Journal of Asthma and Allergy

Dovepress
Taylor & Francis Group

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ORIGINAL RESEARCH

Airway Microbiota Profiles in Children With and Without Asthma: A Comparative Study

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Background: Asthma is a common chronic respiratory disease that affects children and adults and can have a serious impact on their quality of life. Factors contributing to the development of asthma and related exacerbations are multifactorial, with microbial communities colonizing the airways possibly playing a key role.

Methods: The study included asthmatic (79) and healthy children (57) aged 5–16 years. Nasal and throat swabs were collected, and bacterial (16s rRNA) and fungal (18s rRNA) amplicon sequence analysis was performed. Diversity indices and the most abundant microbial genera were estimated accordingly.

Results: At the level of the bacteriome in the nasal samples, the asthma group had significantly lower diversity than the control group ($p = 0.02$). However, the microbiota of the asthma cohort was more evenly distributed, and *staphylococci* were enriched in the control group. Throat samples collected from the asthma cohort revealed significantly lower diversity ($p < 0.0001$), with a significant difference in species composition between the two groups ($p = 0.005$). Enriched bacterial species were different within the asthma subgroups (controlled vs uncontrolled asthma). The fungal microbiome of the nasal and throat samples showed no difference in species richness between the two groups, however, a significant difference in beta diversity (species composition) was detected. The nasal samples from the control group were enriched with *Malassezia* species, while the asthma cases were enriched with *Mucor* species. On the other hand, throat specimens of the asthma group were found to be enriched with *Candida* and *Saccharomyces*.

Conclusion: Our findings suggest that asthmatic samples were less diverse than the control samples with certain microbial genera enriching some study groups. Addressing the biomarkers that influence the progression of asthma could lead to improved care for children suffering from severe asthmatic episodes, possibly by including targeted therapies and prevention strategies.

Keywords: pediatrics asthma, 16s rRNA, 18s rRNA, DNA metabarcoding, microbiome, microbiota, mycobiome

Introduction

Asthma is recognized as one of the most common non-communicable diseases among children with a global burden of 262.41 cases and more death-related reports in low-income countries.[1] In Saudi Arabia, the estimated prevalence of pediatric asthma has been reported to range from 8% to 25%.[2]

The global increase in asthma prevalence and the disease's heterogeneous nature pose challenges for asthma diagnosis and management. Thus, a range of biomarkers have been studied as predictors of asthma severity, including IgE levels, eosinophil count, vitamin D and interleukins.[3] However, the surge of omics research in the past few years has shed light on microbiome dysbiosis and allergic disorders, with asthma frequently being studied as a disease model.[4]

Studies assessing microbial colonization during infancy and the stages of maturation and differentiation have shown that certain bacterial genera are more predominant in specimens collected from children with asthma or respiratory allergies.[5] Evidence suggests a possible link between microbial dysbiosis and a trigger in immune modulation, leading to potential exacerbation.[6]

Received: 30 September 2024
Accepted: 22 February 2025
Published: 5 March 2025

The microbial association with asthma progression may not be restricted to bacterial alteration; other microbial communities such as fungi and viruses could contribute to disease initiation and/or severity, but their role is poorly understood.[7]

Fungi are also known to be a pathological cause of atopy and asthma. A number of studies have described the potential link between asthma and an imbalanced mycobiome, but with a main focus on intestinal rather than airway samples.[8]

Microbial colonization and its relation to asthma development has been evaluated by examining multiple sites, ranging from superficial, easy-to-sample sites such as nose, nasopharynx, oropharynx and gut, to more invasive or difficult-to-collect specimens such as induced sputum or bronchoalveolar lavage (BAL), all revealed a microbial pattern that was altered in individuals with asthma.[9]

Studies have shown that asthma-related microbiome originates from the pharynx (naso/oropharyngeal path) or gut route.[9] While the nasal passage is directly connected to the lungs, the throat (oropharynx) acts as a transition zone between upper and lower respiratory tract. Thus, both sites have the potential to reflect on the microbial communities associated with asthma development. Additionally, swabbing the nose or throat is particularly useful due to its non-invasive nature and the high-level of patient acceptability, especially in pediatric studies.

Different practical magnitudes have also been used to assess potential microbial differences, both at a qualitative level (diversity and richness) and through a more in-depth functional analysis (ie, what do organisms do?), providing a more meaningful explanation of how microorganisms interact with each other and how this interaction modulates the immune reaction that eventually shapes signs and symptoms of asthma among affected children.[9]

In the present study, the aim was to detect the potential differences in the airway bacterial and fungal microbiota associated with asthma. Nasal and throat swabs were collected from children with asthma (controlled and uncontrolled asthma cases) and from a healthy control group, then microbiota profiles were evaluated.

Materials and Methods

Children Recruitment

Patient Inclusion Criteria

The study enrolled Saudi children with physician-diagnosed asthma (aged 5–16 years) with and without asthma exacerbations (controlled and uncontrolled). Controlled asthma was defined as non-urgent, physician-diagnosed asthma with symptoms and/or use of reliever medication less than twice/week, no nocturnal symptoms, and no exercise-induced symptoms. Uncontrolled, or poorly controlled, asthma is considered if the patient had, in the past 4 weeks, at least 3 of the following: self-reported day time symptoms of shortness of breath, or the requirement for the use of short acting bronchodilator, more than twice/week, exercise-induced symptoms, and/or nocturnal awakening due to asthma symptoms.[10] The diagnosis of asthma exacerbation is considered if the patient had worsening of asthma symptoms, including shortness of breath, wheezing, and cough, and the need for systemic corticosteroid therapy or hospitalization.[11] In this study, asthmatic patients with an exacerbation were considered under the category of uncontrolled asthma. Healthy controls without asthma or atopy were recruited and age- and sex-matched with the asthmatic participants.

Sample collection was conducted prospectively from February 2021 till September 2023. In patients with controlled asthma, samples were collected during follow-up visits at least 4 weeks after the resolution of the most recent symptoms of asthma exacerbation.

