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Gut microbiota dysbiosis and metabolic reprogramming in pediatric migraine: a multi-omics analysis revealing diagnostic biomarkers

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Abstract

Background Numerous studies have identified disruptions in the gut microbiota of patients with migraine, and the role of the gut–brain axis in the pathogenesis and development of migraine has been established. The incidence of migraine in children increases with age, yet the intestinal microbiota in pediatric migraine has been inadequately investigated. Therefore, we aim to investigate the composition, functional characteristics, and metabolite profiles of the gut microbiota in children with migraine.

Methods We recruited 30 children with migraine and 30 healthy controls aged 5–14 years from Hebei Province, China, and collected 60 fresh fecal samples. Metagenomic sequencing was performed to obtain species abundance profiles and Kyoto Encyclopedia of Genes and Genomes (KEGG) functional annotations of gene sequences. Non-targeted metabolomic analysis was applied to assess differential changes in gut microbiota metabolites between children with migraine and healthy controls.

Results The abundance of gut microbiota species was significantly reduced in children with migraine ($P=0.001$), and was associated with migraine presence ($R^2=0.051$, $P=0.001$). Bacteria within *Pseudomonadota* were significantly enriched in the gut flora of children with migraine, with *Escherichia coli* being the most abundant species. The propionic acid, the arginine and proline metabolism pathways were significantly upregulated in children with migraine ($P<0.05$). In contrast, the porphyrin metabolism pathway, amino acid biosynthesis pathway, and arginine biosynthesis pathway were significantly down-regulated ($P<0.05$). The intestinal lipopolysaccharide biosynthetic pathway showed diagnostic potential for pediatric migraine, with an Area Under the Curve (AUC) of 0.75 (95% CI: 0.63–0.87). Intestinal metabolites were also dysregulated in children with migraine; notably, Docosahexaenoyl Ethanolamide (DHEA) and kynurenic acid were significantly depleted ($P<0.05$).

Conclusion This study revealed an association between gut microbiota and its metabolite in children with migraine, suggesting that the potential pathogenic role of gut microbiota may be mediated by the functions of

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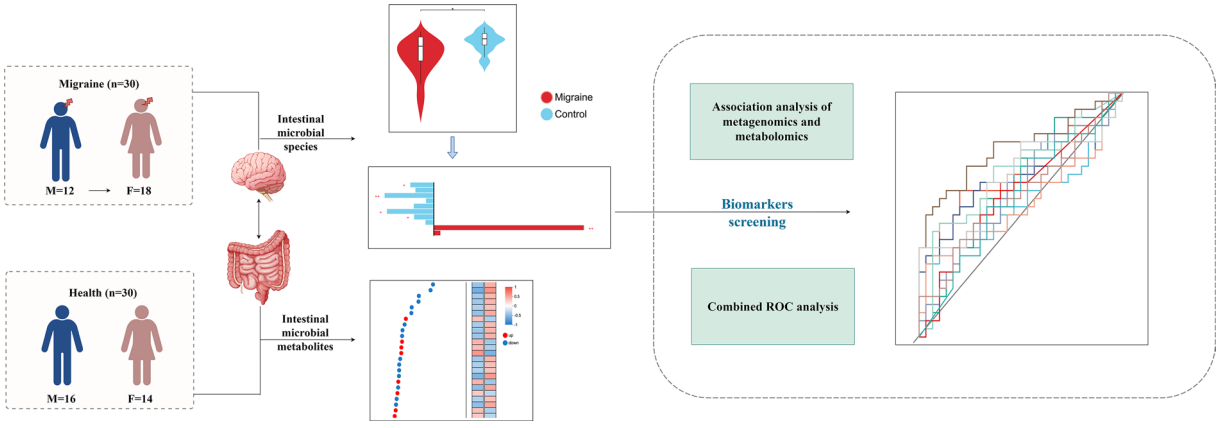
Pseudomonadota and *Escherichia coli* and by the levels of metabolites derived from them. Furthermore, this study provides strong evidence for the diagnostic potential of kynurenic acid in pediatric migraine and supports future targeted metabolite research.

Keywords Mmigraine in children, Metagenomics, Non-targeted metabolomics, Gut microbiome, Tryptophan metabolism

Graphical Abstract



Introduction

Migraine is a common primary headache disorder in children, characterized by recurrent episodes [1]. These headaches are often accompanied by gastrointestinal symptoms such as nausea and vomiting [1]. According to 2021 Global Burden of Disease data, migraine ranks among the leading causes of disability-adjusted life-years (DALYs) in children and adolescents aged 5–19 years [2]. A meta-analysis involving 15,626 children reported an overall migraine prevalence of approximately 11% in this population, with prevalence gradually increasing with age [3]. Although the global standardized prevalence of migraine has remained relatively stable over time, reported rates have increased across all age groups [4]. Notably, migraine can significantly impair both physical development and social functioning in children, during both ictal and interictal periods [5–6]. Migraine adversely affects physical activity, reduces academic performance, limits social and sports participation, and increases the risk of school dropout [7].

