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ORIGINAL RESEARCH

Gut Microbiome and Metabolomics Profiles of Allergic and Non-Allergic Childhood Asthma

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Purpose: This study aimed to investigate the characteristics of gut bacteria and the derived metabolites among allergic asthmatic children, non-allergic asthmatic children and healthy children without asthma.

Methods: Fecal samples were collected from 57 participants, including 20 healthy children, 27 allergic asthmatic children, and 10 non-allergic asthmatic children. 16S rRNA gene sequencing was conducted for analyzing gut bacterial compositions and untargeted metabolomics was used to analyze the alterations of gut microbe-derived metabolites. The associations between gut bacterial compositions and metabolites were analyzed by the method of Spearman correlation.

Results: The results showed that the compositions and metabolites of gut microbiome were altered both in allergic and non-allergic asthmatics compared with healthy controls. Chao1 ($p = 0.025$) index reflected a higher bacterial richness and Simpson ($p = 0.024$) index showed a lower diversity in asthma group. PERMANOVA analysis showed significant differences among the three groups based on unweighted UniFrac distance ($p = 0.001$). Both allergic and non-allergic asthmatics showed a higher relative abundance of *Proteobacteria* and a lower relative abundance of genera from *Clostridia*. More bacteria were altered in non-allergic asthmatics compared with allergic asthmatics. Metabolomics analysis identified that 42 metabolites were significantly associated with allergic asthma, and 58 metabolites were significantly associated with non-allergic asthma (multiple linear regression, $p < 0.05$). Histamine was 4 folds up-regulated only in the non-allergic asthma group. The relative abundance of *Candidatus Accumulib* was significantly correlated with the upregulation of histamine. The relative abundance of genera from *Clostridia* was significantly correlated with the downregulation of lipid and tryptophan metabolism.

Conclusion: The altered gut microbes was associated with the mechanism of asthma attack through metabolites in allergic and non-allergic asthma group, respectively. The result suggested that gut microbiome had an impact on the development of both allergic and non-allergic asthma. The distinct gut microbiome and microbiome-derived metabolites in non-allergic asthma children suggested that gut microbiome might play a critical role in modulation of asthma phenotype.

Keywords: gut microbiome, untargeted metabolomics, allergic childhood asthma, non-allergic childhood asthma, 16S rRNA gene sequencing

Introduction

Childhood asthma is one of the most prevalent chronic pediatric diseases. It affected children's health all over the world.[1] Asthma is a heterogeneous disease with complex pathogenesis, which presents many phenotypes.[2] Allergic and non-allergic asthma are the most common phenotypes of asthma. Approximately 80% of children with asthma were reported to be allergic.[3] Allergic asthma is mainly induced by inappropriate immune responses of type 2 T helper (Th2) cells, which might be triggered by an increased serum level of total immunoglobulin E (IgE) due to allergen exposures. While non-allergic asthma occurs independently of allergen sensitization.[4] Mechanisms of non-allergic asthma development have not been fully elucidated yet.[5,6]

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Apart from allergen triggers, recent studies showed that development of asthma might be modulated by gut microbiome. Hygiene hypothesis firstly suggested a link between microbes and allergy.[7,8] Riedler's study showed that exposures to stables in early lives might have a protective effect not only on allergic but also on non-allergic asthma, which suggested that gut microbiome may be also related with non-allergic asthma based on the hygiene hypothesis.[9] Recent studies showed the association between early life gut microorganism colonization and risk of asthma later in life.[10–13] As the improvements in multi-omic technologies, gut microbiome has been demonstrated to affect the immune responses in the lung by microbes or microbes-derived metabolites. Surviving gut microbes, fragments of dead bacteria or immune cells (activated by gut microbes or their metabolites) may travel to the lung through lymphatic circulation and trigger the host immune responses.[14–16] In addition, metabolites derived from gut microbiome, for instance, short chain fatty acids (SCFAs) could migrate through the bloodstream to modulate lung immune responses.[16] Animal experiments illustrated that some gut microbes could regulate differentiation of regulatory T cells (Tregs), and modulate the balance of Th1 and Th2 cells.[17–21] While, some gut microbes can also induce Th17 responses.[22,23] These findings suggested that a continued cross-talk may exist between gut microbes and immune responses, dysbiosis of gut microbiome may also play a critical role in the recurrent attack of asthma. Furthermore, Zou's study showed that gut microbiome between allergic and non-allergic asthma patients were different.[24] Thus, we hypothesized that gut microbiome has an impact on the attack of both allergic and non-allergic childhood asthma, whereas it plays a different role in allergic and non-allergic asthma.

To confirm our hypothesis, we conducted this study by combining the methods of 16S rRNA gene sequencing and fecal metabolomics to analyze gut microbial compositions and functions in allergic and non-allergic childhood asthma. Fecal metabolomics is the functional readout of gut microbiome.[25]

Materials and Methods

Study Population

Participants in this study were recruited from a project of National Natural Science Foundation of China, Epigenetic Modification Mechanisms of Black Carbon Impacts on Non-allergic Asthma, performed from 2016 to 2019. A total of 57 children met the criteria of sample collection, and agreed to join in this study. All participants aged from 5 to 14 years old. They all lived in urban areas of Beijing. All asthmatic participants were mild asthma. Asthmatic children were diagnosed according to the Global Initiative for Asthma (GINA, http://ginasthma.org) by pediatricians from Children Hospital of Capital Institute of Pediatrics in Beijing. Briefly, asthma was confirmed as repeated attacks of respiratory symptom (wheezing, shortness of breath, chest tightness, and cough), together with variable expiratory airflow limitation. Lung function and fractional exhaled nitric oxide (FeNO) of asthmatic children was measured by pediatricians. Allergic and non-allergic status of asthmatics was identified by skin prick testing. Children without any history of asthma, allergic disease, and chronic inflammation of the respiratory system were recruited as healthy controls. Height and weight were measured by trained professionals. In addition, all participants were asked to offer information about basic characteristics, and records of disease with a questionnaire based on the questionnaire of the International Study of Asthma and Allergies in Childhood. Food consumption of all participants were investigated by a questionnaire based on the food frequency questionnaire of National Health and Nutrition Examination Survey, which mainly including vegetables, mushrooms and eggs. Carbohydrate intake was defaulted to no difference, as all children shared similar carbohydrate consumption in their diet based on the local diet habits. This study was conducted in accordance with the Declaration of Helsinki and approved by the ethical review committee of the Institute for Environmental Health and Related Product Safety, Chinese Center for Disease Control and Prevention (201,501). All participants and their legal guardians signed the written informed consent.

Fecal Sample Collection

A total of 57 fecal samples were collected between December 2017 to March 2018, including 27 samples from children with allergic asthma, 10 samples from children with non-allergic asthma and 20 samples from healthy children. All participants, who offered feces, were asked to meet the following criteria, including no oral drug intake or no vaccination history in the past one month, no injury or diarrhea in the past two weeks, girls were not in the period of the menstrual

cycle when offering feces. Fecal samples of all participants were collected into sterilized tubes and stored at a temperature of −80°C until detected. After removing low quality samples, gut microbiome was detected in 47 samples and metabolomics analysis was detected in 50 samples.