Exclusion Criteria

Individuals with no asthma but who had other diagnoses of pulmonary diseases that may resemble bronchial asthma in terms of symptoms and exacerbations were excluded from the study.

Sample Collection and Processing

From each asthma group and control group participants, two nasal swabs (right and left nostril) and one throat swab were collected and placed in a tube containing DNA–RNA shield (Zymo Research, USA). The tubes were stored at −80°C

until DNA extraction, which was performed using DNeasy *PowerSoil* Pro Kit with bead beating technology (Qiagen, Germany) following manufacturer recommendations.

Beside the swab collection, blood tests and clinical data were gathered for the asthmatic children during the clinic visit, therefore no blood test was done for the control group as their samples (only swabs) were collected randomly from the community.

16s and 18s Amplicon Sequencing and Filtering

16s ribosomal RNA (rRNA) amplification was conducted as described by Krogsgaard et al[12] using primers targeting the V3–V4 region (5'-ACTCCTAYGGGRBGCASCAG-3' and 5'-AGCGTGGACTACNNGGGTATCTAAT-3') of the ribosomal small subunit (16s rRNA) and PCR master mix (Takara Bio Group, San José, CA, USA). Subsequent sequencing was performed on the MiSeq platform (Illumina Inc., San Diego, CA, USA) as described by Eickhardt-Dalbøge et al.[13]

We used three different sets of primers targeting 18s rRNA gene to ensure amplification of as broad a spectrum as possible of eukaryotic organisms, including G3F1/G3R1 (5'-GCCAGCAGCCGCGGTAATTC-3'/5'-ACATTCTTGGCAAATGCTTTCGCAG-3'), G4F3/G4R3 (5'-CAGCCGCGGTAATTCCAGCTC-3'/5'-GGTGGTGCCCTTCCGTCAAT-3') and G6F1/G6R1 (5'-TGGAGGGCAAGTCTGGTGCC-3'/5'-ACGGTATCTGATCGTCTTCGATCCC-3').[10]

Prior to analysis, all sequences belonging to Mammalia and Cyanobacteria/Chloroplast were removed. For 18s rRNA data, only fungal taxa belonging to phyla associated with infestation of human airways were included: Ascomycota, Basidiomycota, Chytridiomycota, Entomophthoromycota, Hyphochytriomycetes, Kickxellomycotina, Mucoromycotina, and Peronosporomycetes.[14]

Statistical Analysis

To identify and visualize taxonomic differences in the bacterial and fungal microbiota between the asthma and control groups, analyses of alpha (the Shannon Diversity Index and observed richness) and beta (principal coordinate analysis (PCoA) of Bray-Curtis distances) diversity were performed, along with linear discriminant analysis effect size (LEfSe). All analyses were performed using the statistical programming software R (v. 4.3.3).[15]

The R package phyloseq (v. 1.46.0) was used for data handling.[16] The seed value was set to 42 throughout the analysis. The package ggplot2 v. 3.5.1 was used for data visualization.[17] Estimated alpha diversity was compared between groups using Wilcoxon rank-sum test. For beta diversity, the Adonis (PERMANOVA) function of the "vegan" v. 2.6–6 R package[18] was used to assess the significance of beta diversity clustering, while the betadisper (ANOVA) function was used to analyze the multivariate homogeneity of group variation. Before ordination analysis, data was transformed using the Hellinger method (sqrt(x)/sum(x)). The R package microbiomeMarker (v. 1.8.0) was used for the LEfSe analysis.[19] P-values less than 0.05 were regarded as statistically significant.

Results

Demographic Data and Laboratory Test results

A total of 136 children (5–16 years old) were recruited in the study (control group = 57, asthma group = 79), and a total of 272 samples were collected. Demographic and clinical data for children with asthma are shown in Table 1.

Assessment of Bacterial Microbiota (16s rRNA Metabarcoding)

Alpha (Shannon Diversity and Observed Richness) and Beta Diversity

Based on nasal samples analysis, Shannon diversity showed that the asthma group had significantly higher diversity than the control group ($p = 0.02$); however, the observed number of species (richness), indicated that the asthma group had significantly lower diversity compared to the control group ($p = 0.02$) (Figure 1A). For the Beta diversity (Bray-Curtis dissimilarity), there was no significant difference in species composition between the two groups ($p = 0.07$) (Figure 2A).

Table 1 Demographic and Clinical Data of Asthmatic Cohort Included in the Study

	Frequency	%
Gender		
Female	29	36.7
Male	50	63.3
Age (Years)		
2–5	14	17.7
6–10	40	50.6
11–16	25	31.6
Asthma control		
Not controlled	24	30.4
Controlled	55	69.6
Allergies		
Single allergy	49	62.0
Multiple allergies	30	38.0
Therapy		
No drug given	4	5.1
Single drug	53	67.1
Multiple drugs	22	27.8
Vitamin D		
Severe deficiency	16	20.3
Mild to moderate deficiency	35	44.3
Optimum level	13	16.5
Increased risk of hypercalciuria	1	1.3
IgE level		
Normal	39	49.4
Abnormal	26	32.9
Eosinophil count		
Normal	8	10.1
Abnormal	62	78.5

In throat samples, the asthma group had significantly lower Shannon diversity ($p = 0.04$) and observed richness ($p \leq 0.0001$) than the control group (Figure 1B). On the other hand, the species composition (Beta diversity) differed significantly between the two groups ($p = 0.005$) (Figure 2B).

Predominant Bacterial Genera in Nasal and Throat Samples

Nasal swabs assessment revealed 466 bacterial genera and the most prevalent bacterial genera are shown in Figure 3A.

