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ORIGINAL RESEARCH

Wheat-Dependent Exercise-Induced Anaphylaxis Patients on a Wheat-Free Diet Exhibit a Gut Microbiota Composition More Similar to Healthy Individuals

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Purpose: There are limited studies on the intestinal microbiome in patients with wheat-dependent exercise-induced anaphylaxis (WDEIA), and changes in the gut microbiome in WDEIA patients after wheat-free diet have not been studied.

Methods: This is a cross-sectional analysis. Fecal samples and clinical data were collected from 26 non-wheat-free patients with WDEIA, 11 wheat-free patients with WDEIA, and 24 healthy controls (HCs). The gut microbiota was evaluated through metagenomic sequencing.

Results: The sequencing revealed differences in the gut microbiome between patients with WDEIA on a non-wheat-free diet and HCs; more specifically, the non-wheat-free group exhibited a downregulation of two families (*Rikenellaceae* and *Odoribacteraceae*), three genera (*Alistipes, Odoribacter*, and *Catenibacterium*), and four species (*Bacteroides_stercoris, Alistipes_putredinis, Bacteroides_intestinalis*, and *Bacteroides_cellulosilyticus*). A wheat-free diet is associated with intestinal flora more similar to the structure of healthy individuals. The species *Bacteroides_stercoris* was negatively correlated with T-IgE, and the genus *Catenibacterium* was negatively correlated with T-IgE, as well as wheat, gluten, or gliadin-specific IgE. The genus *Catenibacterium* was positively correlated with the healthy control-enriched "Apoptosis (ko04210)" pathway and negatively correlated with the non-wheat-free WDEIA group-enriched "Thyroid hormone signaling pathway (ko04919)" pathway.

Conclusion: Patients with WDEIA exhibit a specific gut microbiota signature and function, which demonstrated the potential association between the gut microbiome and WDEIA development. WDEIA patients on a wheat-free diet exhibit a gut microbiome composition more similar to healthy individuals.

Keywords: wheat-dependent exercise-induced anaphylaxis, gut microbiota, metagenomic

Introduction

Wheat-dependent exercise-induced anaphylaxis (WDEIA) is a distinct type of immunoglobulin E (IgE)-mediated food allergy that can be life-threatening and manifests as urticaria, angioedema, and severe allergic reactions resulting in hypotension or anaphylactic shock.[1] Patients with WDEIA can generally tolerate wheat on its own. Allergic reactions occur usually when its ingestion is combined with inducing cofactors, including exercise, alcohol, and nonsteroidal anti-inflammatory drugs.[2] Importantly, allergic reactions can also occur at rest if the allergen concentration is sufficiently high.[3] The Long-term management strategies of WDEIA include dietary modification, exercise modification and education.[4] Dietary management may range from complete wheat exclusion to timing-based avoidance around exercise, with lifelong strict gluten-free diets reserved for concomitant celiac disease or persistent severe reactions. Exercise

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management involves altering the timing and intensity of activity relative to wheat intake and avoiding exertion when additional cofactors are present.[5] Few studies have investigated the pathogenesis of WDEIA; those that have mostly focused on the mechanisms of the triggering factors, demonstrating that cofactor-induced blood flow redistribution, plasma hyperosmolality, increased tissue transglutaminase activity, and enhanced gastrointestinal permeability may play a role in this disorder.[2] However, Scherf et al[2] reported that the absorption of gliadins, a class of proteins found in wheat and other grains, was unaffected by these cofactors in healthy individuals. Thus, hypersensitivity or damage to the intestinal epithelium may be responsible for the intolerance observed in those with WDEIA.[2]

The hygiene hypothesis highlights the potential role of the microbiome in the development of allergic disorders.[6] An increasing number of studies have demonstrated that intestinal dysbiosis is involved in the development of food allergies, including allergies to cow's milk and eggs.[7–9] The gut microbiome plays an essential role in the modulation of mucosal immunity and the programming of oral tolerance.[10,11] Alterations in the intestinal flora can lead to food intolerance and allergic disorders in the host,[12] and increased gastrointestinal permeability appears to be a key factor in the pathogenesis of WDEIA.[2] The gut microbiota can control intestinal barrier function by increasing barrier-promoting interleukin-22 expression and decreasing intestinal permeability.[13,14] Our previous study has identified differences in the gut microbiome composition of WDEIA patients through 16S ribosomal RNA (rRNA) gene sequencing of fecal samples.[15] However, 16S rRNA sequencing is limited to taxonomic and phylogenetic identification, with strain-level detection being challenging.[16] While adherence to a wheat-free diet can prevent anaphylaxis in WDEIA patients, its impact on gut microbiome composition remains unexplored.

Thus, this study aimed to comprehensively analyze the gut microbial signature and function in patients with WDEIA and investigate the effect of a wheat-free diet on their gut microbiome through whole-genome metagenomic shotgun sequencing of fecal samples.

Materials and Methods

Study Design

A total number of 26 non-wheat-free patients with WDEIA, 11 wheat-free patients with WDEIA, and 24 healthy controls (HCs) were recruited at Peking Union Medical Hospital (PUMCH) from 2021 to 2023. This study compares three groups: non-wheat-free WDEIA, wheat-free WDEIA, and healthy controls. Wheat-free refers specifically to avoidance of wheat-containing foods as self-reported by participants. Non-wheat-free refers to the participant's usual diet without intentional wheat avoidance. Among the 11 patients in the wheat-free group, 5 avoided wheat for more than one week and up to two weeks, 1 avoided wheat for more than two weeks and up to four weeks, and 5 avoided wheat for more than four weeks. The WDEIA groups were matched with the control group according to age, sex, and body mass index (BMI). WDEIA was diagnosed via the following criteria:[15] (i) anaphylaxis occurred only when physical activity occurred within 6 hours of wheat ingestion; (ii) gliadin-specific or gluten-specific IgE were positive; (iii) consuming a wheat-free diet prevented anaphylaxis; and (iiii) anaphylaxis could occur in the presence of other cofactors, such as nonsteroidal anti-inflammatory drugs, aspirin, and alcohol. Subjects with current gastrointestinal disorders, infections, autoimmune diseases, cardiovascular disorders, renal disease, or tumors were excluded. Subjects who were administered antibiotics or probiotics within the three-month period before the start of the study were also excluded. Information related to potential risk factors such as pet exposure, classification of the place of residence (rural or urban), the use of antibiotics or proton pump inhibitors and dietary history was acquired via questionnaires at the time of sample collection. Participation in the study was voluntary, and all participants provided written informed consent for inclusion. This study was approved by the Ethics Committee of our hospital (JS-2615).