The gut–brain axis constitutes a bidirectional communication system between the brain and the gastrointestinal tract. Emerging evidence indicates that it plays an important role in the pathogenesis of migraine [8]. Studies in adults with migraine have revealed associations between certain bacterial species (such as Bifidobacterium and Lactobacillus) and brain function [9]. Their metabolites, including short-chain fatty acids (SCFAs), are involved not only in neurotransmitter synthesis [10] but also in metabolic and immune functions [11]. Notably, during migraine attacks, differences in gut microbial diversity have been observed between patients and healthy controls [12], and intestinal SCFA levels are elevated [13]. However, gut microbiota research is susceptible to various confounding factors, such as geographic location [14], body mass index, and dietary habits [15]. Of particular note, current diagnostic criteria for pediatric migraine remain limited. Diagnosis relies primarily on the subjective criteria outlined in the International Classification of Headache Disorders, 3rd edition (ICHD-3) [16], with no pediatric-specific biomarkers available. Therefore, investigating the characteristics of the gut–brain axis in children with migraine is of significant scientific and clinical value.

The 16 S rRNA sequencing used in most existing studies achieves approximately 80% accuracy at the genus level; however, it remains difficult to fully resolve taxonomic profiles at the species or strain level, particularly with short reads [17]. In contrast, metagenomic sequencing enables comprehensive analysis of microbial DNA, allowing for precise taxonomic classification at the species level and providing insight into specific functional pathways [18–21]. Therefore, our study employed metagenomic sequencing combined with non-targeted metabolomics to conduct an in-depth exploration of the composition, functional characteristics, and metabolite profiles of the gut microbiota in children with migraine. This approach aims to elucidate the potential mechanisms by which gut microbiota and their metabolites contribute to the pathogenesis of migraine in children, and to provide a scientific basis for the development of

novel diagnostic biomarkers and intervention targets (Fig. 1).

Methods

Research object

A prospective cohort study was conducted, with all samples collected between May 2024 and January 2025. A total of 30 children with migraine and 30 healthy children aged 5–14 years from inland China were enrolled and assigned to the migraine group ($n = 30$) or the healthy control group ($n = 30$). We required that symptoms had been present for ≥ 1 month prior to enrollment to ensure a stable, recurrent migraine presentation and to reduce the inclusion of transient or sporadic headaches. This criterion was intended to increase the likelihood of detecting microbiome- and metabolome-related features associated with pediatric migraine, rather than acute short-term fluctuations. The diagnosis of migraine was based on the International Classification of Headache Disorders, 3rd edition (ICHD-3). The inclusion criteria for the migraine group were as follows: (1) meeting the ICHD-3 diagnostic criteria for migraine; (2) being of Han ethnicity, history of migraine symptoms for ≥ 1 month prior to enrollment(Counting from the time when the first headache symptoms characteristic of migraine occurred)), and aged between 5 and 14 years; (3) no use of antibiotics, analgesics, sedatives, hormones, immunoglobulins, hemofiltration, or similar treatments within two weeks prior to specimen collection; (4) non-pregnant females; (5) provision of signed informed consent by a parent or guardian. The exclusion criteria for the migraine group were: (1) presence of other types of headache disorders; (2) current or previous use of migraine-specific pharmacological prophylaxis. (3) previous treatment with antibiotics, hormones, immunoglobulins, hemofiltration, or related therapies before specimen collection; (4) comorbid gastrointestinal or underlying systemic diseases, including infectious diarrhea, inflammatory bowel disease, Crohn's disease, or immune system defects; (5) major organ failure.

Based on attack frequency, pediatric patients with migraine were classified into three groups: episodic migraine (0–7 headache days per month), high episodic migraine (8–14 headache days per month), and chronic migraine (headache occurring on 15 or more days per month for more than 3 months, which, on at least 8 days per month, has the features of migraine headache) [16, 22] .This study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Hebei Children's Hospital (Approval No.: 2024132). Informed consent was obtained from all participants' parents or guardians.

Clinical data collection and sample collection

Fresh fecal specimens were systematically collected within 24 h after presentation to our center and prior to the initiation of any migraine-specific treatment. Based on the medical history obtained by the treating clinicians, all patients were naive to migraine-specific pharmacological treatment (acute or preventive) before enrollment. Utilizing aseptic techniques, the core portion of each fecal sample, which remained unexposed to environmental contaminants (air and surface contact), was carefully extracted using a sterile spatula and promptly transferred into pre-sterilized collection tubes. The tubes were then sealed and stored at − 80 °C.