The most prevalent genera inhabiting the nasal samples were *Gemella, Dolosigranulum, Corynebacterium, Rothia, Moraxella, Haemophilus, Prevotella, Staphylococcus, Streptococcus* and Veillonella (Figure 3a). However, the *Staphylococci* genus was enriched in the control group of the 205 bacterial genera detected in throat samples, the top

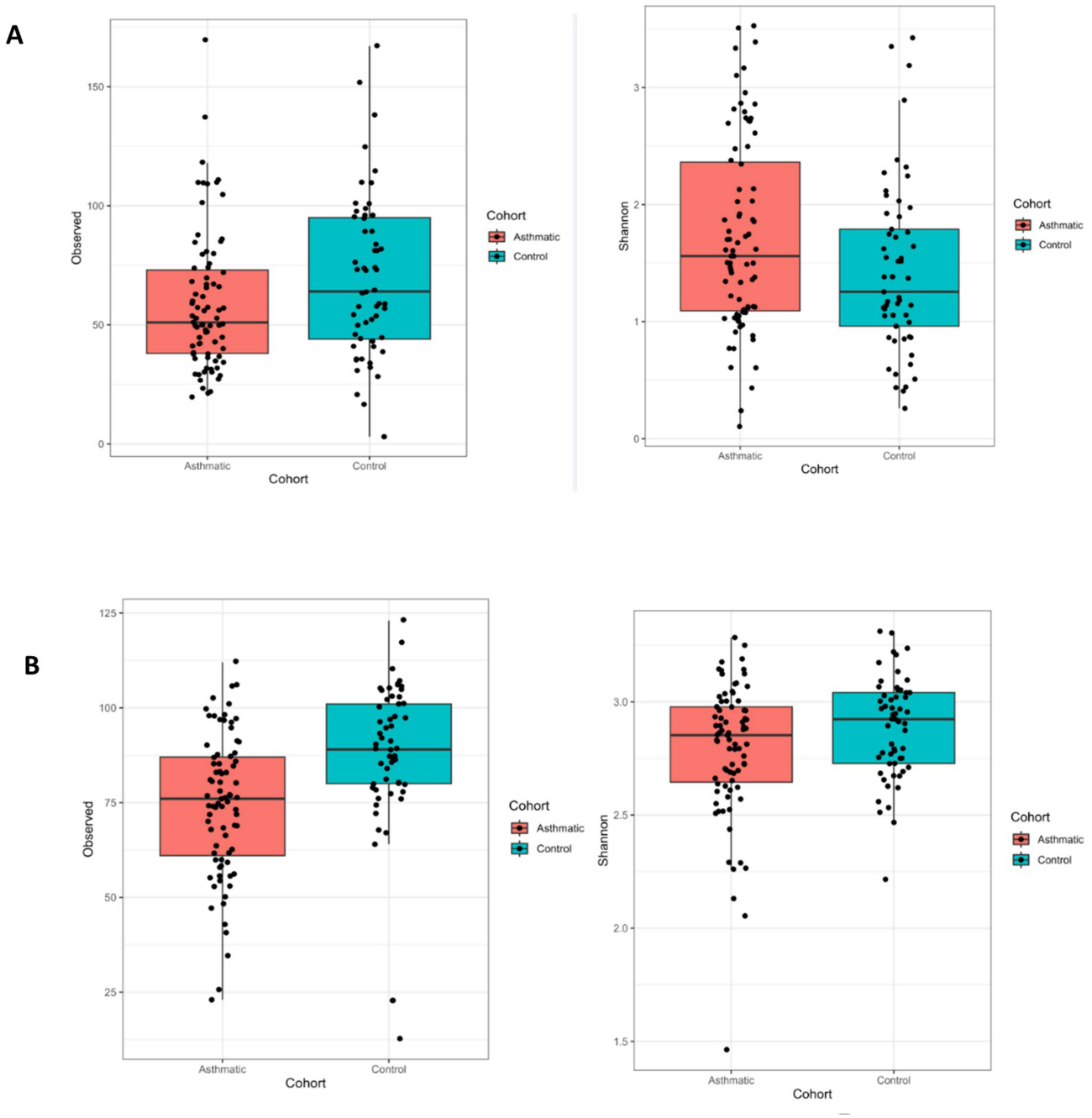

Figure 1 Alpha diversity for samples based on 16s rRNA analysis: observed richness (left) and Shannon diversity (right) indices of nasal (**A**) and throat samples (**B**). Although nasal asthmatic samples appeared to be more diverse, observed number of species was significantly lower than that of the control group. For the samples collected from throat, alpha diversity indices showed a lower diversity profile among asthmatic samples.

10 most prevalent genera are shown in Figure 3B. *Actinomyces, Gemella, Fusobacterium, Leptotrichia, Rothia, Haemophilus, Alloprevotella, Prevotella, Streptococcus* and *Veillonella* appeared to be the most prevalent genera in the throat samples (Figure 3B). Interestingly, in the controlled asthma group, a range of bacterial genera seemed to be enriched, namely *Fusobacterium, Streptococcus, Prevotella, Veillonella, Gemella, Haemophilus, Rothia, Leptotrichia,*

