greatly influenced by socioeconomic factors and regional dietary habits, with both the

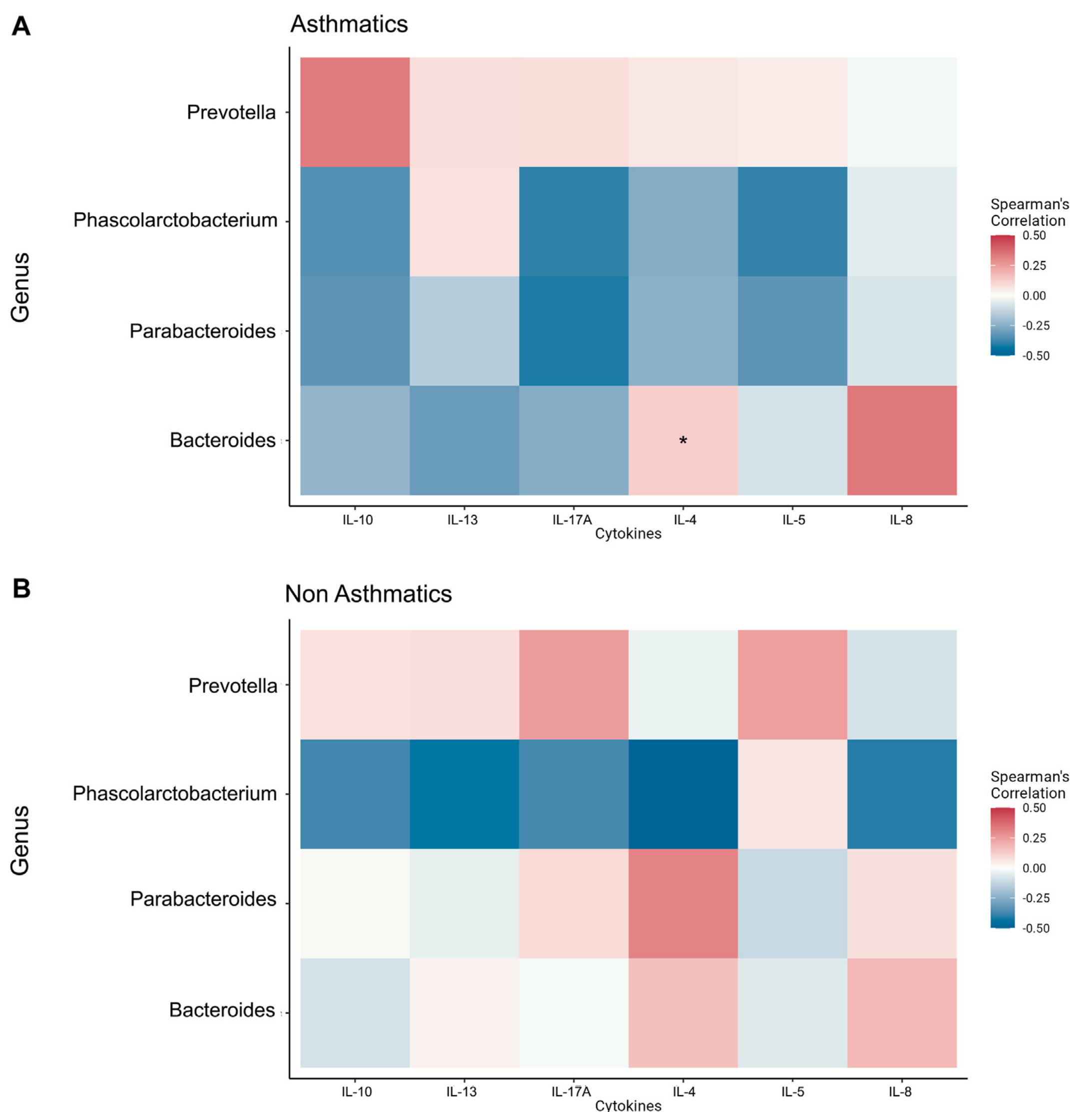

FIG 6. Correlations between asthma (n = 24) and nonasthma (n = 20) gut microbiome relative abundance of genera and nasal lavage concentrations of inflammatory cytokines. Color is according to Spearman coefficient distribution: *red* represents positive correlation; *blue,* negative correlation. **P* < .05.