Gut Microbiome Detection and Analysis

Total genome DNA from fecal samples was extracted using the CTAB (Cetyltrimethylammonium Bromide) method. The V4 region of 16S rRNA genes was amplified by using the specific primers of 515F and 806R. Paired end sequencing (2 × 250 bp) was performed on the Illumina HiSeq 2500 (Illumina Inc., USA) by Novogene Bioinformatics Technology Co.

Paired-end reads were assigned to samples based on the unique barcode and truncated by cutting off the barcode and primer sequence. Then they were merged using Fast Length Adjustment of Short reads (FLASH, v1.2.7) to obtain the raw tags.[26] The demultiplexed data were conducted using QIIME2.[27] Errors were corrected by DADA2.[28] Then a tree of phylogenetic diversity analyses was generated. Taxonomy was classified according to the database of SILVA.[29] Chao1 and Simpson indices were calculated for evaluating the alpha diversity of gut microbiome. Weighted and unweighted unique fraction metric (UniFrac) distances were calculated to estimate beta diversity.[30] Principal coordinates analysis (PCoA) was used to show the clustering of all samples. Linear Discriminant Analysis (LDA) of effect size (LEfSe) was used to discriminate the taxa with statistical and biological difference.[31]

Metabolomics Detection and Analysis

An untargeted metabolomics analysis on fecal samples was carried out at METABOLON's Discovery HD4™ Metabolomics Platform (Calibra (DIAN) - Metabolon Joint Laboratory, Hangzhou, China). Samples were prepared and detected according to the operation process of the platform.[32] Raw data was extracted, peak-identified and QC processed using Metabolon's proprietary software. Compounds were identified by comparison to library entries of purified standards. Peaks were quantified using area-under-the-curve. Missing values in samples were filled in with the minimum observed value for each compound, and raw data were normalized to the median of each compound in all samples. Functional pathways were annotated using Kyoto Encyclopedia of Genes and Genomes (KEGG).

Statistical Analysis

Statistical analysis was carried out using R software (v4.0.2). Characteristics of study populations were analyzed by the method of Chi square test, *t*-test and ANOVA. Differences in gut bacteria diversity and relative abundance among three groups were analyzed by the nonparametric Kruskal–Wallis test. Differences between two groups were analyzed by the Wilcoxon test. Difference of community structure of gut microbiome among groups was analyzed using the method of permutational multivariate ANOVA (PERMANOVA). Metabolomic data were log transformed before statistical analysis. Multiple linear regression was used to identify metabolites that were associated with asthma, by controlling for age, gender and BMI. Spearman correlation was used to analyze the correlation between gut microbes and metabolites. All reported p values were two-tailed and $p < 0.05$ was considered significant.

Results

Characteristics of Study Populations

A total of 57 participants were enrolled in this study, including 27 children with allergic asthma, 10 children with non-allergic asthma and 20 healthy children for control. Of these participants, 54.4% were boys, and 45.6% were girls. The mean age of all participants was 8.0 ± 2.0 years old. All children were Han nationality. Table 1 showed the baseline characteristics of each group. Table 2 showed the details of clinical information of all asthmatic children, including lung function, FeNO and allergens. There was no significant difference in lung function between allergic and non-allergic asthmatic children (*t*-test, $p > 0.05$). Allergic asthmatic children showed a significantly higher value of FeNO (*t*-test, $p = 0.021$). Approximately 59.2% allergic asthma children had two or more allergens.

Table 1 Baseline Characteristics of All Study Populations

Variable	Allergic Asthmatics (n=27)	Non-Allergic Asthmatics (n=10)	Healthy Control (n=20)	P value
Gender				0.080
Boys, n (%)	15 (55.6)	4 (40.0)	9 (45.0)	
Girls, n (%)	12 (44.4)	6 (60.0)	11 (55.0)	
Age (years, mean ± SD)	6.9 ± 1.4	9.3 ± 2.8	8.9 ± 1.5	< 0.010
BMI (kg/m^2, mean ± SD)	16.5 ± 1.8	18.7 ± 2.9	17.9 ± 3.5	0.298
High-fiber food intakes[a] (times/week, mean ± SD)	8.9 ± 3.5	10.6 ± 3.2	11.4 ± 5.6	0.150
High-protein food intakes[b] (times/week, mean ± SD)	4.5 ± 0.6	3.8 ± 1.5	4.5 ± 0.9	0.107

Notes: [a]High-fiber food included vegetables, mushrooms and fruit. [b]High-protein food included eggs and duck eggs.
Abbreviations: BMI, body mass index; SD, standard deviation.

Table 2 Clinical Information of Asthmatic Children

Variable	Allergic Asthmatics (n=27)	Non-Allergic Asthmatics (n=10)
Lung function (%, mean±SD)[a]		
FEV1	96.2±16.1	85.9±21.3
FVC	97.6± 7.0	90.8±19.7
MMEF75/25	75.3± 29.0	61.2±20.8
FeNO (ppb, mean±SD)	39.1± 31.1	21.3±11.9
Allergens foy skin pick testing		
Cat and dog allergies, n (%)	7 (25.9)	None
Mites, n (%)	11 (40.7)	None
Pollens of weeds, trees and grasses, n (%)	13 (48.2)	None
Fungi, n (%)	11 (40.7)	None

Note: [a]Lung function was the percentage of measured value to predicted value.
Abbreviations: SD, standard deviation; FEV1, forced expiratory volume in the first second; FVC, forced vital capacity; MMEF, maximal mid-expiratory flow; FeNO, fractional exhaled nitric oxide; ppb, part per billion.