https://doi.org/10.2147/JAA.S498803

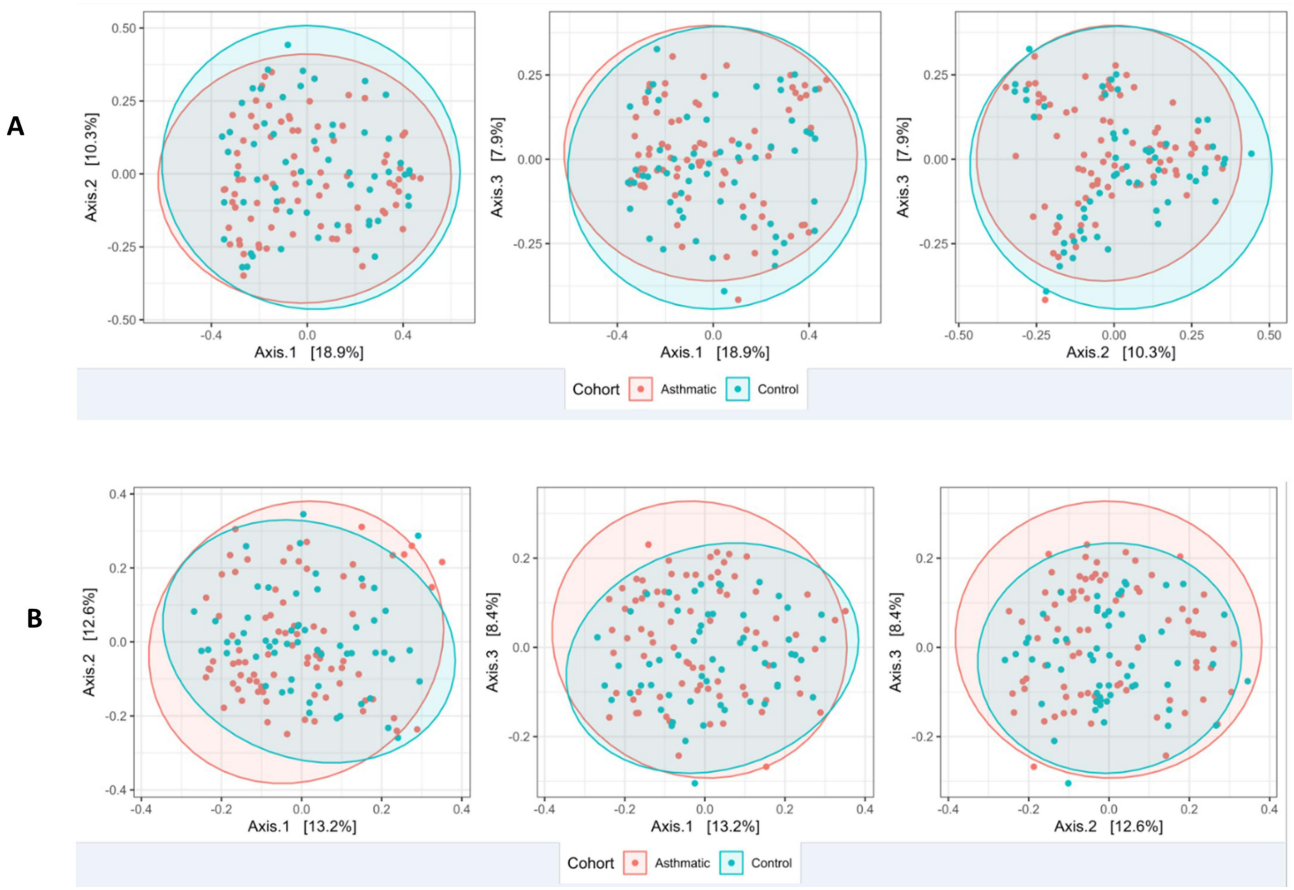

Figure 2 Beta diversity (PCoA plot of Bray-Curtis dissimilarity) of bacterial microbiota associated with asthma and control groups in nasal (**A**) and throat samples (**B**). There was no significant difference in species composition between the two groups in nasal samples (**A**). For throat swabs, the species composition differed significantly between the two groups, with homogeneous dispersion (**B**).

Alloprevotella, Campylobacter, Actinomyces and *Neisseria* (Figure 3C). For the uncontrolled asthma, samples were enriched with *Prevotella, Streptococcus, Veillonella, Lachnoanaerobaculum* and *Granulicatella* (Figure 3C).

Assessment of Fungal Microbiota (18s rRNA Metabarcoding)

Alpha (Shannon Diversity and Observed Richness) and Beta Diversity

For both nose and throat samples, there was no statistical difference in the observed richness of controls and asthmatic cases ($p > 0.05$) (Figure 4A, 4B). Although, the healthy group in our study had a tendency towards an increased richness of the selected fungal genera, the difference was not statistically significant (Figure 4A, 4B). For the beta diversity, we observed a significant difference in species composition (beta diversity) between the two groups in nasal ($p = 0.029$) and throat samples ($p = 0.019$) (Figure 5A, 5B).

Predominant Genera in Nasal and Throat Samples

Out of the 213 fungal genera identified in the nasal swabs, the top 10 most prevalent were found to be *Penicillium, Cladosporium, Malassezia, Mucor, Alternaria, Curvularia, Lewia, Candida, Saccharomyces* and *Aspergillus* (Figure 6A). The genus *Mucor* was enriched in the asthma group, whereas the control cohort was enriched with *Malassezia*.

For the throat samples, the most prevalent fungal genera included *Penicillium, Cladosporium, Malassezia, Alternaria, Candida, Saccharomyces, Trichosporon* and *Aspergillus* (Figure 6B).