Sample Collection and Sequencing

Fecal samples from the participants were collected in 5-mL stool containers, followed by transportation and freezing at −80 °C within 2 hours. Fecal DNA was extracted using an Omega Stool DNA Kit (Omega Bio-Tek, Norcross, GA, United States) according to the manufacturer's instructions. The purity and quality of the genomic DNA were measured by 1.0% agarose gel electrophoresis. The DNA was sheared to 300 base pairs (bp) using a Covaris S220 Focused

Ultrasonicator (Covaris, Inc., MS, United States). Sequencing libraries were constructed using the NEBNext Ultra DNA Library Prep kit (NEB, Beijing, China). Briefly, a sequencing library was prepared by end repair, A tailing, and ligation of Illumina-compatible adapters. The prepared libraries were subsequently sequenced on an Illumina HiSeq4000 platform at Allwegene Technology Co. Ltd. (Beijing, China). After the run, image analysis, base-calling, and error estimation were performed using Illumina Analysis Pipeline Version 2.6 (Illumina Inc., SanDiego, CA, United States).

Sequence Data Processing and Metagenomic Analyses

Quality control was performed using Trimmomatic software, including the elimination of adaptor sequences, host contamination, low-quality reads (N ratio greater than 1%), and content with low-quality bases ($Q \leq 20$) exceeding 50%. Remaining reads with a length of less than 150 bp were filtered out after the quality control steps. Sequencing data were assembled using MEGAHIT (v1.0.6)[17] and contigs less than 500 bp in length were filtered out. To minimize host contamination, each sample was compared with data from a host database to filter out reads that may have come from the host using Bowtie 2.[18] The open reading frames of the assembled contigs were predicted using Prodigal software.[19] Non-redundant gene sets were clustered using CD-HIT.[20] High-quality DNA reads were aligned to the National Center for Biotechnology Information NR database and annotated into different taxonomic groups using Diamond.[21] The Kyoto Encyclopedia of Genes and Genomes (KEGG) database was subjected to the protein-to-protein Basic Local Alignment Search Tool (BLASTP) to determine the KEGG annotations. KOBAS 2.0 was used for functional annotation based on the comparison results.

Analysis of the Microbiome Composition

Alpha diversity was estimated based on the species-level abundance of each sample according to the Shannon index using the vegan diversity() function in R software (v3.6.0), and significant differences were assessed using Wilcoxon's rank sum tests. Very rare taxa were filtered out on the basis of very low median relative abundance (<0.01%by total sum scaling). Partial least squares discrimination analysis (PLS-DA) was preformed to visualize sample distances using the "ade4" package in R. Linear discriminant analysis (LDA) scores, calculated using the linear discriminant analysis effect size (LEfSe) method, were used to identify the most differentially abundant taxa between two groups, with a significance level of alpha = 0.05 and an LDA threshold of 3.0. Correlations between the relative abundance of species, gut microbiome KEGG functional pathways, and WDEIA-related clinical indices were calculated using Spearman's rank correlation analysis and visualized using the ComplexHeatmap package in R software.

Statistical Analysis

Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) software (version 25.0; SPSS Inc., Chicago, IL, USA). Clinical indices are expressed as the mean ± standard deviation (SD) and were compared via 2-way ANOVA. Categorical data are expressed as percentages and were compared using the chi-square test or Fisher's exact test. The relative abundances of the intestinal microbiome were compared between two groups using the Wilcoxon rank-sum test and among three groups using the Kruskal–Wallis test. Multiple testing correction was performed using the Storey FDR (False Discovery Rate) method (Supplemental Table 1). *P*-values < 0.05 were considered statistically significant. Graphical representations were generated using GraphPad Prism (version 9.0) software (GraphPad Software, San Diego, IL, USA).

Results

Clinical Characteristics of the Study Populations

To investigate the signatures of the gut microbiota and their function in patients with WDEIA, we performed metagenomic sequencing of 37 fecal samples from patients with WDEIA (26 non-wheat-free and 11 wheat-free) and 24 age-, sex-, and BMI-matched healthy controls. There were no significant intergroup differences in terms of pet exposure, geographic classification of the place of residence, and the use of antibiotics or proton pump inhibitors. The baseline characteristics of the patients are presented in Table 1.

Table 1 Clinical Characteristics of the Study Populations

Variables	Non-Wheat-Free Patients with WDEIA (n=26)	Wheat-Free Patients with WDEIA (n=11)	Healthy Controls (n=24)	*P*-value
Age (years)	33.3 (10.64)	32.6 (11.7)	32.3 (9.5)	0.659
Male sex	13/26 (50)	4/11 (36.4)	8/24 (33.3)	0.458
BMI (kg/m^2)	23.24 (4.00)	20.81 (3.20)	21.26 (1.96)	0.052
Pet exposure	1/24 (4.2)	2/11 (18.2)	2/22 (9.1)	0.325
Geographic classification				0.792
Rural	1/24 (4.2)	1/11 (9.1)	1/22 (4.5)	
Urban	23/24 (95.8)	10/11 (90.9)	21/22 (95.5)	
Use of antibiotics				0.689
Frequent	4/24 (16.7)	2/11 (18.2)	2/22 (9.1)	
Seldom	20/24 (83.3)	9/11 (81.8)	20/22 (90.9)	
Use of proton pump inhibitors				0.325
Frequent	1/24 (4.2)	2/11 (18.2)	2/22 (9.1)	
Seldom	23/24 (95.8)	9/11 (81.8)	20/22 (90.9)	
T-IgE (kUA/L)	408 (52.5–2197)	239 (27.5–1370)	21.9 (2.03–871)	0.075
Wheat-specific IgE	0.43 (0.09–2.09)	0.25 (0–3.49)	0.01 (0.01–0.03)	0.025
Gluten- specific IgE	0.92 (0.10–8.46)	2.19 (0.06–20.8)	0	0.068
Gliadin-specific IgE	9.89 (0.42–49.4)	4.02 (0.79–32.6)	0	0.021

Alterations in Gut Microbial Populations in Patients with WDEIA

There was no significant difference in the alpha diversity among the three groups (Chao1 richness index, P=0.15; Shannon index, P=0.073). However, compared with the non-wheat-free WDEIA group, the distribution of microbial communities in the wheat-free WDEIA group was more similar to that of HC groups based on the PLS-DA (Figure 1A).

