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ORIGINAL RESEARCH

Changes in the Gut Microbiome is Influenced by the Level of Control and Treatment in Asthma

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Background: The influence of intestinal microorganisms on the development and course of allergic diseases has recently been the subject of intensive research, but studies describing changes in the intestinal microbiome of asthma patients in response to altering factors are still scarce.

Objective: (1) the analysis of eating habits composition of intestinal microbiota and BMI in asthma patients compared to the control group, (2) the comparison of the results of the analyzed parameters in asthma patients and in the control group, (3) the analysis of asthma treatment results depending on the composition of intestinal microbiota.

Methods: Clinical stool isolates were cultured and genetic material was sequenced. The study included 49 subjects with asthma and a control group of 18 healthy volunteers. Clinical data was collected through questionnaires on the most frequently reported symptoms and the FFQ questionnaire. The composition of intestinal microbiota was determined using the traditional breeding method (the serial dilution method was used) followed by 16S rRNA sequencing.

Results: Patients with asthma reported the greatest severity of clinical symptoms in all the body systems examined.The most common cause of the aberrant stool test results was *E. coli*, with titers $<10^6$. The was no difference in the dietary habits between the asthma patients and the control group. Alpha and beta diversity, was significantly lower in asthma patients compared to the control group. Asthma patients had lower abundance of *Faecalibacterium* vs healthy volunteers. Statistically significant depletion of *Oscilospirales, Anaerovoracaceea*, was demonstrated in patients with uncontrolled asthma compared to controlled and partially controlled asthma. In patients taking glucocorticoids (oral and inhaled) enriched intestinal microbiota in *Anaerovoracaceae* and *Christensenellaceae* and depleted *Faecalibacterium* were observed.

Conclusion: Patients with asthma showed less richness and diversity in the composition of their intestinal microbiota compared to the control group.

Keywords: abundance, allergy, asthma, microbiome

Introduction

Asthma is one of the most important medical burdens; the chronic nature and troublesome symptoms limit the quality of life of patients and negatively affect their social, professional, and family life. Recently, an increasing number of microbiological and epidemiological studies confirm the hypothesis that the development of asthma (and allergic diseases in general) may be, at least in part, related to the intestinal microbiota.[1] Herbst et al provided evidence that commensal intestinal bacteria play an important protective role in the development of the allergic inflammatory reaction in the lungs by regulating the Th2-dependent response. Specifically, in the animal model, GF (germ free) mice had higher total numbers of infiltrating eosinophils and lymphocytes in the airways compared to those bred under the SPF (specific pathogen–free). The percentage of eosinophils in the airways correlated with IgE antibodies and the levels of Th2-related

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cytokines. The lack of commensal bacterial flora seemed to lead to abnormal maturation and recruitment of macrophages and plasmacytoid dendritic cells, resulting in an excessive allergic inflammatory response in the airways. Interestingly, the phenotype of conventional dendritic cells could be saved by the introduction of commensal flora in GF mice.[2]

In line with this, a breakthrough human study on the respiratory microflora conducted by Hilty et al in 2010 provided the first evidence of the commensal colonization in the bronchial tree, thus overturning the previously prevailing dogma on its sterility. A pioneering publication using culture-independent genetic techniques to identify taxa of particular groups of microorganisms showed that the microbiota of the respiratory tract of asthmatics is significantly altered in comparison to that of healthy people.[3] This was followed by similar observations on the intestinal microbiome, which in healthy people predominantly consists of commensals such as *Bifidobacterium, Lactobacillus and Eubacterium*, with a small component of opportunistic and pathogenic species (*Bacteroides, Enterobacteriaceace* as well as *Clostridium, Staphylococcus, and Pseudomonas*, respectively).[4] It has been suggested that the influence of the gut microbiota on the allergic inflammatory response may be mediated, at least in part, by bacterial metabolites.[5] While the published data provide a compelling link between the intestinal microbiome on the development of asthma, how the composition of the microbiome is determined in the patients is unclear. To date, most studies have focused on the impact of gut microbiome changes on increased asthma risk in children. Fujimura et al demonstrated that lower abundance of *Bifidobacterium, Faecalibacterium*, and *Akkermansia* was associated with a higher risk of asthma.[6] Another study also confirmed the association between reduced abundance of Lachnospira, Faecalibacterium, and Rothia and increased asthma risk.[7] Fewer studies have examined the gut microbiome in adult asthmatics. A pilot study examining the microbiome of adult asthmatics demonstrated that the gut microbiome differs depending on the asthma phenotype, which may translate into different treatment responses.[8] Further research in this area offers significant value in incorporating gut microbiota data to link asthma severity and treatment response. Since the microbiota in the gut undergoes dynamic changes in response to the intestinal contents, which comprises both beneficial macro- and micronutrients, as well as xenobiotics such as medications, both the diet and treatment could have an influence and dietary interventions may help develop new preventive and therapeutic strategies to aid asthma control. Here, we investigated the effect of the diet and medication on the species composition in the patient group, as well as the subsequent effect of the altered flora on the treatment efficacy.