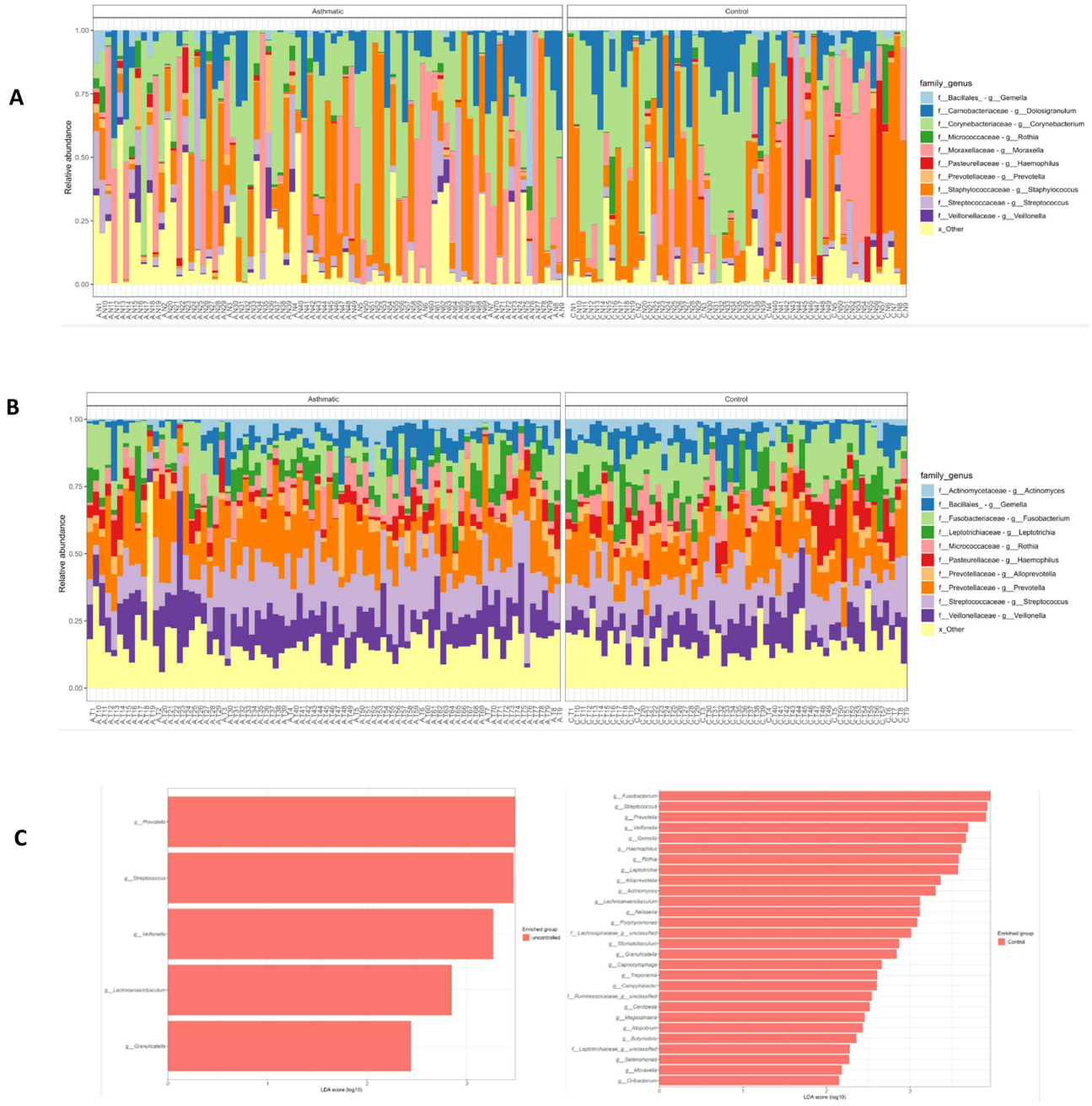

Figure 3 The relative abundance of the 10 most prevalent bacterial genera classified using 16s rRNA sequencing, in nasal (**A**) and throat (**B**) samples. Each bar represents a sample, with low abundance taxa or unclassified microorganisms grouped under "other". Linear discriminant analysis effect size (LEfSe) shows enriched genera (**C**) within throat samples of asthma sub-groups: uncontrolled (left) and controlled (right). "g" is genus and "f" is family.

Assessment of throat samples revealed that the family *Saccharomycetaceae* and the genus *Candida* were more abundant in the asthma group compared to the control group.

Additionally, no significant difference was identified at the alpha and beta diversity levels between the asthma sub-groups (controlled vs uncontrolled cases).

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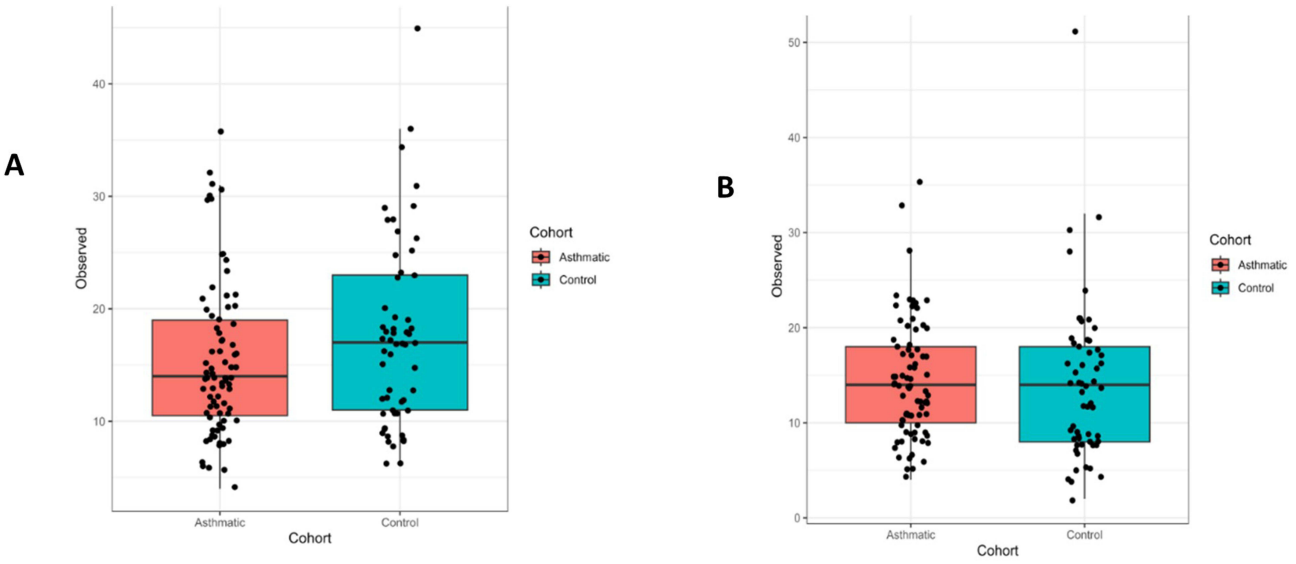

Figure 4 Alpha diversity based on 18s rRNA analysis: observed richness of nasal (**A**) and throat (**B**) samples. For both nose and throat samples, there was no statistical difference in the observed richness between control and asthma cases.