case and control groups coming from the same geographic area and sharing similar dietary patterns. To provide a proxy for diet in our study population, we analyzed the weekly consumption of prevalent foods among the participants, which included beans, milk, goodies, and vegetables (see Fig E3 in the Online Repository at www.jaci-global.org).

Environmental variables evaluated, such as humidity, exposure to domestic animals, and tobacco use, in our population did not show significant differences in terms of microbiota profiling (data not shown).

It is well known that the gut microbiota plays several important roles in the development, regulation, and maintenance of healthy immune responses. In our study, we examined the microbiome profiles in the T2 asthma endotype and found a link between the gut microbiota and asthma-related immune responses. The results can help us gain a complete and comprehensive description of the bacterial community that can be associated with asthma in our population. In Brazil, a previous study has focused on the relationship between obesity and microbiome development in childhood in connection to delivery mode and socioeconomic class.[34] In addition, Melli et al[35] showed the relationship between the gut microbiota and atopic dermatitis in school-age children from Brazil; in accordance with our study, Bacteroidetes and Firmicutes were also found in all the samples examined.

Bacteroides was the most abundant genus among those with asthma. This genus has been implicated in the development of asthma through the production of short-chain fatty acids (SCFAs), which regulate several leukocyte functions, including production of cytokines (TNF-α, IL-2, IL-6, and IL-10).[36,37] Higher prevalence of *Bacteroides* was observed in the early microbiota of children who later developed allergies,[38] in people with asthma,[39] and in patients with atopic dermatitis.[40] T2 asthma is characterized by upregulation of the T_H2 cytokine profile and eosinophilic airway inflammation,[8] which we also observed in our study, where *Bacteroides* had a positive correlation with IL-4 production, increasing the T2 asthma risk through the activation of T_H2 cells

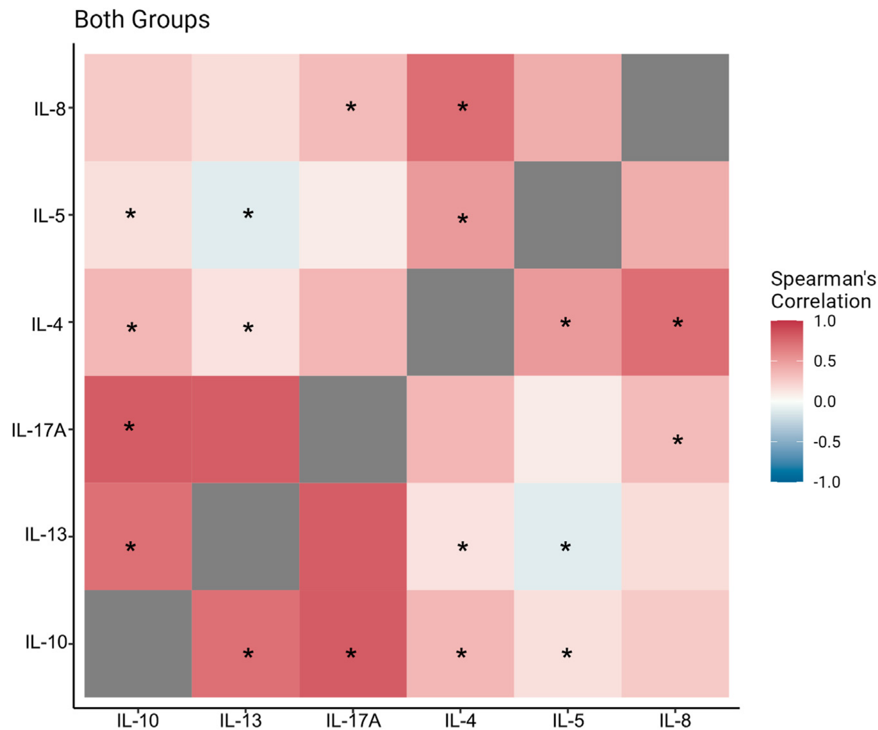

FIG 7. Correlations of nasal lavage cytokines levels with each other. Color is according to Spearman coefficient distribution: *red* represents positive correlation; *blue,* negative correlation. $*P < .05$.