Compared to that of the HCs, differences in 273 gut flora species were identified in the non-wheat-free WDEIA group based on the Wilcoxon test (Figure 1B). To assess differences in bacterial taxon biomarkers between the non-wheat-free WDEIA and HC groups, LEfSe analysis was conducted to identify flora that differed significantly in abundance between the groups. The greatest differences in fecal microbiota taxa between the non-wheat-free WDEIA patients and HCs were identified based on an LDA score > 2 and a P-value < 0.05 (Figure 1C). The number of different taxa at each taxonomic level included two families, three genera, and four species, all of which were downregulated in the non-wheat-free WDEIA group, including *Rikenellaceae* and *Odoribacteraceae* at the family level, *Alistipes, Odoribacter*, and *Catenibacterium* at the genus level, and *Bacteroides_stercoris, Alistipes_putredinis, Bacteroides_intestinalis*, and *Bacteroides_cellulosilyticus* at the species level.

Our previous study[15] demonstrated that the bacterial genera *Blautia, Erysipelatoclostridium, Akkermansia*, and *Lachnospiraceae NK4A136* group were increased in patients with WDEIA, whereas *Lactobacillus* and *Dialister* were decreased. Thus, we focused on identifying the relevant species. Compared with that of the HCs, the relative abundance of the bacterial species *Blautia_coccoides* (P=0.026) was significantly increased, whereas those of *Lactobacillus_apis* (P=0.033), *Lactobacillus_mali (P*=0.040), and *Lactobacillus_buchneri* (P=0.017) were significantly decreased in the non-wheat-free WDEIA group (Figure 2).

A Wheat-Free Diet May Help Normalize the Intestinal Flora Structure of Patients with WDEIA

The heatmap of the top 30 intestinal bacterial species among the three groups showed that, compared to that of the non-wheat-free WDEIA group, the intestinal flora of the wheat-free WDEIA group was more similar to that of the HC group (Figure 3A).

Compared with that of the non-wheat-free WDEIA group, the intestinal bacteria that were enriched in the HC group were also increased in the wheat-free WDEIA group, including the families *Rikenellaceae, Odoribacteraceae*, the genera *Alistipes, Odoribacter*, and *Catenibacterium*, and the species *Bacteroides_stercoris, Alistipes_putredinis,*

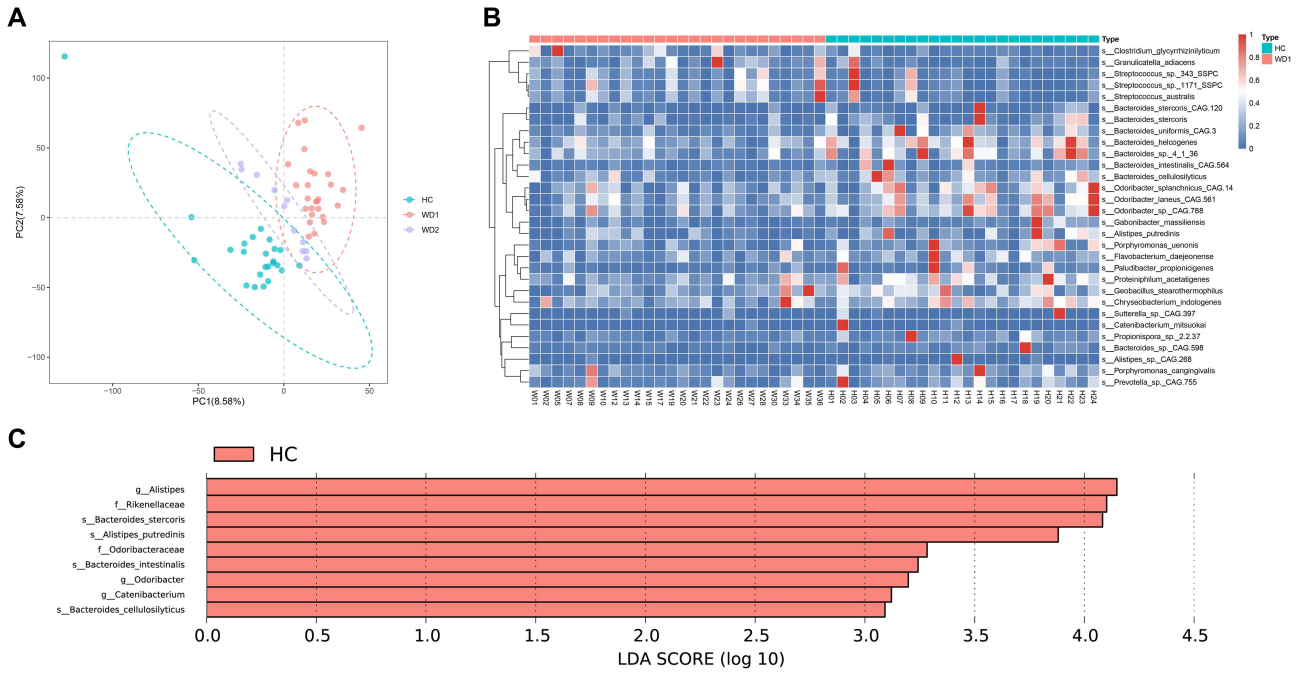

Figure 1 Taxonomic analyses of the fecal microbiota in patients with WDEIA. (**A**) β diversity (Bray–Curtis similarity index) analyses of microbial species between patients with WDEIA and HCs. (**B**) Heatmap of the top 30 species that differed between the non-wheat-free WDEIA patients (WD1) and HC based on the Wilcoxon test. (**C**) Histogram of the LDA scores between the WD1 and HCs (LDA score > 2).

Abbreviations: HCs, healthy controls; LDA, Linear discriminant analysis; WD1, non-wheat-free WDEIA patients; WD2, wheat-free WDEIA patients; WDEIA, wheat-dependent exercise-induced anaphylaxis.

Bacteroides_intestinalis, Bacteroides_cellulosilyticus, Lactobacillus_mali, and *Lactobacillus_apis*. The bacterial species *Blautia_coccoides* decreased in both the HC and wheat-free WDEIA groups (Figure 3B).

Associations of Gut Microbiota with Clinical Indices

Correlations between intestinal microorganisms at the species level (abundance > 0.1% by total sum scaling) and clinical characteristics were determined via Spearman's rank correlation analysis. Four species, including *Bacteroides_sp._3_1_33FAA, Bacteroides_sp._4_1_36, Bacteroidesstercoris*, and *Bacteroidesstercoris_CAG.120* were negatively associated with T-IgE, and 14 species, including *Blautia_massiliensis, Clostridioides_difficile*, and *Eubacterium_rectale*, were positively associated with T-IIgE, wheat-specific IgE, gluten-specific IgE, or gliadin-specific IgE (Figure 4).