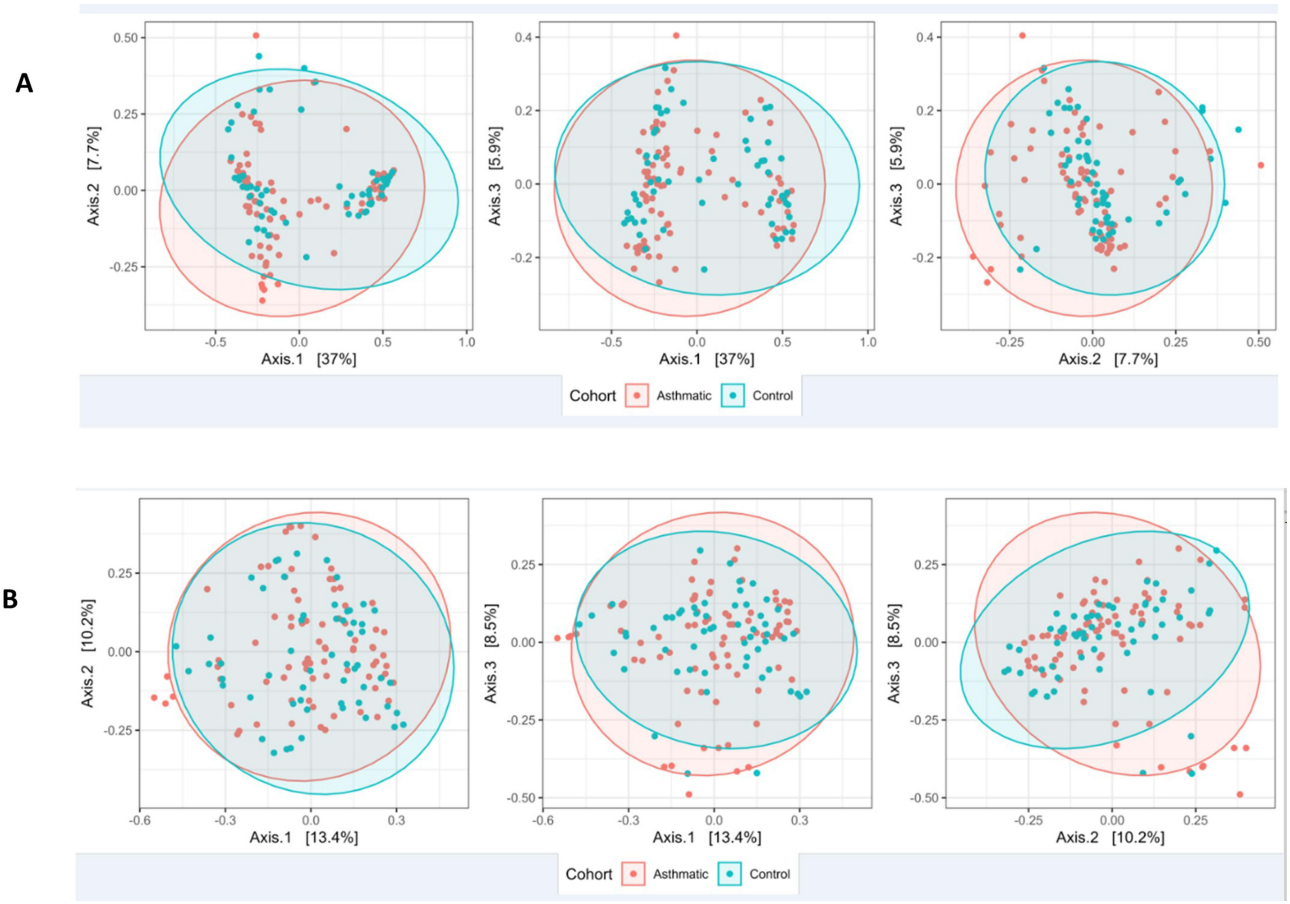

Figure 5 Beta diversity (PCoA plot of Bray-Curtis dissimilarity) of Fungal microbiota related to control and asthma cases. A significant difference in species composition between the two groups in nasal (**A**) and throat samples (**B**) was detected.