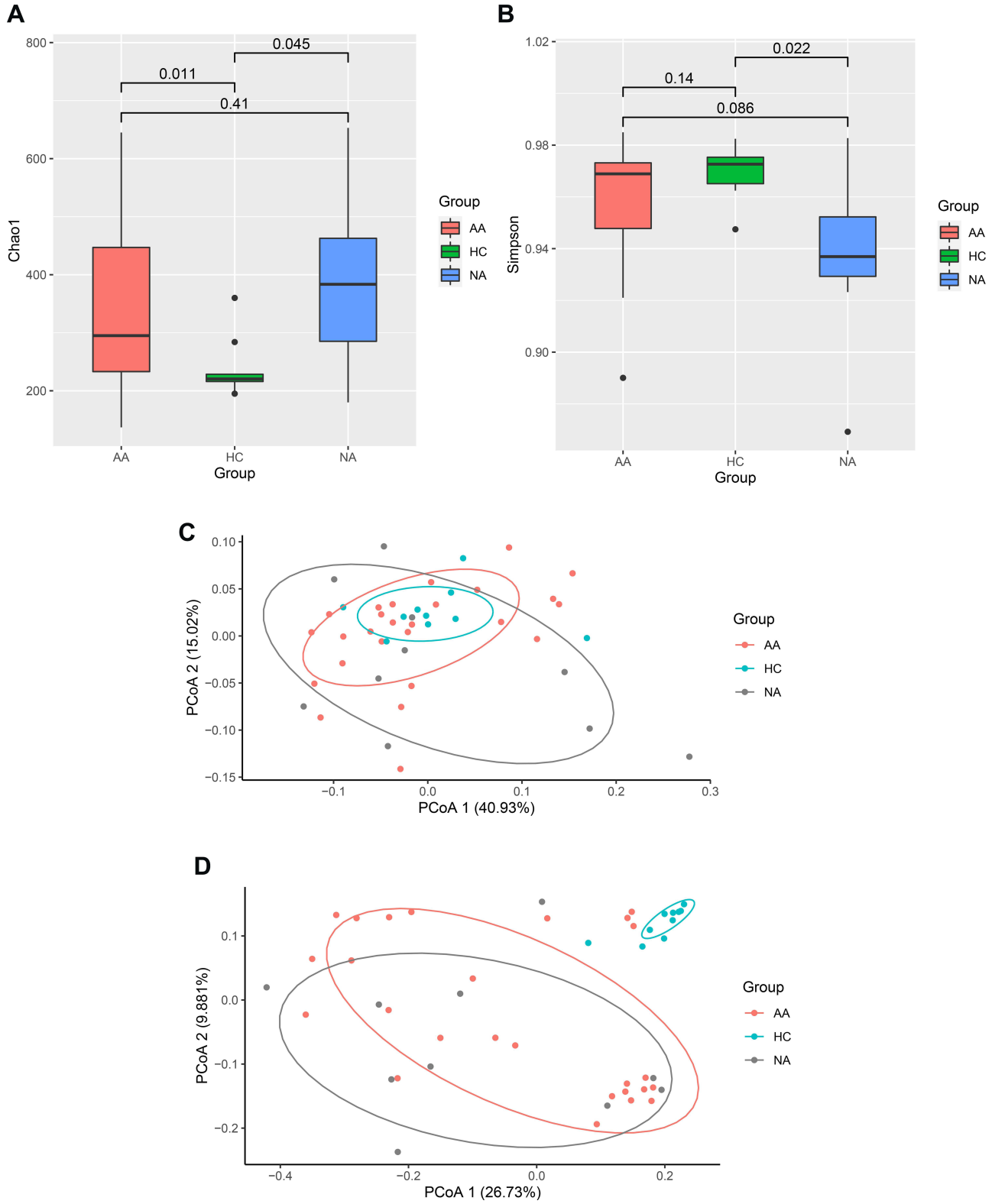
Characteristics of Gut Microbiome

Diversity of Gut Microbiome

We performed a 16S rRNA genes sequencing to investigate characteristics of gut bacteria in allergic and non-allergic asthmatics. Chao1 (p =0.025) and Simpson (p =0.024) indices showed significant differences among the three groups (Kruskal–Wallis test). Figure 1A and B showed the parameters of alpha diversity by groups. These results reflected a higher bacterial richness and a lower diversity in asthma group. We assessed beta diversity of gut microbiome based on weighted and unweighted UniFrac distance. Figure 1C and D showed beta diversity with the PCoA plot among the three groups. PERMANOVA analysis showed a significant difference among the three groups based on unweighted UniFrac distance (p = 0.001).

Differential Gut Microbes Among Groups

All of the observed sequences were assigned to 35 phyla. Figure 1E showed the relative abundance of top 10 phyla among the three groups. *Firmicutes, Bacteroidetes, Actinobacteria*, and *Proteobacteria* were the dominant phyla of all samples. *Proteobacteria* showed significant difference among the three groups (Kruskal–Wallis test, p = 0.008). Both



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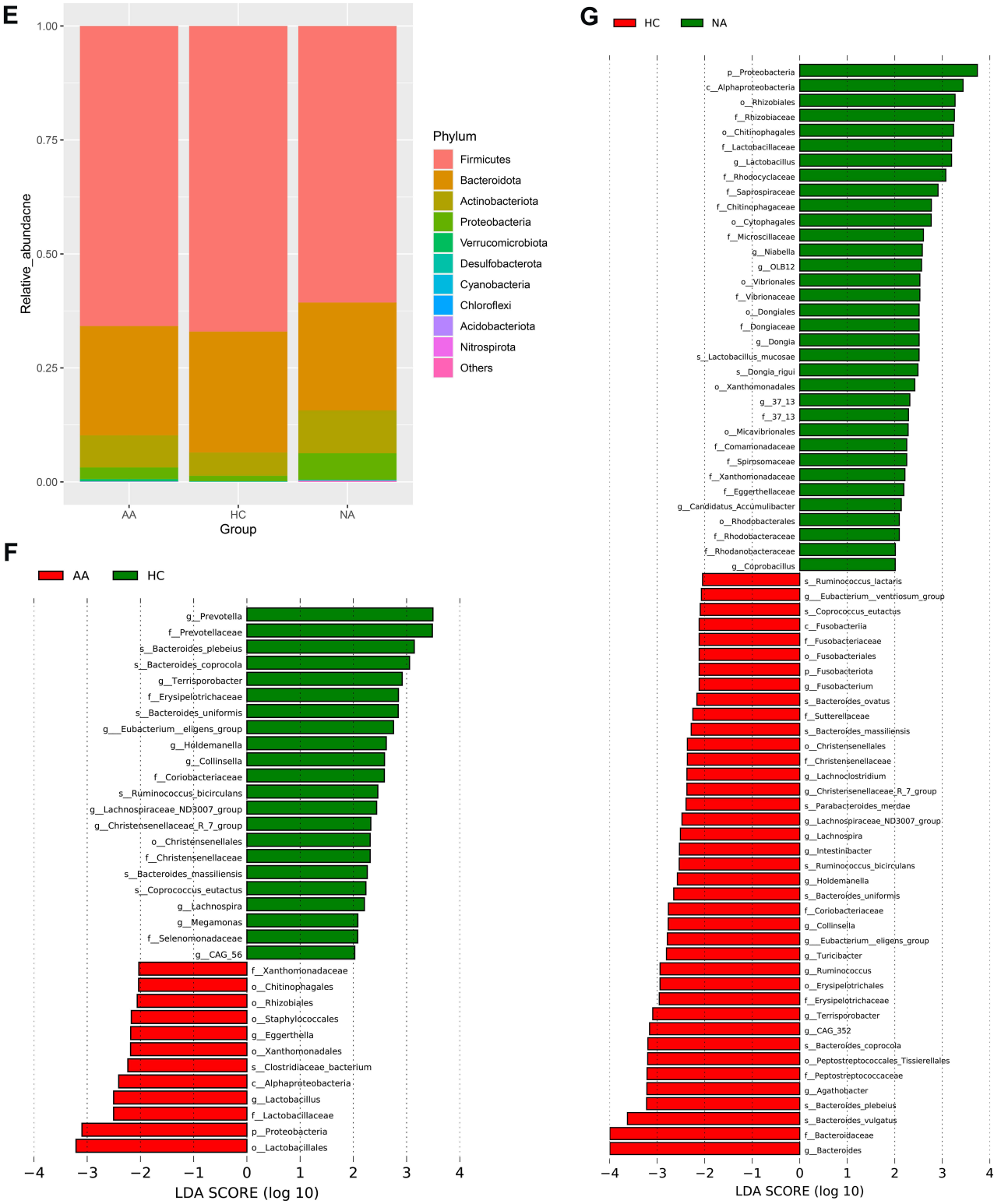

Figure 1 Characters of gut microbiome. (**A** and **B**) Alpha diversity (Chao1 and Simpson parameter) in allergic, non-allergic asthma and healthy control groups. (**C**) PCoA plot based on weighted UniFrac distance, (**D**) PCoA plot based on unweighted UniFrac distance. (**E**) Relative abundance of top 10 phyla by groups. The height of bars represents relative abundance. (**F**) LEfSe analysis between allergic asthma and healthy control groups. (**G**) LEfSe analysis between non-allergic asthma and healthy control groups. The graph of LEfSe distinguished the microbial communities of each group with LDA > 2 and $p < 0.05$.
Abbreviations: AA, allergic asthma group; NA, non-allergic asthma group; HC, healthy control group.

allergic (p =0.007) and non-allergic (p =0.019) asthma groups showed significant increases compared with healthy group (Wilcoxon test).