Methods

Patients and the Study Groups

The study included 49 asthma patients, 15 men and 34 women, their average age was 51 years. Patients diagnosed with asthma in Allergology Clinic of the Medical University of Gdańsk were divided into three groups depending on the level of asthma control based on assessment over the last 4 weeks before examination: controlled, partially controlled, and uncontrolled (based on the criteria – eTable 5).[9] The control group consisted of 18 healthy volunteers (eTables 1 and 2). The study was approved by the Independent Bioethics Committee for Scientific Research of the Medical University of Gdańsk (consent no. NKBBN/374/2016). All the participants gave informed consent to participate in the study. The exclusion criteria were: infections of the digestive system, heart, kidney and liver dysfunction, cancer, pregnancy and taking probiotics or antibiotic in the last month before stool collection.

The study consisted of 3 parts. Firstly we analyzed the questionnaires regarding the most frequently reported symptoms and the Food Frequency Questionnaire (FFQ) questionnaire.[10] FFQ-6 is the questionnaire gathers information on the frequency of consumption of the eight main food groups, ie, cereal and dairy products, fat-containing food, eggs, vegetables, fruits, fish, meat, snacks, sweets and drinks. The study groups were then compared in terms of the occurrence of symptoms related to the skin (itching, flush, urticaria, angioedema), respiratory system (cough, shortness of breath, wheezing, frequent colds) and gastrointestinal tract (diarrhea nausea, vomiting, constipation, flatulence, abdominal cramps). The frequency of occurrence of a given symptom was scored as follows: 0 - never, 1 - sometimes, 2 - often, 3 - very often; the higher the score, the greater the frequency of the reported symptoms.

In the second stage we tested the intestinal microbiota using the traditional breeding method. In the third stage we analyzed 16S rRNA sequencing in 16 randomly selected asthmatics who participated in the previous stages of the study (eTables 3 and 4) and compered results to control group.

Stool Samples and Detection of Bacterial Species

Stool samples were collected into sterile containers, and stored at −80°C until further processing, instruction for patients. Quantitative stool culture was performed in the Clinical Microbiology Laboratory of the University Clinical Center, using the traditional culture method. To determine the number of cells of individual bacterial species in the stool samples the serial dilution method was used. From test tubes containing fecal suspension in dilutions from 10^5 to $10^{10,}$ 1/100 mL was inoculated into Columbia Agar base medium, Mac Conkey, Sabouraud. The species of microorganisms were determined by an automated biochemical method using the Vitek 2 Compact automatic bacteriological analyzer (Biomerieux) and the mass spectrometry method using the MALDI Biotyper apparatus (Bruker).

Sequencing

The Genomic Mini AX Bacteria + kit (A&A Biotechnology) was used for bacterial DNA isolation. V3-V4 amplicons were checked for quality and quantity. Then, 5′ and 3′ adapters were ligated. PCR was used to amplify adapter-ligated fragments. The Herculase II Fusion DNA Plymerase Nextera kit was used for library preparation. The Illumina qPCR Quantification Protocol Guide12 was used to check library quality. Sequencing using the Illumina MiSeq 2×300 bp platform was performed at the Laboratory, IT and Business Headquarters & Support Center in the Republic of Korea.

Microbiome Analysis

Microbiome bioinformatics was performed using QIIME 2 version 2024.5.[11] Raw sequence data were demultiplexed and quality filtered using the q2-demux plugin, followed by denoising with DADA2[12] via q2-dada2. Alpha diversity metrics, which describe ecological diversity within a sample, were calculated to assess richness (Faith's Phylogenetic Diversity, PD), evenness (Evenness index), and combined metrics that account for both richness and evenness (Shannon Index and Simpson's Index). Beta diversity metrics, which describe differences in taxonomic composition between samples, were evaluated using both unweighted methods (Jaccard distance and unweighted UniFrac, which focus on the presence or absence of taxa) and weighted methods (Bray-Curtis dissimilarity and weighted UniFrac, which account for the relative abundance of taxa). PERMANOVA was used to assess the statistical significance of beta diversity differences between groups. These diversity metrics were estimated using the q2-diversity plugin after samples were rarefied to 40,000 sequences per sample.