Altered Gut Microbial Function in Patients with WDEIA

Regarding intergroup differences in gut microbial function, 25 KEGG metabolic pathways were significantly altered in the non-wheat-free patients with WDEIA compared with those of the HCs (Figure 5).

The group-wise distributions of T-IgE and wheat, gluten, or gliadin-specific IgE were in Table 1.

To further investigate the relationships between the clinical indices and differences in the gut microbiome and KEGG functional pathways in the non-wheat-free WDEIA, wheat-free WDEIA, and HC groups, correlation coefficients were calculated using the method described by Pedersenet et al.[22]

Two HC-enriched gut microbiomes and five HC-enriched pathways were negatively correlated with T-IgE, as well as wheat, gluten, or gliadin-specific IgE (Figure 6). In the HC group, the enriched pathways included those related to the "Renin-angiotensin system (ko04614)", "Lipopolysaccharide biosynthesis (ko 00540)", "Cationic antimicrobial peptide CAMP resistance (ko01503)", "Apoptosis (ko04210)", and "Huntington's disease (ko05016)"; these pathways were negatively correlated with wheat, gluten, or gliadin-specific IgE, and were positively correlated with 10 HC-enriched species. Notably, *Bacteroides_stercoris* was negatively correlated with T-IgE, and the genus Catenibacterium was negatively correlated with T-IgE, as well as wheat, gluten, or gliadin-specific IgE. The genus Catenibacterium was

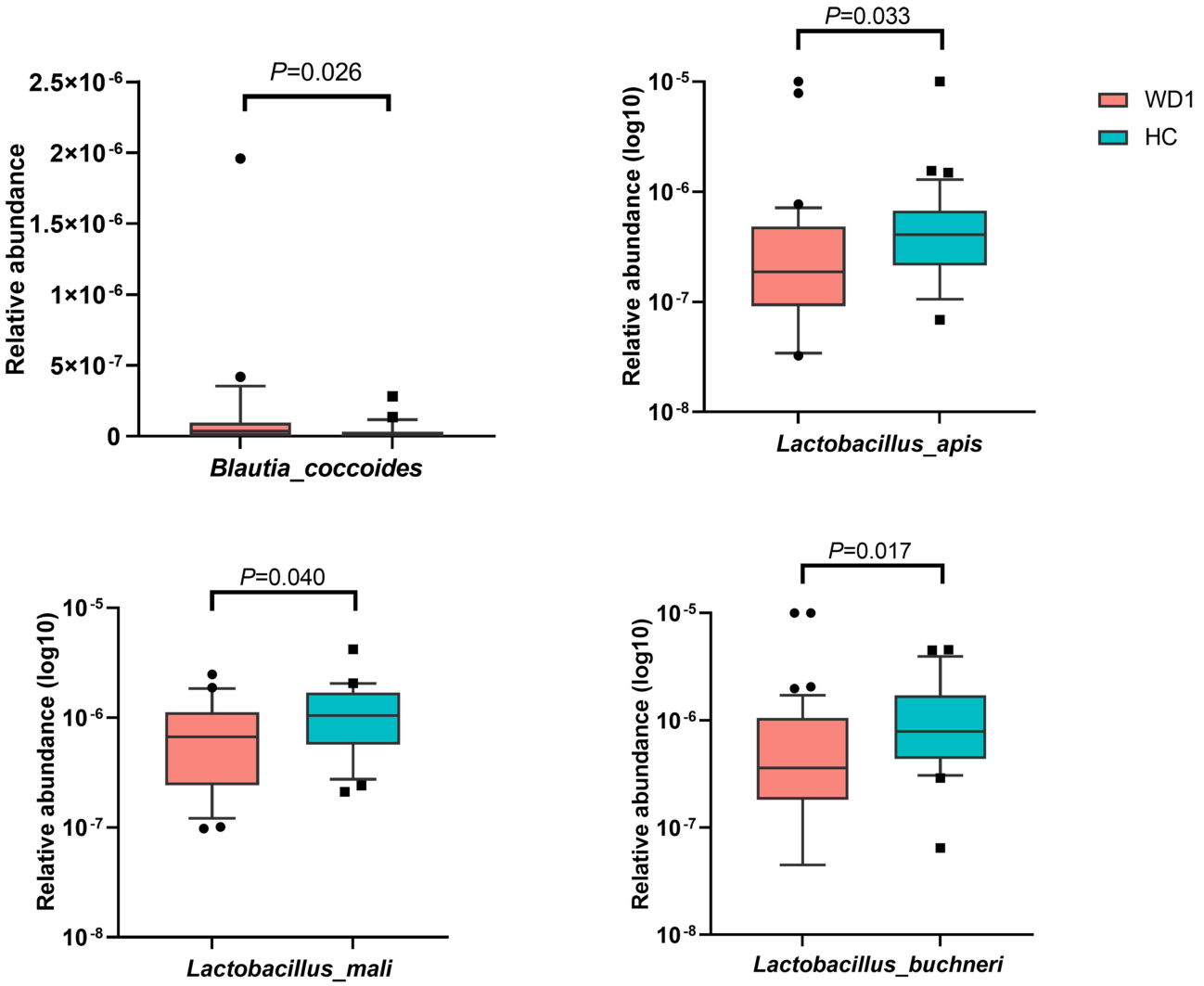

Figure 2 Comparation of relative abundance of the bacterial species by Wilcoxon rank sum test. The black dot, The outliers in non-wheat-free WDEIA group that exceed the 95% confidence interval; The black square, The outliers in healthy control group that exceed the 95% confidence interval.
Abbreviations: HC, healthy control group; WD1, non-wheat-free WDEIA group; WDEIA, wheat-dependent exercise-induced anaphylaxis.

positively correlated with the healthy control-enriched "Apoptosis (ko04210)" pathway and negatively correlated with the non-wheat-free WDEIA group-enriched "Thyroid hormone signaling pathway (ko04919)" pathway. Thus, gut microbes may affect metabolic pathways, thereby influencing the clinical indices.

Discussion

In this study, we conducted an in-depth characterization of the compositional and functional changes of the gut microbiota in WDEIA using metagenomic sequencing. We confirmed the association between the intestinal microbiota and WDEIA, and we discovered correlations between gut microbial function and clinical indicators of WDEIA. Furthermore, we compared gut microbiota traits between the non-wheat-free WDEIA, wheat-free WDEIA, and healthy controls and demonstrated, that WDEIA patients on a wheat-free diet exhibit a gut microbiome composition more similar to healthy individuals.