Concurrently, baseline clinical characteristics including age, sex, height, weight, and medication history were obtained from medical records. Migraine severity was assessed using the Visual Analogue Scale (VAS), the Pediatric Migraine Disability Assessment Score (Ped MIDAS), and the Headache Impact Test-6 (HIT-6) scale.

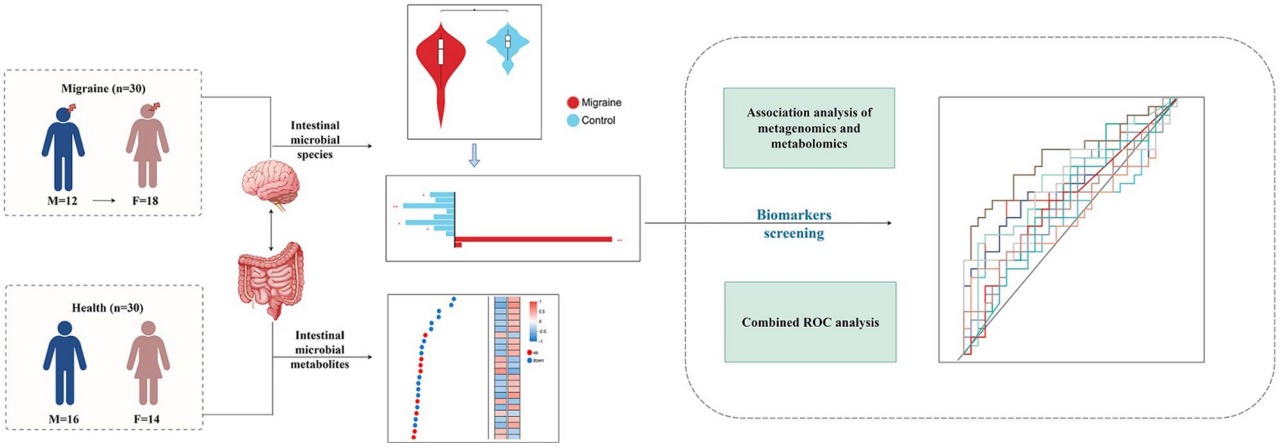


Fig. 1 Flowchart

Intestinal microbial analysis

Metagenomics analysis

DNA was extracted from the collected fecal samples, and the integrity of the extracted DNA was verified using 1% agarose gel electrophoresis. A 350 bp DNA fragment was selected for the construction of a paired-end (PE) library. High-throughput sequencing was then performed on the Illumina platform using the NovaSeq™ X Series 25B Reagent Kit (300 Cycle) to generate raw sequencing reads. Quality control of the data was carried out with fastp [23] and BWA [24]. Non-redundant gene sets were assembled using MEGAHIT and Cluster Database at High Identity with Tolerance (CD-HIT), and gene abundance calculations were performed with SOAPaligner.

Finally, the amino acid sequences of the non-redundant gene set were aligned with the Diamond software (v2.0.13; key parameter: blastp; e-value ≤ $1e^{-5}$) to obtain taxonomic abundance information at each level (genus, species) per sample. Functional annotation of genes was obtained by comparison with the KEGG database (KEGG_v94.2).

Non-targeted metabolomics analysis

A 100 mg aliquot of each sample was placed in a 2 mL centrifuge tube, to which a 6 mm grinding bead and 800μL of extraction solvent (methanol: water = 4:1, v/v) were added. The mixture was homogenized using a cryogenic tissue grinder and subsequently subjected to low-temperature ultrasonic extraction. The processed samples were then incubated at − 20 °C for 30 min and centrifuged for 15 min (4 °C, 13,000×g). The supernatant was collected for Liquid Chromatography–Mass Spectrometry (LC–MS) analysis. Additionally, a quality control (QC) sample was prepared by pooling equal volumes of metabolites from all samples. Throughout the analytical sequence, one QC sample was inserted after every 5–10 experimental samples to monitor the reproducibility and stability of the analysis. LC–MS was performed using a Thermo Fisher Scientific UHPLC-Q Exactive HF-X system. Raw data were processed with Progenesis QI software (Waters Corporation, Milford, USA). The stability and performance of the metabolic model were evaluated using orthogonal least squares-discriminant analysis (OPLS-DA) implemented in the R package *ropls* (version 1.6.2).

Table 1 Basic information of children with migraine and healthy control children

	Migraine($n=30$)	Healthy controls($n=30$)	*P* value
Sex			
Female, n	18(60%)	14(46.7%)	0.305
Male, n	12(40%)	16(53.3%)	
Age, years	10.47 ± 2.53	9.33 ± 2.12	0.073

Statistical analysis

Statistical analysis of baseline data was performed using SPSS 26.0. The Shapiro–Wilk test was applied to assess whether the data followed a normal distribution. For data not conforming to a normal distribution, the Mann–Whitney U test (Wilcoxon rank-sum test) was used for intergroup comparisons.