Taxonomy was assigned to features using the q2-feature-classifier plugin[13] with the "human stool weighted Silva 138 99% OTUs full-length sequences" reference database.[14] Differential abundance testing was conducted using the Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC) method[15] via the q2-composition plugin. ANCOM-BC is an advanced method for identifying true differences in taxa abundance across groups, correcting for compositional bias in microbiome data by accounting for factors like sequencing depth and sampling variation, thereby providing more accurate results.

Results

Clinical Characteristics

In our study there were no statistically significant differences between the study groups in terms of age, gender, BMI and the occurrence of food allergies and food hypersensitivities (eTable 1). The food frequency questionnaire FFQ-6 was used to determine dietary habits.[10] We did not find statistically significant differences in the diet between the study groups ($p < 0.05$), which confirmed that the eating habits of the asthma patients and the control group were similar (eTable 6). However, we noticed that the patients with asthma reported significantly more symptoms from skin, respiratory system and gastrointestinal tract compared to the control group $p<0.05$ (eTable 7).

Stool Test Results Using Culture Methods

Quantitative stool culture was performed in 49 asthma patients; we observed abnormal stool test results in 40 asthma patients, as determined based on the published characteristics,[16] ie an *Escherichia coli* titer < 10^6, undetectable probiotic flora (ie below the adopted cut-off point for bacterial detection - <10^5 in 1 g of stool), and the presence of pathogenic

microorganisms. The *E. coli* titer $<10^6$ was the most frequent reason of stool test results. *Klebsiella pneumoniae* and *Clostridium perfringens* were detected among microorganisms with the highest pathogenic potential in the asthma patients.

Stool Test Results Using NGS Methods

The alpha diversity, assessed using the Simpson index, was significantly lower in asthma patients compared to the control group (p-value 0.048, Figure 1). Evaluations with the Shannon Index and Faith's Phylogenetic Diversity (PD) did not show statistically significant differences between the groups (eFigures 1 and 2)). Beta diversity analyses revealed significant differences in gut microbial composition between asthma patients and controls. Significant results were observed for Bray-Curtis (p-value 0.006), Jaccard (p-value 0.007), and weighted UniFrac (p = 0.034), reflecting changes in taxa abundance. In contrast, unweighted UniFrac analysis showed a p-value close to significance (p = 0.054), suggesting minimal shifts in species presence or absence. These patterns were further supported by 3D visualizations of beta diversity plots, which showed distinct clustering between the groups. These findings indicate that asthma-associated microbiomes are dominated by specific taxa (Figures 2, 3, eFigures 3 and 4).

Differential abundance analysis (DAA) used to identify differences in the abundances of individual taxa showed a significantly lower abundance of *Faecalibacterium* and a significantly higher abundance of the *Christensenellaceae* taxa family in the patient group vs healthy volunteers (Figure 4). DAA of the intestinal microbiome was performed depending on the level of asthma control. Statistically significant depletion of *Oscilospirales, Anaerovoracaceea*, Family XIII was demonstrated in patients with uncontrolled asthma compared to controlled asthma (both partially and well-controlled, Figure 5. In the next stage, a DAA analysis was performed comparing glucocorticosteroids (GKS) intake. Among asthma patients, 12 people were taking ICS, 1 person with asthma was taking ICS and OCS, 3 people with asthma were not taking any glucocorticosteroids (ICS, OCS). In patients taking glucocorticoids (inhaled, orally), statistically significantly enriched intestinal microbiota in *Anaerovoracaceae* and *Christensenellaceae* and depleted *Faecalibacterium* were observed, Figure 6.

Discussion

The study showed reduced biodiversity of intestinal microflora in a group of asthma patients.