We used LEfSe analysis to discriminate taxa with statistical and biological differences among the three groups, stratified by allergy and non-allergy status. Figure 1F showed the taxa with significant discriminations between allergic asthma and healthy control groups. Figure 1G showed the taxa with significant discriminations between non-allergic asthma and healthy control groups. All discriminated taxa were from phyla of *Firmicutes, Bacteroidetes, Actinobacteria* and *Proteobacteria*, except for *Fusobacteriota*. Difference of *Fusobacteriota* was induced only by *Fusobacterium* at genus level, which decreased significantly in non-allergic asthma group. The richest taxon in allergic asthma was *Lactobacillales*. The next one was phylum of *Proteobacteria*, including *Alphaproteobacteria, Rhizobiales*, and *Xanthomonadales* (*Xanthomonadaceae*). While in non-allergic asthma group, more taxa (allergic/non-allergic:12/34) ranging from phylum to species were significantly overrepresented, especially more taxa (allergic/non-allergic:5/19) from *Proteobacteria* showed significant differences. In healthy control group, most of the discriminated taxa were from *Bacteroidetes* and *Firmicutes* at phylum level. For phylum of *Bacteroidetes, Prevotella* and *Bacteroides* showed significant richness. For phylum of *Firmicutes*, more bacteria from class of *Clostridia* showed significant richness, *such as Terrisporobacter, Eubacterium eligens group, Lachnospira, Lachnospiraceae ND3007 group*, and *Christensenellaceae R-7 group*.

Then we assessed the alterations in relative abundance at genus level. We found 9 genera of the top 50 genera showed significant differences between allergic asthmatics and healthy controls (8 genera decreased, 1 genus increased), and 14 genera of the top 50 genera showed significant differences between non-allergic asthmatics and healthy controls (13 genera decreased, 1 genus increased) (Wilcoxon test, $p < 0.05$). Table 3 presented details of the genera with significant differences. We noted that 10 genera among the significantly decreased genera of all asthmatics belonged to *Clostridia*.

Based on the altered genera obtained from LEfSe and Wilcoxon test analysis, we compared the altered gut bacteria in allergic and non-allergic asthma. Among the significantly increased genera in asthmatics, allergic and non-allergic asthma shared 1 genus (*Lactobacillus*), while *Eggerthella* increased only in allergic asthma, *Coprobacillus, Niabella, Chitinophagales|37-13, Microscillaceae OLB12, Candidatus Accumulib*, and *Dogia* increased only in non-allergic asthma. Among the significantly decreased genera in asthmatics, allergic and non-allergic asthma group shared 7 genera, including *Collinsella, Christensenellaceae R 7 group, Eubacterium eligens group, Lachnospira, Lachnospiraceae ND3007 group, Terrisporobacter*, and *Holdemanella. Megamonas, Lachnospiraceae CAG-56*, and *Prevotella* only decreased in allergic asthmatics. *Intestinibacter, Lachnoclostridium, Ruminococcus, Agathobacter, Eubacterium ventriosum group, Ruminococcaceae CAG-352, Turicibacter, Parasutterella, Bacteroides*, and *Fusobacterium* only decreased in non-allergic asthmatics.

Characteristics of Gut Metabolomics

To further study the functions of gut bacteria, we conducted an untargeted metabolomics analysis. A total of 811 metabolites were found in all samples. We analyzed the associations of asthma status with the metabolites by the method of multiple linear regression, controlling for age, gender and BMI. Among all metabolites, 42 metabolites were significantly associated with allergic asthma (14 of them elevated and 28 of them down regulated), and 58 metabolites were significantly associated with non-allergic asthma (9 of them elevated and 49 of them down regulated) ($p < 0.05$). Only 6 metabolites showed significantly associations with both allergic and non-allergic asthma. Table 4 showed the pathway of the significantly altered metabolites involved in allergic and non-allergic asthma groups. Supplementary Table 1 showed details of the significantly altered metabolites in allergic and non-allergic asthma groups.

The altered metabolites mainly involved in amino acid and lipid metabolism. More metabolites of amino acid and lipid metabolism showed significant alterations in non-allergic asthmatic group. For amino acid metabolism, 8 metabolites were significantly altered in allergic asthmatics, while 15 metabolites were significantly altered in non-allergic asthmatics. Only tryptophan and lysine metabolism showed significant alterations in both allergic and non-allergic asthmatic groups. Especially, the metabolites of histidine metabolism, including histamine, N-acetylhistamine, and 4-imidazoleacetate, showed significant increases only in non-allergic asthma group. For fatty acid metabolism, 17 metabolites were significantly decreased in asthmatic groups (p <0.05). Among them, only 4 metabolites were significantly changed in allergic asthmatic group, while 15 ones were significantly changed in non-allergic asthmatic group. Fatty acids metabolism in non-

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Table 3 Genera with Significant Differences in Relative Abundance Between Asthmatic Group and Healthy Control Group (Wilcoxon Test)

Genus	Affiliated Phylum	Affiliated Class	Affiliated Order	Affiliated Family	Asthmatics	Healthy Control	P value
					Mean ± SD[a]	Mean ± SD[a]	
Allergic asthmatic group vs Healthy control group							
Prevotella	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	1.27±5.26	4.19±9.41	<0.001
Lactobacillus	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	0.42±0.42	0.09±0.12	0.006
Lachnospiraceae ND3007 group	Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	0.53±0.50	0.79±0.28	0.040
Collinsella	Actinobacteriota	Coriobacteriia	Coriobacteriales	Coriobacteriaceae	0.54±0.88	0.90±0.62	0.011
Terrisporobacter	Firmicutes	Clostridia	Peptostreptococcales. Tissierellales	Peptostreptococcacea	0.42±0.80	1.32±1.90	0.002
Megamonas	Firmicutes	Negativicutes	Veillonellales. Selenomonadales	Selenomonadaceae	0.15±0.17	0.26±0.19	0.005
Eubacterium eligens group	Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	0.26±0.30	0.82±0.35	<0.001
CAG.56	Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	0.26±0.35	0.38±0.27	0.017
Lachnospira	Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	0.30±0.54	0.44±0.17	0.003
Non-allergic asthmatic group vs Healthy control group							
Bacteroides	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	10.08±6.28	20.35±7.09	0.003
Agathobacter	Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	1.72±1.64	3.38±2.16	0.043
CAG.352	Firmicutes	Clostridia	Oscillospirales	Ruminococcaceae	1.31±1.00	2.82±1.61	0.029
Ruminococcus	Firmicutes	Clostridia	Oscillospirales	Ruminococcaceae	0.95±0.85	1.82±0.80	0.029
Intestinibacter	Firmicutes	Clostridia	Peptostreptococcales. Tissierellales	Peptostreptococcaceae	0.81±0.74	1.14±0.33	0.029
Lachnoclostridium	Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	0.42±0.14	0.68±0.20	0.003
Lactobacillus	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	1.86±4.00	0.09±0.12	0.012
Turicibacter	Firmicutes	Bacilli	Erysipelotrichales	Erysipelotrichaceae	0.40±0.35	1.03±1.42	0.005
Lachnospiraceae ND3007 group	Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	0.46±0.55	0.79±0.28	0.023
Collinsella	Actinobacteriota	Coriobacteriia	Coriobacteriales	Coriobacteriaceae	0.27±0.30	0.90±0.62	0.009
Terrisporobacter	Firmicutes	Clostridia	Peptostreptococcales. Tissierellales	Peptostreptococcaceae	0.12±0.10	1.32±1.90	<0.001
Parasutterella	Proteobacteria	Gammaproteo-bacteria	Burkholderiales	Sutterellaceae	0.23±0.25	0.34±0.24	0.043
Eubacterium eligens group	Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	0.18±0.14	0.82±0.35	<0.001
Lachnospira	Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	0.11±0.09	0.44±0.17	<0.001