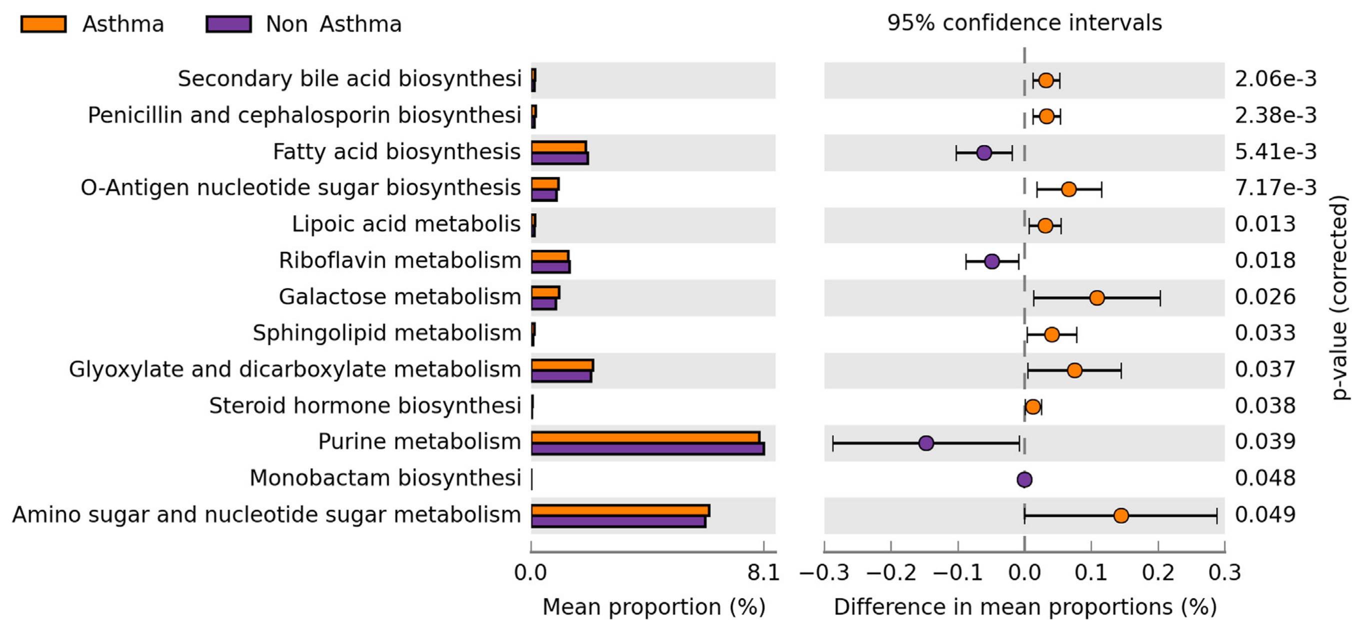

FIG 8. Prediction of differential pathways presented in asthmatic and nonasthmatic groups with PICRUSt2 analysis. Extended error bar plot for each pathway indicating differences in mean proportions for each pair of groups. Dot plots at right show differences in mean proportions between 2 indicated groups (asthma in *orange,* nonasthma in *purple*) using *P* values.

and their effector mechanisms. Our study is the first to observe a higher abundance of *Parabacteroides and Phascolarctobacterium* in T2 asthma.