The significant difference observed in the Simpson index, but not in Shannon or Faith's PD, suggests that asthma patients may have a less evenly distributed microbiota, with certain taxa dominating. Beta diversity analyses further demonstrated significant differences between asthma patients and controls in the structure of the microbial

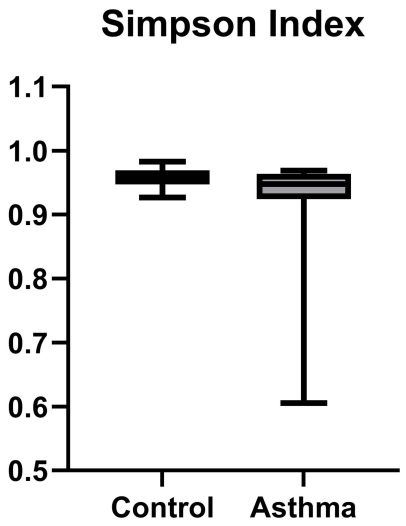

Figure 1 Alpha diversity measured by Simpson Index across study groups (asthma vs controls). The alpha diversity was significantly lower in asthma patients compared to the control group (p-value 0.048).

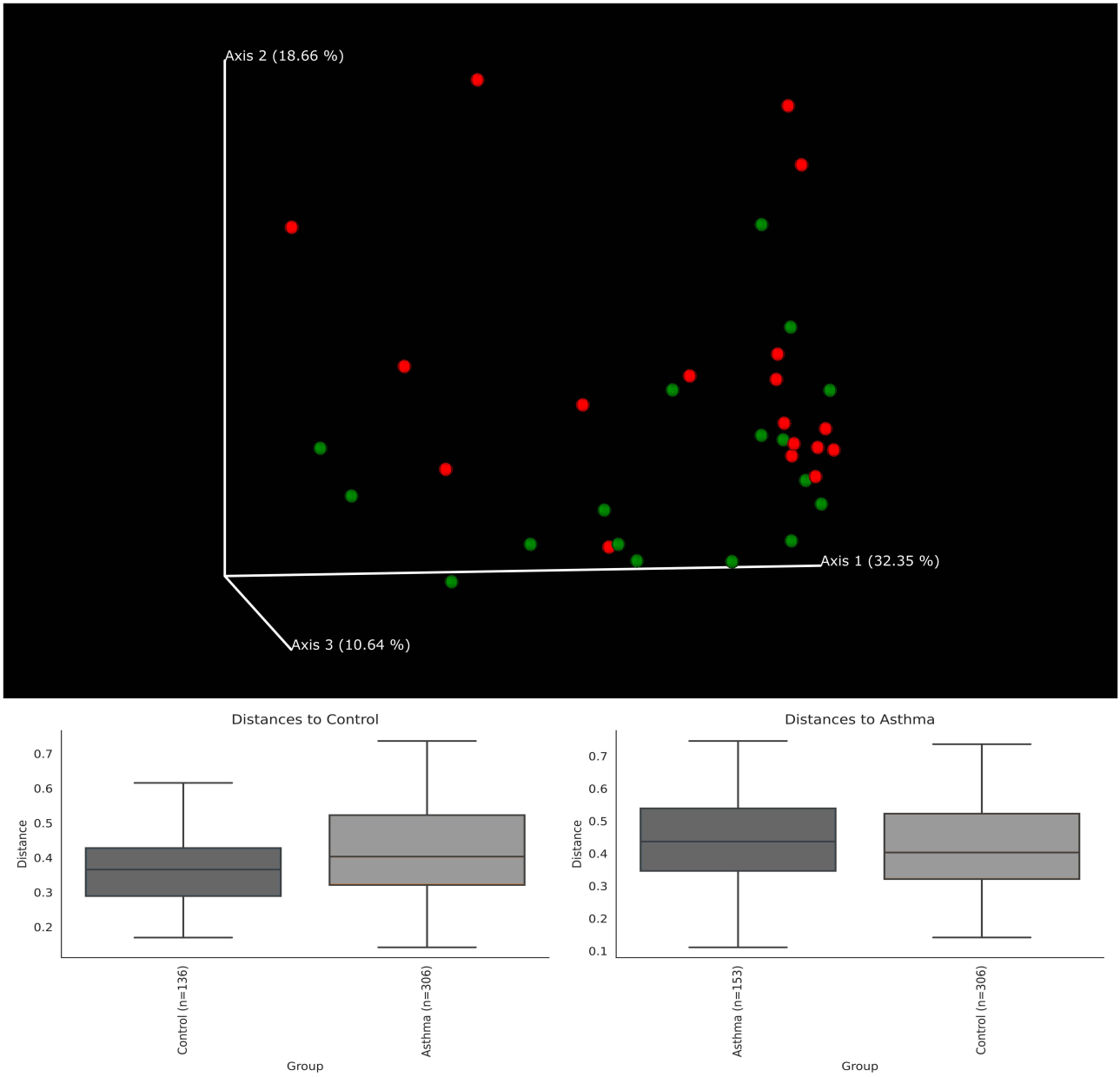

Figure 2 Beta analysis of the species diversity in the study groups. Beta diversity analysis of gut microbiota using weighted UniFrac. The 3D visualization (Emperor plot) shows clustering patterns between asthma (red) and control (green) groups. These patterns are statistically supported by PERMANOVA analysis, which demonstrates significant differences between the groups (p-value 0.036).