Note: [a]Relative abundance, %.
Abbreviation: SD, standard deviation.

Table 4 The Involved Metabolic Pathways of the Altered Metabolites in Allergic and Non-Allergic Asthma Groups

Pathway	Allergic Asthmatics (Up, Down)	Non-Allergic Asthmatics (Up, Down)
Amino Acid	8 (1,7)	15 (4,11)
Lipid	10 (5,5)	19 (1,18)
Peptide	7 (0,7)	2 (0,2)
Carbohydrate	0	1 (0,1)
Energy	2 (2,0)	0
Nucleotide	5 (1,4)	4 (0,4)
Cofactors and Vitamins	2 (1,1)	4 (2,2)
Xenobiotics	8 (4,4)	13 (2,11)

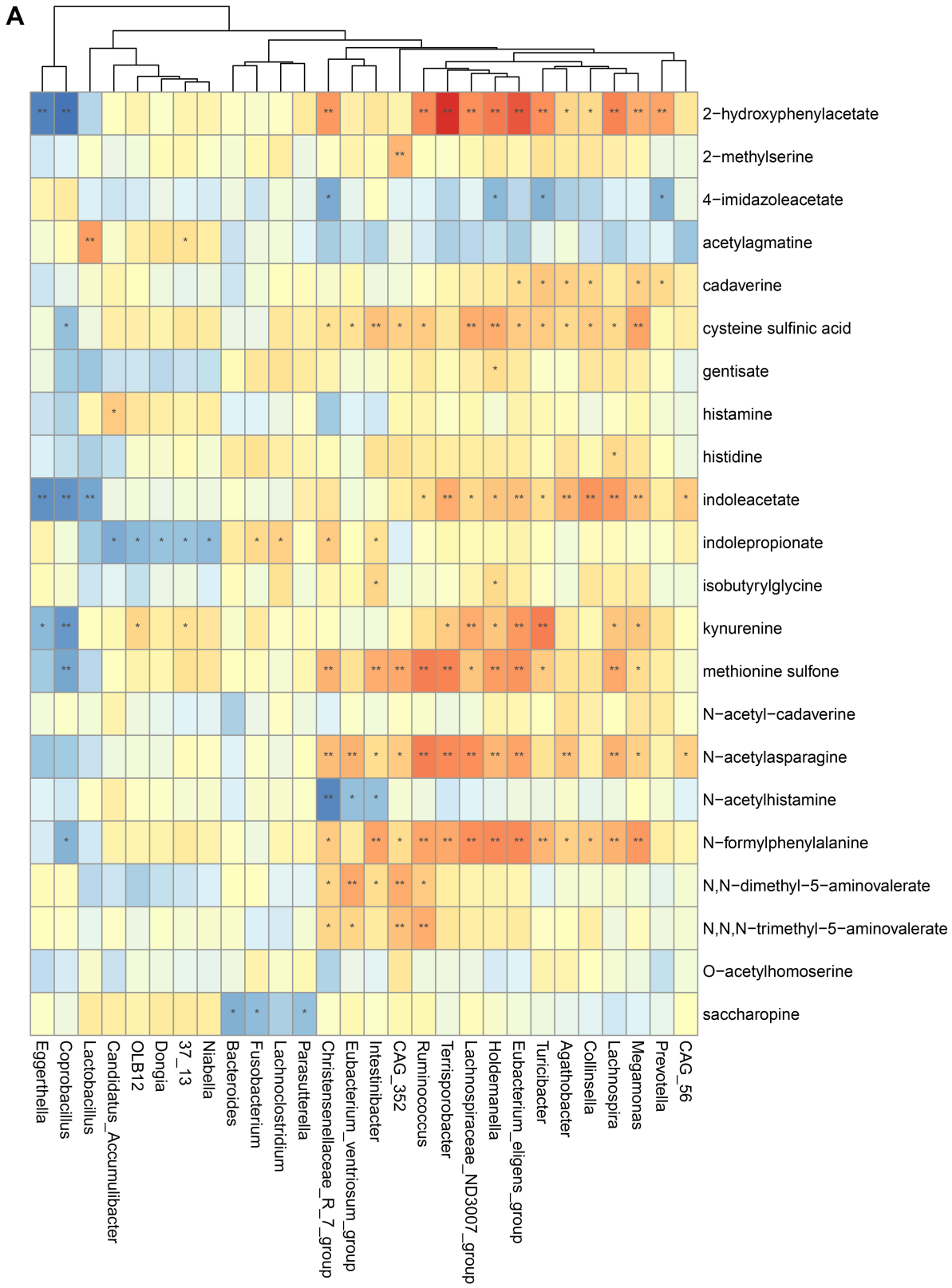
allergic asthmatic group presented more alterations. A marginally significant decrease of SCFAs (p =0.089), and significant decreases in medium chain fatty acids and long chain fatty acids (p <0.05) were observed in non-allergic asthma compared with healthy control group.