The previous study[15] compared gut microbiota between WDEIA patients and healthy controls, without considering the influence of dietary factors on the microbiome. IAdditionally, we employed whole-genome metagenomic shotgun sequencing, which provides species- and strain-level resolution along with functional gene annotation, yielding more detailed insights than the limited taxonomic data from 16S rRNA sequencing. Consistent with the findings of our

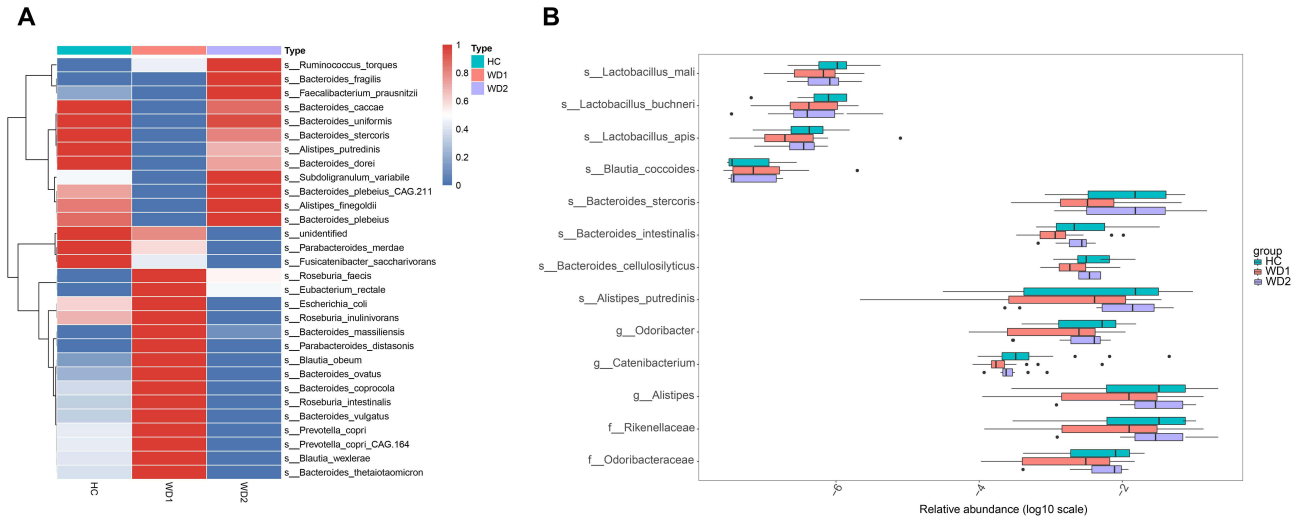

Figure 3 Comparison of the taxonomy of the fecal microbiota between the WD1, WD2, and HC groups. (**A**) Heatmap of the top 30 intestinal bacteria species. (**B**) A comparison of the relative abundance of the differential intestinal microbiota between the HC, WD1 and WD2 group.
Abbreviations: HC, healthy control; WD1, non-wheat-free WDEIA group; WD2, wheat-free WDEIA group; WDEIA, wheat-dependent exercise-induced anaphylaxis.

previous study,[15] there was no difference in the microbiome diversity between the patients with WDEIA and HCs. Our study of patients with WDEIA that employed 16s rRNA sequencing methods[15] revealed significant enrichment of the bacterial genera *Blautia, Erysipelatoclostridium, Akkermansia*, and *Lachnospiraceae_NK4A136_group*, whereas *Lactobacillus* and *Dialister* levels were significantly decreased in patients with WDEIA. The major bacterial taxa previously found significantly altered in WDEIA patients are not prominent in our new findings, such as *Akkermansia, Erysipelatoclostridium, Lachnospiraceae* and *Dialister*. There are some possible explanations. Metagenomic sequencing and 16S rRNA gene sequencing target different regions of DNA and have different biases. Metagenomics provides broader functional insight, while 16S rRNA sequencing offers limited taxonomic and phylogenetic identification. This could have skewed previous results based on methodology. The recruitment period of participants is different. While we attempted to match the groups by age, sex, and BMI, subtle differences in other factors may have contributed to the observed differences.

In this study, nine different taxa were identified between the non-wheat-free WDEIA and HC groups, all of which were downregulated in the former, including *Rikenellaceae* and *Odoribacteraceae* at the family level, and *Alistipes, Odoribacter*, and *Catenibacterium* at the genus level. *Rikenellaceae* levels are decreased in those with an allergy to cow's milk,[23] but it was increased in ovalbumin-sensitized interleukin 4 receptor antagonist $(Il4ra)^{F709}$ mice.[24] Both *Rikenellaceae* and *Alistipes* are downregulated in the peanut-allergic BALB/c mouse model.[25] The genus *Alistipes*, which includes the species *Alistipes_putredinis*, decreases in children with food sensitization.[26] Thus, *Rikenellaceae* and *Alistipes* may play important roles in the modulation of food allergies. The levels of *Odoribacteraceae*, which is a family of succinate-consuming bacteria,[27] were higher in the HCs than in the non-wheat-free patients with WDEIA. Succinate may contribute to the sensitivity and severity of food allergies by activating tuft cells in the epithelial lining of the gut.[28] The genus *Odoribacter*, which belongs to the family *Odoribacteraceae*, has been shown to exert beneficial effects in those with food allergies[29] by producing short-chain fatty acids (SCFAs), and promoting the proliferation and differentiation of regulatory T cells (Tregs), consequently enhancing the integrity of the intestinal barrier.[30] At the species level, *Bacteroides_stercoris, Alistipes_putredinis, Bacteroides_intestinalis, and Bacteroides_cellulosilyticus* were enriched in the HCs compared with the respective levels in the non-wheat-free WDEIA group in this study. And *Bacteroides_sp._3_1_33FAA, Bacteroides_sp._4_1_36, Bacteroides_stercoris*, and *Bacteroides_stercoris_CAG.120* were negatively associated with T-IgE levels. *Bacteroides*, which is dominant after natural delivery, was shown to be correlated with caesarean section-discriminative microbial metabolites, suggesting that maternal microbial transmission during birth helps regulate the metabolism of newborns and may protect children from asthma.[31] *Bacteroides stercoris*