community. The results from Bray-Curtis, Jaccard, and weighted UniFrac metrics highlight notable shifts in the microbial composition and taxa abundance. In contrast, the borderline significance of unweighted UniFrac suggests these differences may be primarily driven by the changes in abundance rather than the presence or absence of specific taxa.

Greater diversity and richness of the gut microbiome is a more covetable state and is attributable with good health.[17] Wang et al analyzed the intestinal microbiome of asthma patients and the control group and found no statistically significant differences in the alpha-diversity scores.[18] In turn, a larger cross-sectional study conducted by Zou et al showed that the alpha diversity in the group of asthma patients was significantly lower,[19] which is in line with our observations. The discrepancy between results may be due to confounding factors such as diet, geographic region, and environmental contaminants. Another limitation could be related to the high complexity of the asthma phenotypes,

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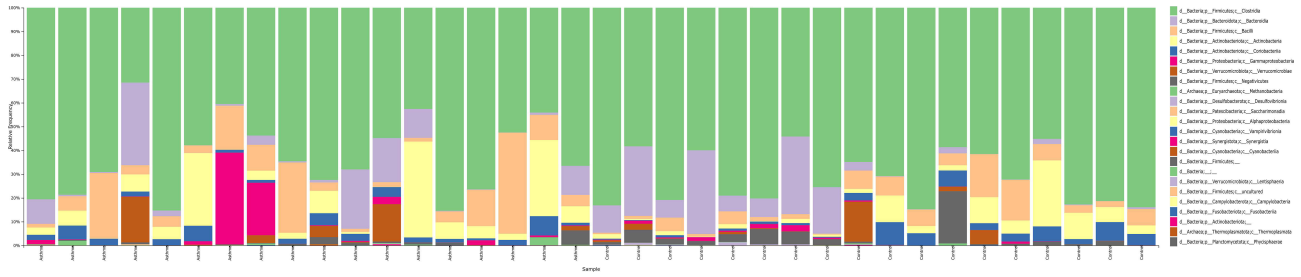

Figure 3 Operational Taxonomic Units alignment in asthma and control group.

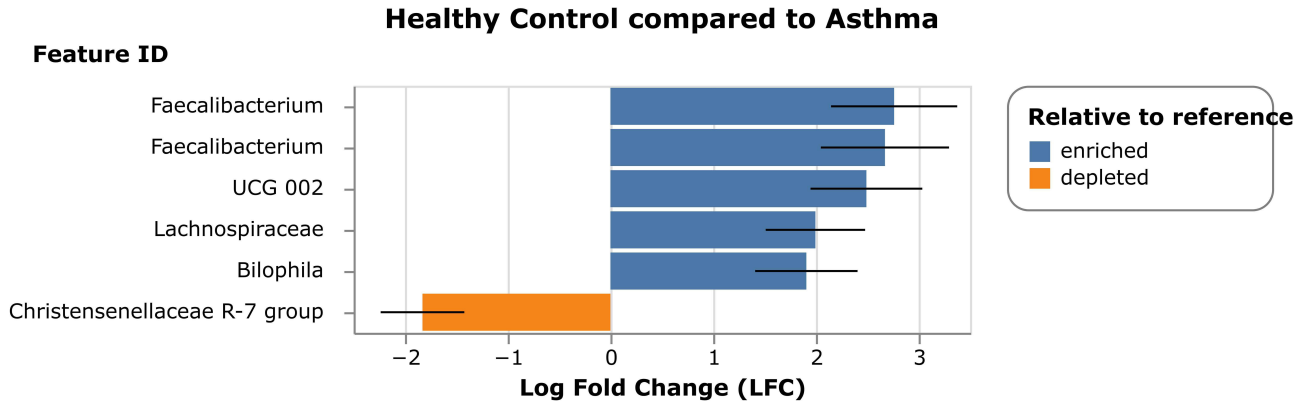

Figure 4 Differential abundance Analysis in control group. Faecalibacterium represent genus-level or near-genus-level assignments, with "group" indicating a placeholder name used in microbiome datasets.