In contrast, *Prevotella* was the most abundant genus among those without asthma. The abundance of the *Prevotella* genus was reduced in a study that evaluated the lung microbiota of patients with asthma and chronic obstructive pulmonary disease compared to healthy controls.[41,42] Larsen[41] reported that *Prevotella* has a capability of driving T_H17 immune responses *in vitro,* and increased abundances in this genus have been

associated with enhanced T_H17 response mediated by mucosal inflammation. Although the correlation between the relative abundance of *Prevotella* and the levels of IL-17A was not significant (Fig 5), it was possible to observe a higher production of this cytokine in people without asthma.

Our results have shown that people with asthma had decreased IL-10 production in nasal lavage samples. The powerful inflammatory response regulator IL-10 is essential for regulating allergic airway inflammation. According to previous reports, people with asthma had lower amounts of IL-10 in their bronchoalveolar lavage fluid and less IL-10 being secreted by their alveolar macrophages.[43] Previously, a lower production of IL-10 has been associated with increased T2 asthma risk.[44] We observed that the production of proinflammatory cytokines is positively correlated with the production of IL-10 in nonasthmatic individuals. This interaction demonstrates the importance of the production of these cytokines in modulating the immune system, ensuring an appropriate regulatory response.

IL-10 interacts directly with $CD4^+$ T cells and suppresses neutrophils, eosinophils, and mast cells in lungs; it also inhibits production of IL-4 and IL-5 by T_H2 cells.[45,46] As a result, IL-10 plays a crucial role in asthma and lung inflammation;[46] it has been shown that microbiota metabolites, such as SCFAs, can promote IL-10 production to maintain intestinal homeostasis.

Additionally, we have previously observed a link between childhood exposure to high levels of microbes and the development of regulatory mechanisms, leading to increased IL-10 production.[47] This information provides further context for the potential role of the gut microbiome in modulating immune responses in individuals with asthma. Moreover, using predicted pathway analysis, we found that fatty acid biosynthesis–related pathways were more common in people with asthma compared to people without asthma. Despite the caution required when interpreting 16S data to infer the metabolic composition of microbial communities, several studies show that fatty acid signaling plays an important role in the pathogenesis of asthma (Table E3).[48] For instance, SCFAs have been recognized for their important role in regulating the immune system by regulating host immune homeostasis. Specifically, they are crucial for promoting the development of regulatory T cells in the colon, which help to prevent an excessive T_H2 response that could potentially contribute to the development of allergic asthma. Previous studies have demonstrated that the *Bacteroides* and *Prevotella* genera are linked to the production of SCFAs.[49,50] Thus, additional studies should be conducted to further investigate this.

This study had a couple limitations. First, this was a cross-sectional study, so we cannot determine whether changes in the microbiota occurred before or after asthma onset. As a result, it is challenging to conclude a cause-and-effect relationship between microbiota changes and asthma development. Second, the sample size was small, so our findings may not adequately represent the entire population of interest. Third and last, we focused on T2 asthma, so we cannot generalize our findings to other phenotypes of the disease, leading to incomplete insights into overall disease complexity. The traditional understanding of asthma has evolved over time, and recent research has highlighted asthma's heterogeneity. Asthma is now recognized as a complex syndrome with diverse pathophysiologic mechanisms.[51]

Nevertheless, to our knowledge, our study is the first to evaluate the profile of intestinal microbiota, nasal lavage cytokine levels, and their relationship with T2 asthma phenotype in Brazilian children and young adults. Our data strongly suggest that the composition of microbes that colonize the gut can significantly influence chronic lung disorders such as asthma.

In conclusion, in young people from Brazil, there were observable differences in the stool microbiota between asthmatic and nonasthmatic subjects. Specifically, those who had asthma also had higher levels of *Bacteroides* present in their stool. Interestingly, this abundance of *Bacteroides* was positively correlated with nasal IL-4 concentration, indicating a potential association between gut microbiota and the immune system's response in individuals with asthma.

DISCLOSURE STATEMENT

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