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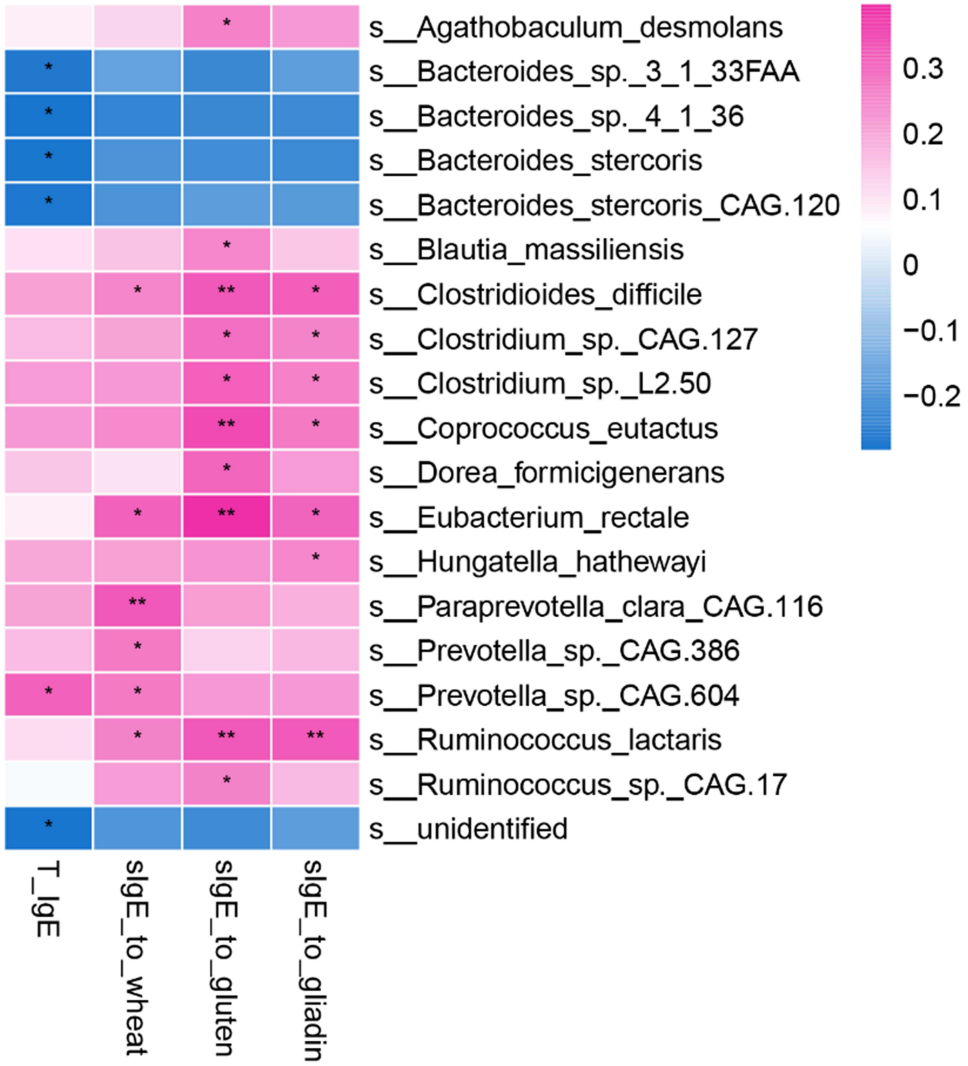

Figure 4 Heatmap of the correlations between the gut microbiota of patients with WDEIA and clinical indices based on Spearman correlation analyses. *$P < 0.05$,**$P < 0.01$.
Abbreviation: WDEIA, wheat-dependent exercise-induced anaphylaxis.

was previously found to be more enriched in controls than in patients with asthma,[32] and Odamaki et al[33] demonstrated a significant correlation between *Bacteroides_intestinalis* and Japanese cedar pollinosis. Therefore, it is possible that the reduction in beneficial flora may be involved in the pathogenesis of WDEIA and that different microflora play beneficial roles through various mechanisms that are yet to be fully elucidated.

The biological effects of microbiota vary among strains at the same taxonomic level;[34] therefore, we conducted an in-depth investigation of the gut species related to the different taxa identified in our study. Compared to that of the HCs, the relative abundance of the bacterial species *Blautia_coccoides* was significantly increased, whereas that of *Lactobacillus_mali, Lactobacillus_buchneri*, and *Lactobacillus_apis* was significantly decreased in the non-wheat-free WDEIA group. The genus *Blautia* is positively associated with asthma,[35] with higher levels in those with severe allergic conjunctivitis than in those with a mild case.[36] *Blautia_coccoides* prevents weight gain by regulating the production of SCFAs,[37] which may suppress immediate hypersensitivity responses in mast cells by regulating the size and function of the colonic Treg pool.[38] *Lactobacillus_apis* can enhance the antioxidant activity of fermented wheat bran.[39] *Lactobacillus_apis* increases tryptophan and tyrosine levels during fermentation, which exert positive health effects.[39] *Lactobacillus_mali K8* can inhibit α-glucosidase activity in vitro and was selected as a candidate probiotic.[40] The