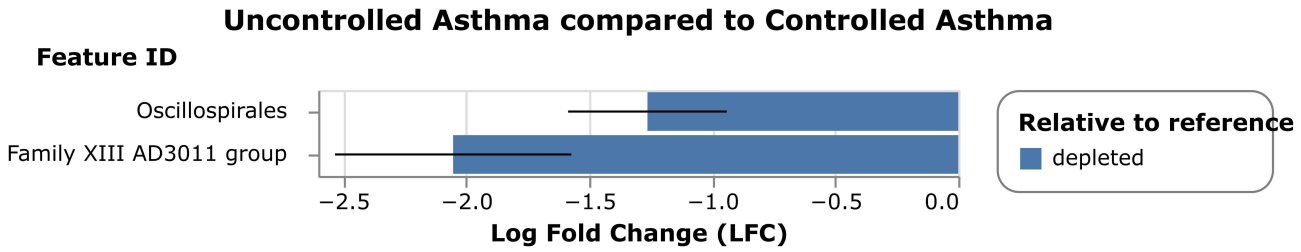

Figure 5 DAA results comparing uncontrolled asthma to controlled asthma (both partially and well-controlled).

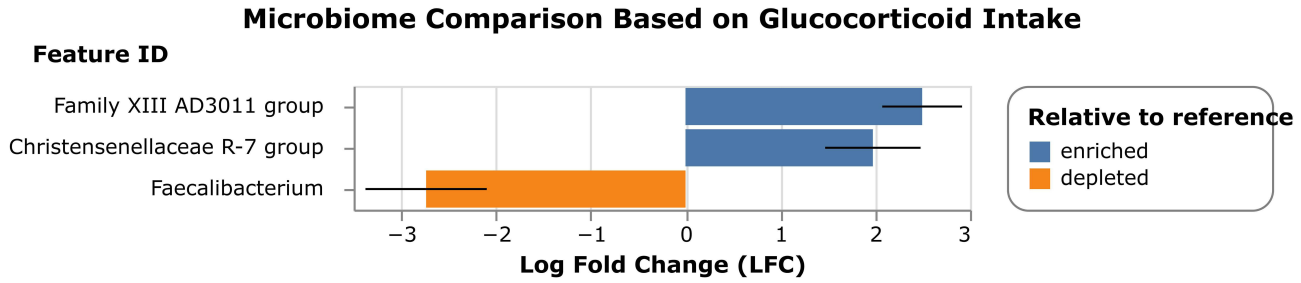

Figure 6 DAA results comparing glucocorticoids (GKS) intake.

which may hamper the interpretation of the results further. Large multicenter studies of the gut microbiome in homogeneous groups with specific asthma phenotypes could be a more informative, but are limited by time and resource constraints.

Adequate symptoms control poses a significant clinical challenge, contributing to the asthma burden with high personal and socioeconomic cost. Since the intestinal microbiome has been shown to impact the pathogenesis and disease severity, identification of the differences and the factors affecting microbial diversity and species involvement could suggest strategies for patients. However, there is only a limited number of publications using next-generation sequencing of genetic material to identify the gut microbiome alterations in asthmatic patients. We have attempted to comprehensively approach the problem by assessing dietary habits of the patients and correlating with the microbiological data obtained from the collected stool samples. The use of bacterial 16S rRNA gene sequencing methods is in line with the current trends in research on the human microbiota. An important observation of the clinical part of this study was the occurrence of the largest number of gastroenterological symptoms (diarrhea nausea, vomiting, constipation, flatulence, abdominal cramps) in patients with asthma, which is in line with previous studies and an interesting observation in the context of similar dietary habits in both analyzed groups.[20–22] As for the effect of the diet, the majority of the previously published studies focus on the beneficial effects of a Mediterranean diet rich in fruits, vegetables, fish and seafood on the degree of asthma control.[21,22] There is evidence of a beneficial effect of frequent (defined as daily) consumption of fruit and vegetables on the course of asthma.[23–25] Conversely, a "Western diet" high in saturated fatty acids, red meat, simple sugars and salt may promote chronic inflammation and intensify an abnormal immune response.[26] Another mechanism is the impact of a high-calorie diet on obesity, which has an unfavorable additive effect on the worsening of asthma.[23,26] We compared the dietary habits of asthmatics and healthy individuals, which showed that the diets of both groups did not differ statistically significantly. On the one hand, we are confident that this factor does not confound the results. On the other hand, it encourages dietary interventions in asthmatic patients.