Correlation Between Gut Microbiome and Metabolites

To validate the association between the altered gut microbiome and the altered metabolites in the pathogenesis, we performed a Spearman correlation analysis with the samples tested for both gut microbiome and metabolomics. We analyzed the correlations between the altered genera and the altered metabolites, except xenobiotics metabolites. Figure 2A–E presented the correlations between the altered gut microbes and metabolites. We found that the metabolites of amino acid and lipid metabolism showed more correlations with gut microbes. Figure 2A showed that 28 genera were significantly associated with 20 metabolites of amino acid. Decreased bacteria of *Clostridia* mainly showed significantly positive correlations with metabolism of tryptophan, tyrosine, lysine, methionine and cysteine. For histidine metabolism, decreased *Lachnospira* showed a significantly positive correlation with histidine, increased *Candidatus Accumulibacter* showed a significantly positive correlation with histamine, decreased *Christensenellaceae R 7 group, Eubacterium ventriosum group*, and *Intestinibacter* all showed significantly negative correlations with N-acetylhistamine. *Christensenellaceae R 7 group, Holdemanella, Turicibacter*, and *Prevotella* showed significantly negative correlations with 4-imidazoleacetate. Figure 2B presented that 24 altered genera showed significant correlations with 26 metabolites of lipid metabolism. Among them, 12 genera from *Clostridia* showed significant correlations with fatty acid metabolism. *Microscillaceae OLB12* was negatively associated with valerate, *Eggerthella* and *Coprobacillus* were positively associated with valerate. *Candidatus Accumulibacter* and *Niabella* were negatively associated with medium chain fatty acid, *Lachnoclostridium* was positively associated with medium chain fatty acid. *Eggerthella, Coprobacillus, Fusobacterium* and *Lachnoclostridium* showed positive correlations with Long Chain Fatty Acid, *Microscillaceae OLB12* and *Terrisporobacter* showed negative correlations with long chain fatty acid. Figure 2C showed that the altered genera were less associated with peptide metabolism. Figure 2D showed that 22 altered genera were significantly positively associated with 7 nucleotide metabolites, only *Eggerthella* and *Coprobacillus* were significantly negatively associated with 4 nucleotide metabolites. Figure 2E presented 11 genera were significantly negatively associated with TCA cycle, 9 genera were significantly positively associated with biocytin.

Discussion

In this study, we combined 16S amplicon sequencing and untargeted metabolomics analysis to explore the gut microbiome association with childhood asthma by allergic status. We found that allergic asthmatic children and non-allergic asthmatic children shared some alterations in gut microbes and microbiome-derived metabolites. In addition, we also



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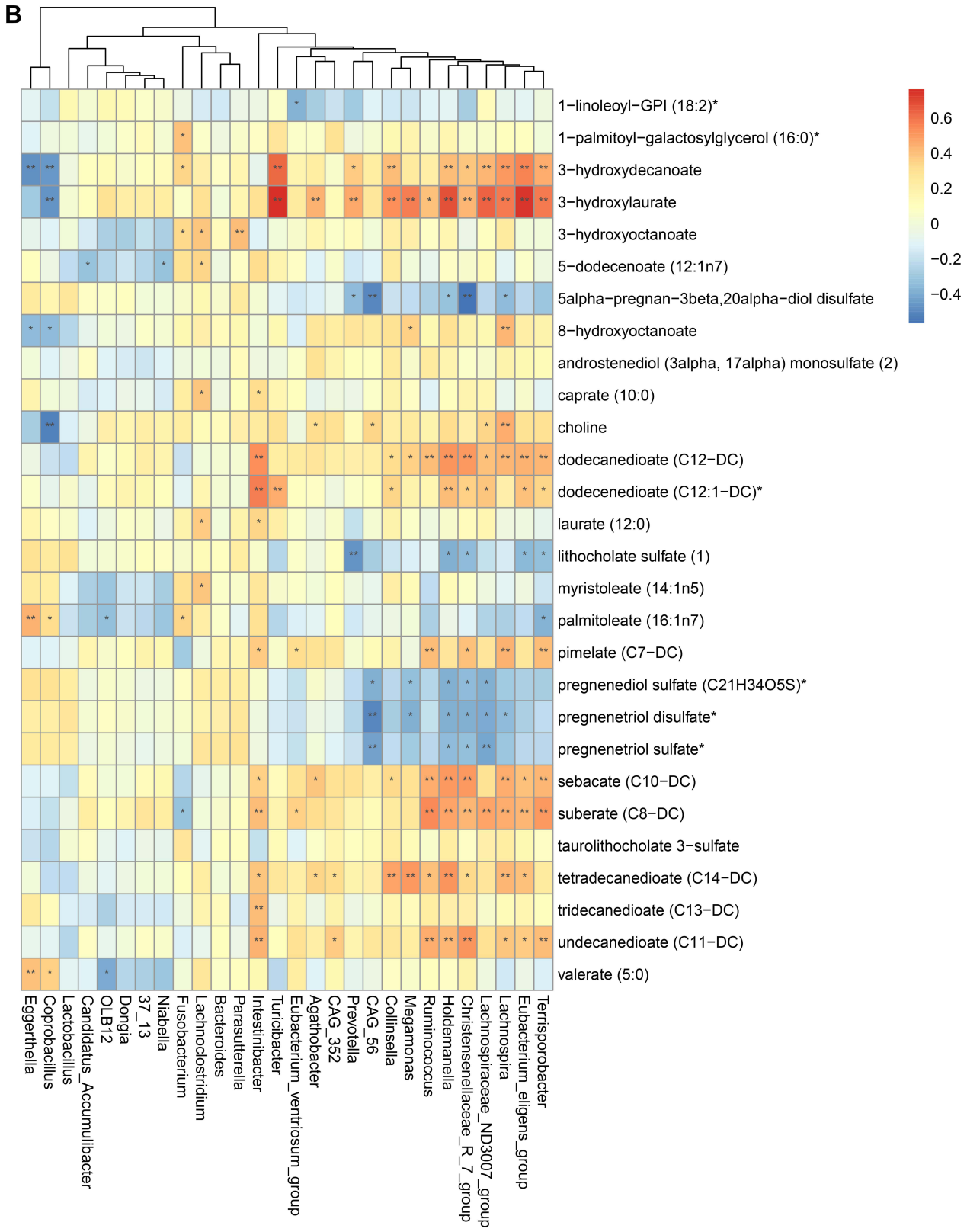

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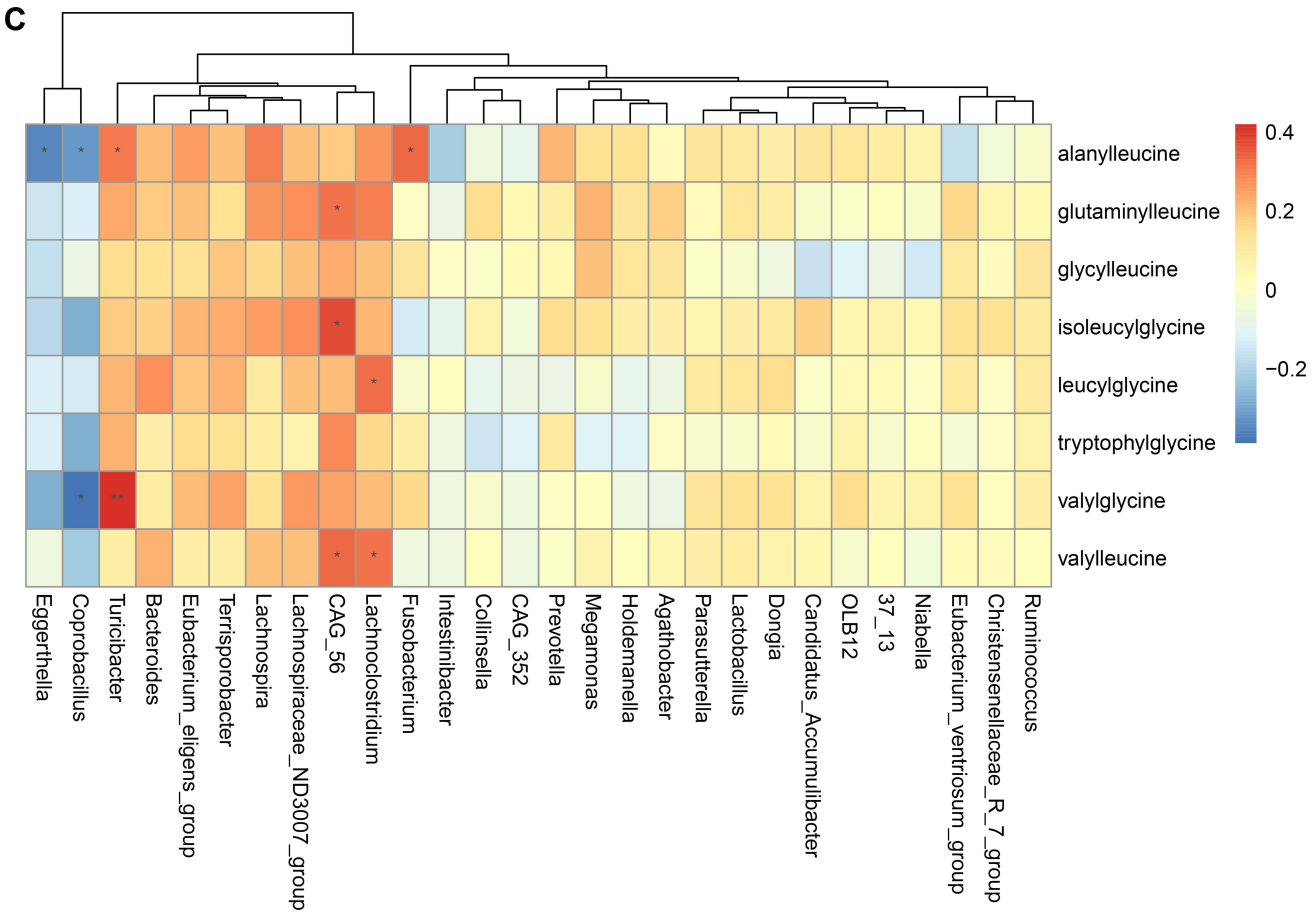