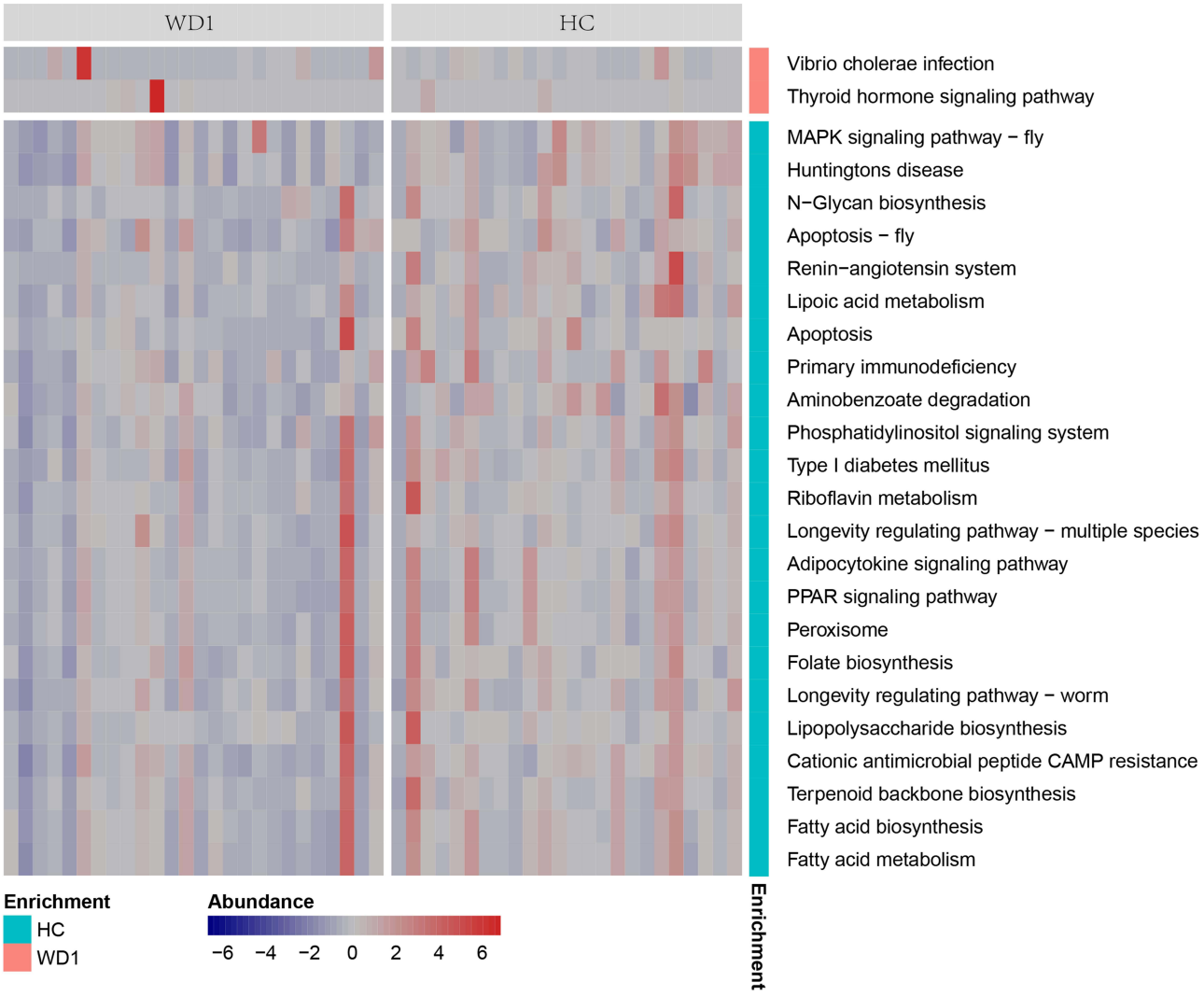

Figure 5 Differences in KEGG metabolic pathways between the non-wheat-free patients with WDEIA and HCs.
Abbreviations: HC, healthy control group; KEGG, Kyoto Encyclopedia of Genes and Genomes; WD1, non-wheat-free WDEIA group; WDEIA, wheat-dependent exercise-induced anaphylaxis.

biological effects of the microbiota may occur at the strain level; therefore, accurate studies investigating the effects of this strain would have high clinical value.

We confirmed that the gut microbiome was associated with the clinical indices of WDEIA. At the species level (abundance > 0.1% by total sum scaling), 15 species, including *Blautia massiliensis, Clostridioides difficile*, and *Eubacterium rectale*, were positively correlated with wheat, gluten, or gliadin-specific IgE. The frequency of amplicon sequence variants has been shown to be higher in *Blautia* from individuals suffering from non-celiac gluten sensitivity.[41] *Clostridium difficile* can cause potentially life-threatening diarrheal illnesses in individuals with dysbiosis,[42] although a Type-2 immune response, which is most often associated with helminth infections and allergies, may protect against *Clostridioides difficile* infection.[43] However, the effect of *Clostridioides difficile* on WDEIA remains unclear. In infants with a cow's milk allergy, administration of an amino acid formula containing synbiotics was shown to be associated with an increase in bifidobacteria and a reduction in *Eubacterium rectale* and *Clostridium coccoides*, and may be associated with improved clinical outcomes.[44]

The renin-angiotensin-aldosterone system (RAS) is vital for maintaining arterial blood pressure and becomes dysregulated during shock.[45] A metagenomic study of children with allergies demonstrated an enrichment of genes involved in the production of bacterial lipopolysaccharides.[46] However, RAS and lipopolysaccharide biosynthesis are

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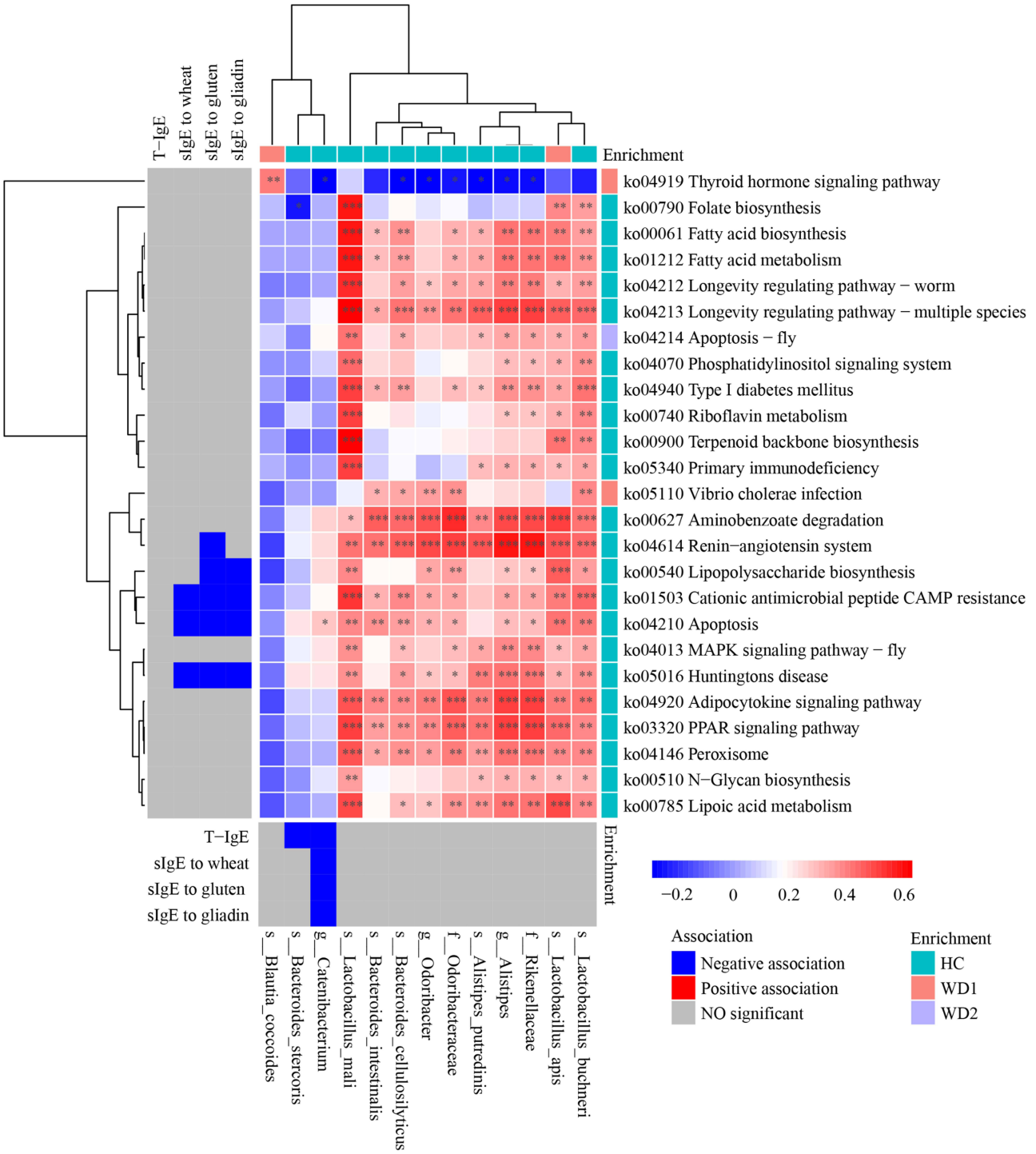