In the control group, 5 types of bacteria predominated: *Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria*, and *Verrrucomicrobia*; which is consistent with studies on the microbiome of healthy people.[27,28] Specifically, a significantly higher percentage of *Proteobacteria* was observed in the asthmatic patients compared to the control group with an overrepresentation of the *Gammaproteobacteria* class. Previous reports examining stool samples from infants showed that the presence of *Proteobacteria* was associated with IgE-mediated contact dermatitis[29,30] Liu et al suggested that a higher level of *Proteobacteria* is associated with an increase in the lipopolysaccharide produced by the bacteria, which intensifies the allergic inflammatory reaction in the respiratory tract and increases the risk of allergic rhinitis.[31] Another study conducted by Wang et al, who analyzed stool samples from both severe and well-controlled asthmatic patients showed positive correlation between the presence of bacteria of the *Proteobacterial* type and the levels of total IgE in the group of asthma patients with high IgE.[18] Interestingly, the above observations on the gut microbiome to some extent reflect the results of the analysis of the respiratory microbiome in asthma patients, where *Proteobacteria* are highly overrepresented.[3,5] Further studies provide evidence that changes in the gut microbiome may also occur in other allergic diseases such as allergic rhinitis.[32,33] Recently, a link between the lung and gut microbiome in the pathogenesis of COPD has been discovered.[34] Bowerman et al's study confirmed that the gut microbiome in COPD patients differs significantly from the gut microbiome of healthy individuals, demonstrating the association of Eubacteriaceae, Bifidobacteriaceae, Streptococcaceae, and Veillonellaceae with COPD.[34,35]

Our study showed a decreased abundance of *Faecalibacterium* and an increase in *Christensenellaceae* in the asthma group. Moreover, statistically significantly enriched intestinal microbiota in *Christensenellaceae* and depleted in *Faecalibacterium* were demonstrated in patients taking glucocorticosteroids. The latter results are consistent with one of the latest microbiome studies, which confirmed a strong positive correlation between the abundance of the *Christensenellaceae* family and asthma.[36] The above conclusions indicate that the depletion of microbiota in *Christensenellaceae* may have a protective function in the context of asthma. *Faecalibacterium*, on the other hand, are commensal bacteria that modulate the intestinal microbiota through the production of SCFA, which causes their anti-asthmatic effect.[37,38] Our study showed a significant reduction in *Faecalibacterium* in patients taking glucocorticoids, which is consistent with the latest observations of Qin Zhang.[39]

The immunomodulatory properties of probiotic bacteria have become the subject of intensive research on the possibility of using probiotics to prevent the development of allergic diseases. Sütas et al furnished evidence of the beneficial effect of *Lactobacillus casei* in reducing protein hypersensitivity reactions in patients with food allergy.[40]

Currently, there is an increasing number of studies supporting the idea that probiotics may have a positive effect on the immune system in the asthma patients.[41–44.]

The latest research provides evidence that the administration of probiotics to asthma patients has a beneficial effect on lung function, improving FEV1 and FVC values compared to the placebo group. Moreover, patients receiving probiotics showed significant improvement in ACT Asthma Control Test and AQLQ Quality of Life Questionnaire scores compared to baseline.[45] Another placebo-controlled and double-blind large study (PROPAM) conducted in 422 children with asthma showed that the probiotic strains *Ligilactobacillus salivarius* and *Bifidobacterium breve* significantly reduced the number of asthma exacerbations.[46] One study showed that four-week use of a symbiotic containing *Bifidobacterium breve* reduced systemic Th2 cytokine production after allergen challenge and improved peak expiratory flow.[47] In addition, Lee et al observed a beneficial effect of the supplementation with capsules containing probiotic bacteria *L. salivariu*, fish oil, and fruit and vegetable extract on improving lung function, reducing the need for short-acting inhaled bronchodilators and improving asthma control in children.[48] Although there are currently no recommendations regarding the use of probiotics in either prevention or treatment of asthma, the findings are promising and may pave the way for further clinical interventions using probiotic bacteria supplementation as a possible therapeutic strategy. The study showed that bacteria that are difficult to cultivate (such as *Faecalibacterium prausnitzii)* are beneficial and, according to the consensus of experts from the International Scientific Association for Probiotics and Prebiotics, are included in the list of new probiotic candidates.[49]

The studies showed a statistically significant reduction in the number of *Oscilospirales* in patients with uncontrolled asthma compared to controlled and partially controlled asthma. *Oscillospira* is an uncultured bacteria, currently described only in high-throughput NGS studies of the intestinal microbiota, the abundance of which is closely related to the health of the host.