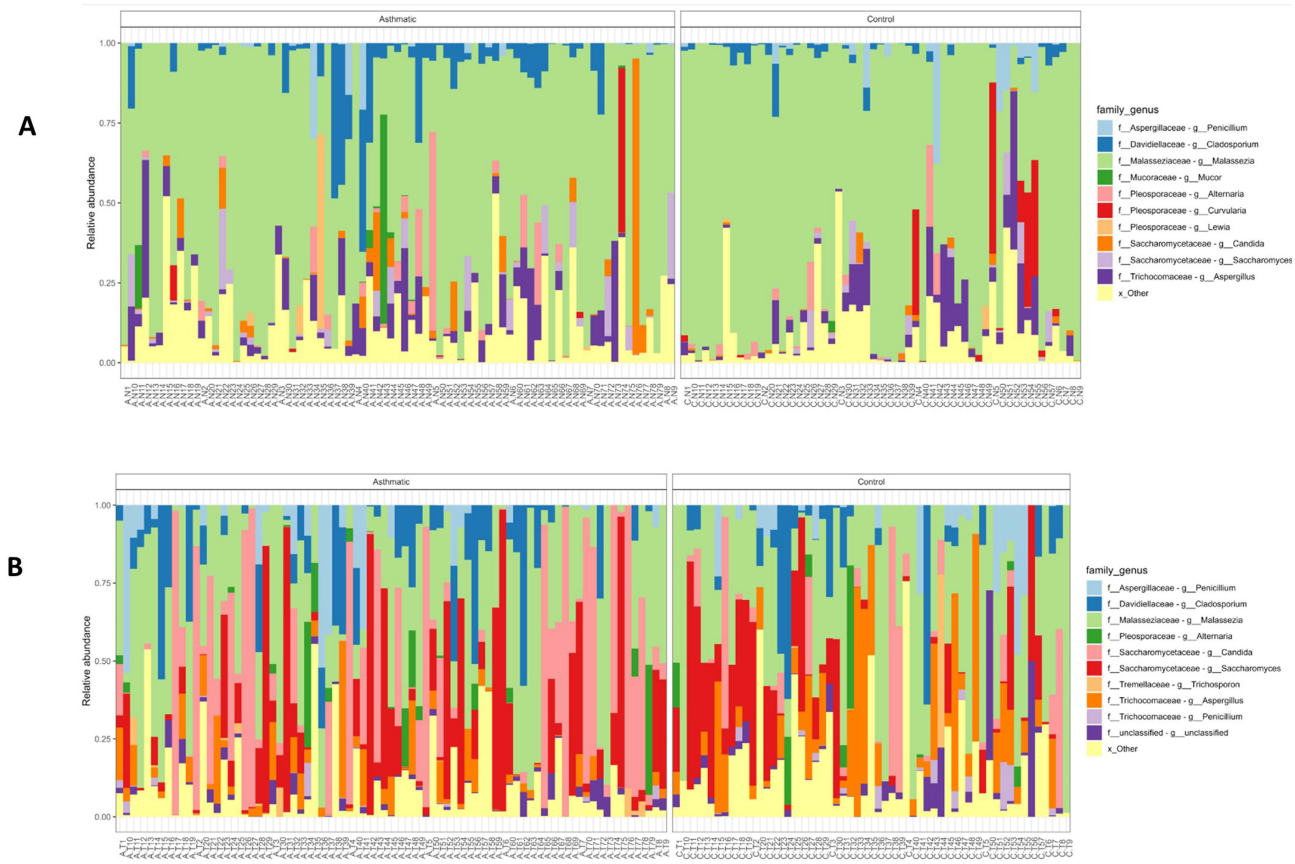

Figure 6 The relative abundance of the 10 most prevalent fungal genera classified by 18s rRNA sequencing, in nasal (**A**) and throat (**B**) samples. Each bar represents a sample, with low abundance taxa or unclassified organisms grouped as "other". "g" is genus and "f" is family.

Discussion

The current study assessed potential differences in microbial diversity and composition between children with asthma and their healthy counterparts. In our study, nasal samples showed significant differences in alpha diversity between the control and the asthma group. The control group showed increased observed richness, while the asthma group had higher Shannon diversity (evenness). This could be attributed to the fact that even though the asthma group consisted of fewer taxa, the Shannon diversity is still higher as the taxa were more evenly distributed, compared to the control group. In some studies, both observed richness and Shannon diversity were found to be lower among the asthma cohort.[6]

The most prevalent bacterial genera (Figure 3A) identified in our work are similar to those detected by other cross-sectional and longitudinal studies that described abundance of *Proteobacteria, Actinobacteria, Bacteroidetes and Moraxella, Corynebacterium, Dolosigranulum* and *Prevotella* in their samples.[20,21]

We found that the observed number of species were significantly lower among asthmatic samples with lower abundance of *Staphylococci* species compared to the control group. This is consistent with studies describing the *Staphylococci* as a common colonizer in anterior nares among healthy individuals.[22] On the other hand, Tang et al reported the presence of a *Staphylococcus*-dominant microbiome in individuals with asthma.[16] Early life studies had also linked the dominance of *Staphylococcus* in the airways with asthma development later in childhood.[23,24] Similarly, other studies have reported asthmatic microbiome enriched with certain bacterial communities such as *Moraxella, Streptococcus*, and *Haemophilus*.[9,25,26]

With regards to the throat swabs analysis, our findings suggest that the asthma group samples had significantly lower diversity indices than the control specimens (Figure 1B and figure 2B), with a range of bacterial genera colonizing the throat samples (Figure 3B). There was also a significant difference between the microbial communities of the two groups

(beta diversity), and, remarkably, the enriched species differed within the asthma cohort (controlled vs uncontrolled asthma cases).

In a multi-center European study that evaluated 241 oropharyngeal swab samples from school-age and preschool children, *Veillonella, Haemophilus, Prevotella* and *Rothia* appeared to be the most common genera identified, with no significant difference in the microbial composition between children with severe and mild asthma.[27] Similar microbial clusters were found in the throat samples collected from both groups in our study.

In the current study, *Streptococci, Prevotella*, and *Veillonella* were enriched in throat samples collected from both controlled and uncontrolled asthma cases. However, other different genera were enriched in both categories (Figure 3B). Our uncontrolled asthma cohort was enriched with *Fusobacterium, Gemella, Haemophilus, Rothia, Actinomycetes and Neisseria* consistent with Pérez-Losada work, who identified similar microbial genera in the oral cavity of patients when analyzing cases with allergic rhinitis with and without asthma.[28]

On the other hand, Van Beveren et al noted that certain genera dominate the asthma cases (with and without exacerbation) including *staphylococci, Neisseria*, and anaerobes with minimal differences in microbial abundance between mild and severe asthma cases, thus questioning the association between microbiota and exacerbation severity.[29,30]

The modest differences in microbiota seen in our study between asthma subcategories could be related to clinical factors such as the presence of other atopic condition or treatment plan that exposes uncontrolled asthma cases to steroids and antibiotics[31] and more important the sample size included in our work (24 not controlled vs 55 controlled cases); therefore, more work is needed to explore this direction.