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found that non-allergic asthma had some distinct commensal gut microbes and microbiome-derived metabolites relative to that of allergic asthma.

Firstly, as previous findings we found asthma children had lower gut microbiome diversity than healthy controls.[24,33,34] Variation of gut bacterial diversity has been found closely associated with allergic disease including food allergies, diabetes, asthma, even cancer.[34–38] Apart from the diversity difference between asthmatic children and healthy controls, we found distinguished Simpson diversity (marginally significant) between allergic and non-allergic asthma children. The heterogeneity of gut microbiome might be related with phenotypic features.[39] Lifestyle and diet are important impacting factors of gut microbiome. However, all participating children in this study came from Beijing. They were all students and they were similar in self-reported diets. Therefore, our findings of diversity difference in asthma children suggested that gut microbiome might play a role in asthma phenotype development.

The pathological mechanism of non-allergic asthma has not been fully elucidated yet. Recent studies showed that the human gut microbiome would be an etiological factor of various diseases.[39] In this study, gut microbiome was especially altered in asthma children including *Clostridia* and *Proteobacteria*. While the altered genera and counts of *Clostridia and Proteobacteria* were quite different between allergic and non-allergic children. Existing studies have proved that *Clostridia* and *Proteobacteria* were related to the pathogenesis of asthma. *Clostridia* was reported to produce SCFAs which may stimulate the differentiation of Tregs.[34–36] Tregs might mediate immunomodulation during asthma development.[40] Impaired Tregs would predispose to severe viral bronchiolitis in infancy which was thought to be a major risk of asthma.[41] Gut *Proteobacteria* has been reported to significantly increase in asthmatics.[42] Several studies showed that *Proteobacteria* was higher in the airway of asthmatic subjects.[43–45] *Brevundimonas vesicularis* of *Proteobacteria* was found enriched in non-allergic asthma.[24] More serious dysbiosis in *Clostridia and Proteobacteria*

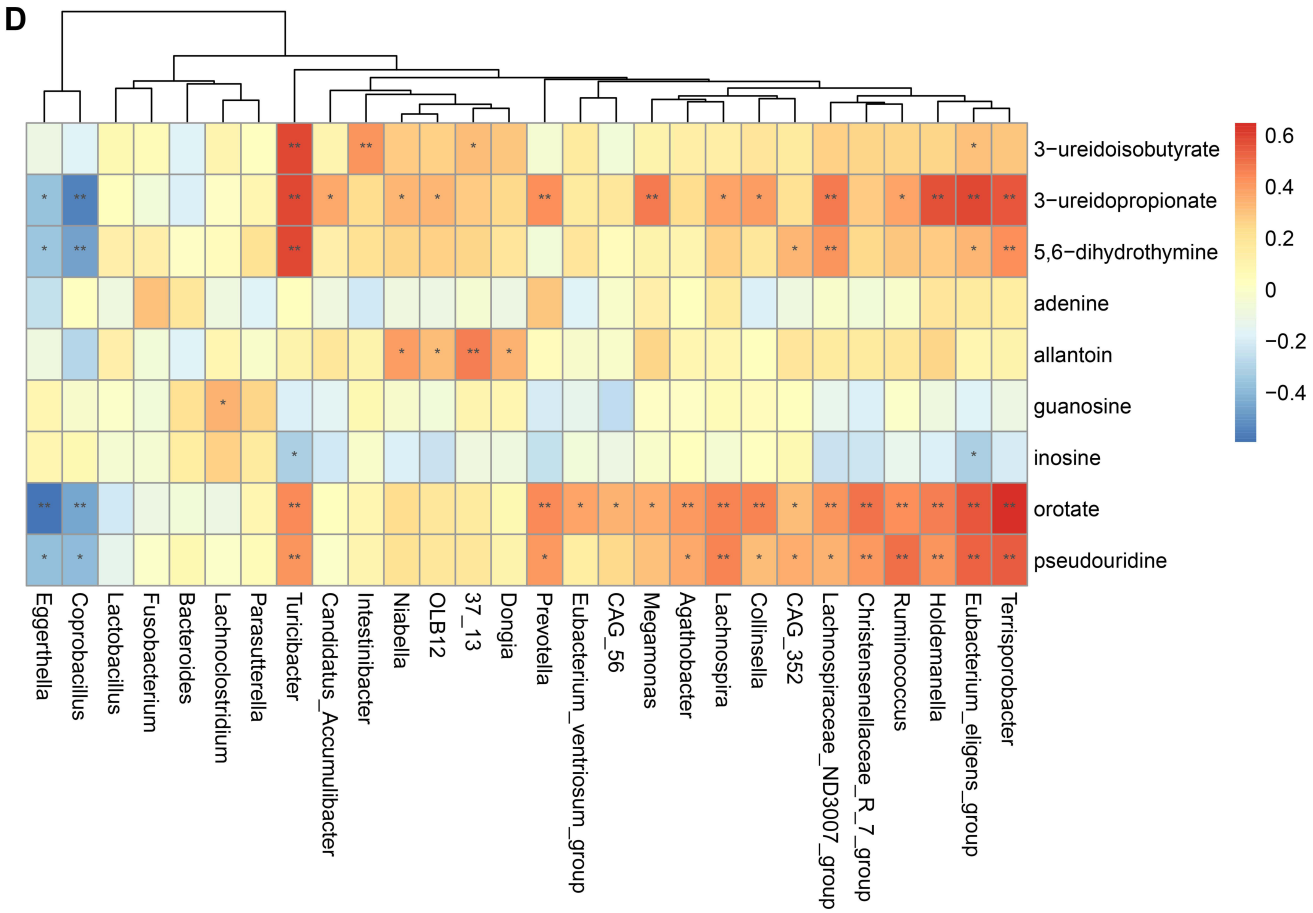

Figure 2 Continued.

in non-allergic asthma suggested that they might also play a role in asthma phenotype development. However, few studies explored the host-microbe association by allergic status of asthma. Some certain gut microbes, including *Coprobacillus, Niabella, Chitinophagales|37-13*, and *Microscillaceae OLB12*, were enriched only in non-allergic children. The potential roles of these distinct microbes in the development of asthma need to be further studied.