Oscillospira is capable of producing short-chain fatty acids (SCFAs) such as butyrate and has already been characterized as one of the next generation probiotic candidates, and therefore has great potential for development and application in future food and biopharmaceutical products.[50]

An interesting observation when analyzing the commensal microbiota was that in the majority of the asthma patients *Escherichia coli* was present at a titer $<10^6$. *Escherichia coli* is nonpathogenic in most cases. Its beneficial effects on the host include vitamin synthesis and immunomodulatory properties. Lower abundance of non-pathogenic *E. coli* strains is associated with allergic diseases.[28,29] Pang et al proved that oral administration of a non-pathogenic *E. coli* strain to mice resulted in a statistically significant reduction in allergic symptoms in both the upper and lower respiratory tract.[29] The mechanism of suppression of the allergic reaction was to reduce the production of pro-inflammatory cytokines by Th-2 lymphocytes and to increase the activity of Treg lymphocytes secreting anti-inflammatory IL-10.[29] The researchers suggest that the above observations are consistent with the current "hygiene theory" and that the reduction of commensal microbiota is unfavorable in the context of achieving immunological tolerance. This is one of the first studies comparing Escherichia coli titers in asthma patients compared to the control group. Based on previous reports of the importance of Escherichia coli in allergic diseases, is a prelude to expanding research in the future on the role of this commensal bacterium in asthma patients.

This is one of the few studies to assess the gut microbiota not only using NGS but also using stool culture. While next-generation sequencing (NGS) is the gold standard in human microbiome research, it has certain limitations. Using PCR techniques to detect the genetic material of microorganisms also includes dead bacterial strains, which can falsely affect the actual total number of viable microorganisms. Conversely, using only the traditional stool culture method prevents the identification of approximately 93% of unculturable microorganisms and does not reflect the overall structure, dynamics, and spatial distribution of microorganisms residing in the gut. The optimal approach is to study the gut microbiome using both methods, as was used in this study.

In summary, we found significant differences in Alpha and Beta diversity in asthma patients compared to controls. Moreover, we found statistically significant differences in DAA depending on the degree of asthma control and glucocorticosteroid intake.

Despite the fact that the dietary habits and BMI in both study groups were similar, patients with asthma had lower richness and diversity of intestinal microbiota compared to the control group on a similar diet. However, we noted

differences in the microbiome composition which could be linked to the disease severity and medication use, and suggest a potential influence of microbial taxa on asthma severity in patients.

Conclusions

Despite the fact that the dietary habits and BMI in both study groups were similar, patients with asthma had lower richness and diversity of intestinal microbiota in Simpson index compared to the control group on a similar diet. However, we noted differences in the microbiome composition which could be linked to the disease severity and medication use, and suggest a potential influence of microbial taxa on asthma severity in patients. These observations provide important evidence of significant changes in the gut microbiome in asthma patients and require confirmation in future studies with larger patient populations. Analyzing the impact of changes in the gut microbiome on metabolic pathway disruptions in asthma patients may also be extremely valuable.

Abbreviations

ACT, Asthma Control Test; AQLQ, Asthma Quality of Life Questionnaire (AQLQ); BMI, Body Mass Index; COPD, Chronic obstructive pulmonary disease; DAA, Differential Abundance Analysis; FEV_1, forced expiratory volume in 1 second; FFQ-6, Food Frequency Questionnaire; FVC, forced vital capacity; GF, germ free; GINA, Global Initiative for Asthma; GKS – glucocorticosteroids; HMP, Human Microbiome Project; ICS, Inhaled Corticosteroids; IgE, immunoglobuline E; NGS, next generation sequencing; OCS, Oral Corticosteroids; OTU, Operational Taxonomic Units; PCR, polymerase chain reaction; SCFAs, short-chain fatty acids; SPF, specified pathogen free; 16S rRNA, 16S ribosomal RNA.

Ethics Statement

Study is complies with the Declaration of Helsinki.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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