In the current study, the presence of fungal genera belonging to selected phylae in the airways was also evaluated. At the alpha diversity level, nasal samples did not show any significant variation; however, genera composition (beta diversity) appeared to be significantly different between the two groups (Figures 4–6). Unfortunately, assessment of fungal composition in patients with asthma has been described in a limited scope, with a focus on gut-lung access. Such research has linked a predominance of certain fungal genera like *Candida* with increased risk of asthma in children and adults.[32]

In our cohort, the nasal mycobiota showed abundance of a range of fungal genera including *Penicillium, Cladosporium, Malassezia* and *Saccharomyces*, however, the control cohort showed increased abundance of *Malassezia* (a genus in the phyla of Basidiomycota). This contrasts with studies that have linked *Malassezia* colonization to certain respiratory conditions such as chronic rhinosinusitis and asthma.[33] In another study, 499 fungal genera were identified in asthmatic samples collected from nasal blow samples, with certain species of *Malassezia* being the most dominant.[34] Interestingly, a shift between the different *Malassezia* species has been observed in episodes of controlled and exacerbated asthma conditions, however this study did not include healthy control samples.[34]

We found that among asthma cases, certain fungal clusters were enriched, namely the family: Mucoraceae and the genus *Mucor*, consistent with studies linking *Mucor* presence in the environment with the risk of asthma exacerbation in children.[35] Contrary, Yuan et al reported that the mold *Mucor* was negatively correlated with asthma exacerbations.[34]

Similar to the nose samples, the throat samples from both categories showed common fungal genera such as *Penicillium, Cladosporium, Malassezia, Alternaria, Candida, Saccharomyces, Trichosporon* and *Aspergillus* (Figure 6A); however, the asthma cohort was enriched with *Saccharomyces* and *Candida*. Although the former genera are known to be part of the developing mycobiome since infancy[36] and among healthy adults with higher intraindividual variability,[37] other studies showed an elevation in *Candida* species in asthmatic airway samples relative to a healthy control.[38] On the other hand, work by Da Liu et al showed that certain species of *Saccharomyces* can alleviate the asthma condition.[39] Yet, their work was based on samples collected from the gut. *Saccharomyces* dominance was also linked to symptoms severity in a study evaluating mycobiome differences among other respiratory conditions, known as chronic obstructive pulmonary disease (COPD).[7]

Notably, both nose and throat samples evaluated for fungal microbiota revealed significant differences in species composition, and this could relate to the notion that the airways are frequently exposed to fungal elements colonizing household environments, leading to a wide variability in mycobiota composition between individuals.[40]

Although studies have shown that certain fungi are frequently linked to asthma and to other allergic diseases, the underlying process remains unclear. For instance, it is not clear whether early exposure leads to sensitization followed by

consequent hypersensitivity or directly by imposing immunomodulatory effects.[8] Taken together, studies have reported variable findings concerning the microbial diversity and its relation to asthma exacerbations. Some studies had identified no difference or lower diversity and others found that higher diversity is linked to loss of asthma control and prolonged wheezing episodes.

One aspect to consider in the current study is the use of Bray-Curtis for beta-diversity in the 18S fungal dataset. Although commonly used in microbiome studies,[41] this metric may be influenced by data filtering to exclude mammalian sequences. Therefore, alternative metrics less affected by filtration may be more suitable for future analyses.

Factors influencing microbiota differences could also be related to the anatomical sampling site within the upper respiratory tract (e.g, nasopharynx, hypopharynx, oropharynx, nasal cavity swabbing or wash), time of sample collection across the year, age, treatment received (antibiotics in particular), variation in sample size, genetic background and living environment.[40] Additionally, technical steps involved in the process of microbiome study play a major role in result variation including sample storage, DNA extraction methods, metabarcoding technique, sequencing platform, and to the bioinformatic pipeline used.[42]

In the current study, Vitamin D levels were low and eosinophil count was elevated among the asthma group samples, these findings are in agreement with previous studies targeting biomarkers of asthma severity.[3,43] However, since data were only available for the asthma group, no further work was done to link vitamin D level or other allergy biomarkers with microbiome disturbance between control and cases.

Although, in our study, certain bacterial and fungal community types have been detected in the patients' samples, more *s*tudies are needed to understand the relationship between certain mycobiome–bacteriome interactions and factors influencing this partnership in the different asthma phenotypes, such as dietary, drug and environmental aspects. Our study examined the microbiome differences at a single point estimate, and this may be considered a limitation, as studies addressing microbial differences on a longitudinal scale demonstrated variation in microbial dominance relative to the patient condition.[26]

Though difficult to collect from children, more invasive samples (sputum, BAL) and higher sample size may reveal different microbiome profiles and provide more insights into how microbiomes evolve and respond to changes that shape the severity of asthma.

To the best of our knowledge, this is the first study in Saudi Arabia assessing the bacterial and fungal microbiota among children with asthma.

Conclusion

The airway microbiota changes extensively from birth. In the past decade, researchers have directed increasing attention to evolving microbiomes and the related risks of allergies and asthma progression. In the current study, the asthmatic airways were found to be significantly less diverse with enrichment of certain genera in both the asthmatic children and the control group. Although the current report focuses on the potential alteration of the airway's microbiota, represented by nose and throat samples, combining a multi-omics approach (proteomics, transcriptomics, and metabolomics) could improve our understanding of asthma initiation and progression.

Data Sharing Statement

The datasets used and/or analyzed in the current study are available from the corresponding authors upon reasonable request.

Ethical Approval and Consent to Participate

The ethical approval for this study was granted by the Institutional Review Board of Imam Abdulrahman bin Faisal University (IAU), Dammam, Saudi Arabia (IRB-2020-03-408).

Informed consent was obtained from parents before the children's enrollment and sample collection.

The work described has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans.

Consent for Publication

Participants received an explanation about the study's objective and were informed about the anonymous and voluntary nature of the study. Written and informed consent was obtained for their contribution to the study and to publish the data.

Funding

The authors extend their appreciation to the Deputyship for Research and Innovation, Ministry of Education in Saudi Arabia for funding this research work through the project number IF-2020-016-CAMS, at Imam Abdulrahman bin Faisal University, College of Applied Medical Sciences, Saudi arabia.

Disclosure

The authors declare that they have no competing interests or relationships that could influence the work reported in this paper.

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