Figure 6 Associations among the phenotypes (WDEIA-related clinical indices), gut microbiome, and gut microbiome functions (KEGG pathways) in the WD1, WD2, and HC groups. The left and bottom panels show significant correlations between the WDEIA-related clinical indices and either the KEGG pathways or certain bacteria, respectively (blue indicates a negative association; red, indicates a positive association; grey indicates no significant association). The top and right panels show differences in enrichment of the intestinal microbiota and KEGG pathways, respectively (green, HC-enriched; yellow, WD1-enriched; purple, WD2-enriched). The middle panel shows significant associations between clinical index-related intestinal microbiota and clinical index-related KEGG pathways (blue, negative association; red, positive association; gray, no significant association). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, according to the Wilcoxon rank-sum test.

Abbreviations: HC, healthy control group; IgE, immunoglobulin E; KEGG, Kyoto Encyclopedia of Genes and Genomes; sIgE, specific IgE; T-IgE, total IgE; WD1, non-wheat-free WDEIA group; WD2, wheat-free WDEIA group; WDEIA, wheat-dependent exercise-induced anaphylaxis.

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downregulated in patients with WDEIA, and the underlying mechanisms remain unclear. In our study, the apoptotic pathway that was upregulated in the HC group was positively correlated with the HC-enriched genus *Catenibacterium* and negatively correlated with the disease-related clinical indices, whereas the genus *Catenibacterium* was negatively correlated with them. It was reported that,[47] in the gut microbiota of mice with pulmonary fibrosis, the relative abundances of *Catenibacterium* and *Lactobacillus* decreased dramatically after induction by bleomycin, and rate of apoptosis of pulmonary cells increased significantly. Thus, *Catenibacterium* may influence the clinical index by modulating the apoptotic pathway through different mechanisms in different diseases.

Interestingly, we found that a wheat-free diet is associated with intestinal flora more similar to the structure of healthy individuals. Correction of the shift toward abnormal intestinal flora may be related to the improvement of WDEIA, although the specific mechanisms must be verified through animal or functional experiments. This normalization may contribute to clinical improvement by reversing wheat-associated dysbiosis: for example, reducing wheat-driven expansion of pro-inflammatory or proteolytic taxa, restoring SCFA-producing commensals that support Treg induction and intestinal barrier integrity, and lowering metabolites that can prime epithelial tuft cells or type-2 immune responses.

This is a cross-sectional analysis and the need for prospective, longitudinal or interventional studies to confirm whether wheat avoidance causes the microbiome shifts observed. The cross-sectional design may introduce selection bias. A longitudinal or randomized controlled study design would be more appropriate to establish causality and minimize selection bias in future. Wheat-free refers specifically to avoidance of wheat-containing foods as self-reported by participants, and is not identical to strict gluten-free diets which exclude other gluten-containing grains. LEfSe was used primarily for biomarker discovery rather than strict hypothesis testing, which may generate false positives, particularly in high-dimensional microbiome data. Although FDR correction reduced the number of significant taxa, overall patterns remained consistent, and we prioritized an inclusive approach to capture broad microbial differences among WDEIA patients on wheat-free and non–wheat-free diets and healthy controls. PLS-DA was employed to visualize clustering patterns, but it does not provide formal statistical evidence, and additional tests such as PERMANOVA were not performed; future studies could include these analyses to validate clustering. Correlation analyses were exploratory, and strict multiple testing correction was not applied to avoid masking biologically meaningful trends, especially given the relatively small sample size. Readers are advised to interpret these findings cautiously and consider them for validation in independent cohorts. Furthermore, whether a causal relationship exists between changes in flora and the incidence of WDEIA remains unknown. Further animal and cellular experiments are required to clarify the mechanism of action through which the intestinal microbiome modulates the pathogenesis of WDEIA. Meanwhile, it is worthwhile to further study the role of probiotics or intestinal flora transplantation in WDEIA.

Conclusion

In conclusion, this study identified differences in gut microbiome compositions and functions between patients with WDEIA and HCs. This study found that a wheat-free diet is associated with an intestinal flora structure that is similar to healthy individuals. The study is helpful for establishing a comprehensive understanding of the composition and functions of the intestinal flora. This study provides insights for further investigation into the potential mechanisms linking wheat intake, microbial changes, and the pathophysiology of WDEIA.

Ethics Approval

This study complies with the Declaration of Helsinki. This study was approved by the ethics committee of Peking Union Medical College Hospital (JS-2615).

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Disclosure

The authors report no conflicts of interest in this work.

The abstract of this paper, titled "Wheat-free diet alters the intestinal microbiome structure of patients with wheat-dependent exercise-induced anaphylaxis", was presented as a poster at both the EAACI Congress 2024 and the 2025 AAAAI/WAO Joint Congress, reporting interim findings. The poster's abstract has been published in the Journal of Allergy and Clinical Immunology as part of its "Poster Abstracts" section: https://www.jacionline.org/article/S0091-6749(23)01952-8/pdf.

Separately, the study titled "Clinical profiles of patients with wheat-induced anaphylaxis at various ages of onset" focuses on the clinical characteristics of WDEIA patients, rather than intestinal microbiome, and has been published in the World Allergy Organization Journal.

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