We then further analyzed the metabolome of the gut microbiome for a better view of the microbe-host association. The most interesting finding was that histamine significantly increased only in non-allergic asthma children. Histamine is an important immunomodulator involved in allergic reactions and inflammatory responses. Its functions are determined by the histamine receptor subtypes. It can activate H1R to enhance Th1 and Th2 type immune responses, activate H4R in human immune cells to release pro-inflammatory cytokine and chemokines to modulate immune responses, such as IL-6, TNF-α, IL-8, and also can induce smooth muscle contraction via activating H1R.[46–48] Our finding suggested gut bacteria-derived histamine may be related with non-allergic asthma development. Gut histamine was derived from histidine decarboxylation under the action of histidine decarboxylase enzyme (HDC). Some bacteria can secrete HDC, such as *Oenococcus oeni* and *Lactobacillus hilgardii*.[49,50] Bacterial HDC gene level has been reported significantly elevated in asthmatics, *Morganella morganii* levels were correspondingly elevated in the gut microbiome.[51] We compared relative abundances of the altered microbes with microbe-derived metabolites. We did not find *Morganella morganii*'s correlation with histamine, however, we only found *Candidatus Accumulibacter* was positively correlated with histamine. It may be influenced by the sample size. To our knowledge, there was no study reported the relation of *Candidatus Accumulibacter* and histamine. *Candidatus Accumulibacter* has been widely used in enhanced biological phosphorus removal (EBPR). At the anaerobic phase of EBPR, SCFAs were absorbed. The role of *Candidatus Accumulibacter* in non-allergic asthma needs to be further studied.

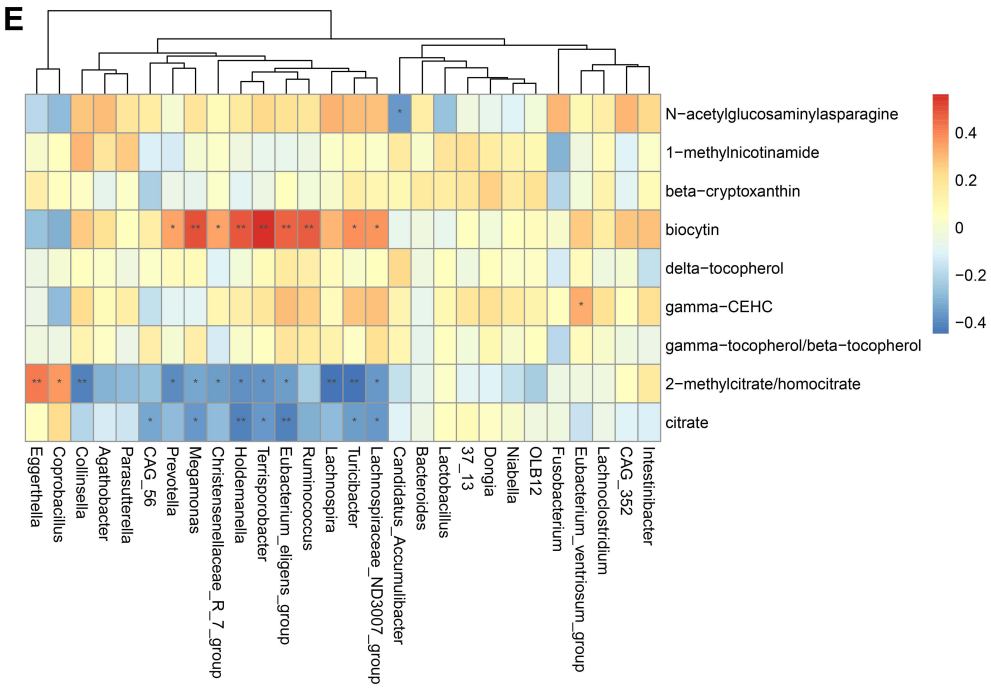

Figure 2 Spearman correlation heatmap for significantly altered metabolites and genera in asthmatics. *p <0.05, **p <0.01. (**A**) Correlation heatmap for amino acid. (**B**) Correlation heatmap for Lipid. (**C**) Correlation heatmap for peptide. (**D**) Correlation heatmap for nucleotide. (**E**) Correlation heatmap for others, including carbohydrate, energy, cofactors and vitamins.

Apart from the distinct increase of histamine in non-allergic asthmatic children, we found some genera of *Clostridia* were positively correlated with lipid and tryptophan metabolism. Lipid metabolism were altered in asthmatics in this study, which was consistent with previous finding.[52] Lipids have been reported to play an important role in the development of asthma through inflammatory responses.[53] Previous study showed that abnormal lipid metabolism was correlated with the severity and IgE levels in asthmatic patients.[54] Some microbes of *Clostridia* have been reported to correlated with asthma by producing SCFAs.[19] More lipid metabolites were changed in non-allergic asthma in our study. Combining analyzed with *Clostridia* dysbiosis in non-allergic asthma, it suggested that *Clostridia* may also play a role on asthma phenotype development by this pathway. Then we analyzed the metabolites in tryptophan metabolism, kynurenine down regulated in both allergic and non-allergic asthma children. Kynurenine plays an important role in immune responses and has been shown to modulate the systemic cytokine network in asthma patients.[55] It is a potent immunomodulatory molecule that may control T-cell immune responses.[56–59] Kynurenine can modulate Tregs generation to influence the immune responses.[60] Kynurenine pathway metabolism decreased in germ-free mice that lack of gut microbiota.[61] Our finding supported that *Clostridia* might also play a role in the development of asthma by kynurenine pathway.

To our knowledge, few studies explored host-microbe association of childhood asthma by allergic status and fewer studies explored the association with multi-omics research. Our findings in this study added evidence on that host-microbe association might be the etiological factor for the allergic phenotype development of asthma. There were still limitations in this study. Firstly, it was only an association analysis between gut microbiome and metabolomic. It is lacked of direct evidence, such as animal experiment or in vitro study. In addition, the sample size was not big, which was also a limitation of this study.

Conclusion

In this study we observed composition and microbial metabolites of gut microbiome of asthma children by allergic status. Dysbiosis of gut microbiome in allergic and non-allergic asthma suggested that gut microbiome play a critical role in

both types of asthma. The distinct altered gut microbes and the microbial derived metabolites among non-allergic asthma children suggested that gut microbiome might play a role in the modulation of asthma phenotype.

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Disclosure

The authors report no conflicts of interest in this work.

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