Metagenomic data analysis

To examine differences in gut microbiota composition between the migraine and healthy groups, analyses were conducted using the *boot* (v1.3.18) and *stats* (v3.3.1) packages in R (v3.3.1). Wilcoxon rank-sum test, followed by false discovery rate (FDR) correction for multiple testing, and two-tailed statistical methods were applied. Alpha diversity was assessed using the Chao1, ACE, observed species, Shannon, and Simpson indices to evaluate species richness and evenness. Beta diversity was visualized through principal coordinate analysis (PCoA) based on Bray–Curtis distance, using analysis of similarities (ANOSIM) with 999 permutations. Additionally, PERMANOVA was employed to compare species-level abundance profiles between the migraine and healthy groups [20].

Non-targeted metabolomics data analysis

The two-tailed Wilcoxon rank-sum test was employed to identify metabolites with differential abundance. Differential metabolites were screened based on the variable importance in projection (VIP) score derived from partial least squares-discriminant analysis and the p-value from Student's t-test. Metabolites with VIP > 1 and $P < 0.05$ were considered statistically significant. These differential metabolites were annotated using the KEGG database. Pathway enrichment analysis was performed using the scipy.stats package in Python, and the most relevant biological pathways were identified using Fisher's exact test.

Finally, a random forest model was constructed using the *randomForest* package in R (v3.3.1) by integrating the differential microbial species and metabolites. All association analyses were conducted using Spearman's rank correlation method.

Results

Data characteristics of migraine children and healthy children

A total of 60 subjects were included in this study, with 60 fecal samples prospectively collected (30 children with migraine and 30 healthy children). No significant differences were observed in age, gender, between the two groups (Table 1). All participants were from the same province (Hebei, China) to ensure similar dietary habits, lifestyles, and environmental exposures, thereby minimizing the influence of non-migraine-related factors.

After quality-controlled sequencing and clustering, a non-redundant gene set was constructed, comprising 2,213,084 genes. This gene set was used for subsequent functional analyses.

Intestinal microbial diversity decreased in children with migraine

Subsequently, we analyzed the species-level composition of the gut microbiota in children with migraine and the control group. By aligning sequences to the NR database, 10,451 species were annotated in the migraine group and 10,475 in the healthy control group. Based on the differences in species counts between the groups, we performed comparative analyses of α-diversity and β-diversity. Significant differences were observed in the Shannon index ($P = 0.023$) and Simpson index ($P = 0.004$) in children with migraine (Fig. 2A, B), indicating a notable reduction in both species richness and evenness of the gut microbiota compared to healthy children, and suggesting a shift in dominant microbial species. β-diversity analysis further confirmed a significant difference in species composition between the two groups ($P = 0.001$) (Fig. 2C). Subsequently, using multivariate PERMANOVA (Bray–Curtis distance, 999 permutations), we found that migraine status was significantly associated with variation in microbial composition ($R^2 = 0.051$, $P = 0.001$).

Comparative analysis of gut microbiota composition reveals statistically significant differences in species diversity and abundance between pediatric migraine patients and their healthy counterparts

To further compare the distribution of core microbiota between the two groups, we performed LEfSe analysis across different taxonomic levels (LDA > 2, $P < 0.05$). The results revealed significant enrichment of *Pseudomonadota* in the gut microbiota of children with migraine. In contrast, no significantly enriched phylum-level bacteria were identified in the healthy control group (Fig. 3A, B). Subsequent analysis identified differentially abundant taxa between the two groups at the genus and species levels. The genera *Escherichia*, *Providencia*, and *Enterococcus*, along with the species *Escherichia coli*, were significantly enriched in children with migraine. Conversely, probiotics including *Ruminococcus*, *Roseburia*, *Phocaeicola*, *Agathobacter*, *Clostridium*, and *Phocaeicola dorei* were significantly more abundant in healthy children (Fig. 3A, B). Notably, the consistent enrichment of *Clostridia* in the healthy group aligns with previous findings from mouse models [25], pediatric cohorts [26], and human nutritional studies [27], suggesting that Clostridia may serve as a health-associated biomarker. Our results are consistent with these reports. We also compared the average relative abundance of each species between the groups and found that *Escherichia coli* exhibited the greatest abundance difference between children with migraine and healthy controls (Fig. 3C).

There were significant differences in the functional composition of intestinal microorganisms between migraine children and healthy children

Through KEGG pathway analysis (classification level: Pathway Level 3), we observed significant up-regulation of multiple metabolic pathways in children with migraine ($P < 0.05$). The analysis identified several metabolic pathways associated with carbohydrate metabolism and energy production, including glycolysis/gluconeogenesis, pyruvate metabolism, fructose and mannose metabolism, the pentose phosphate pathway, and peptidoglycan biosynthesis. These pathways are known to be involved in

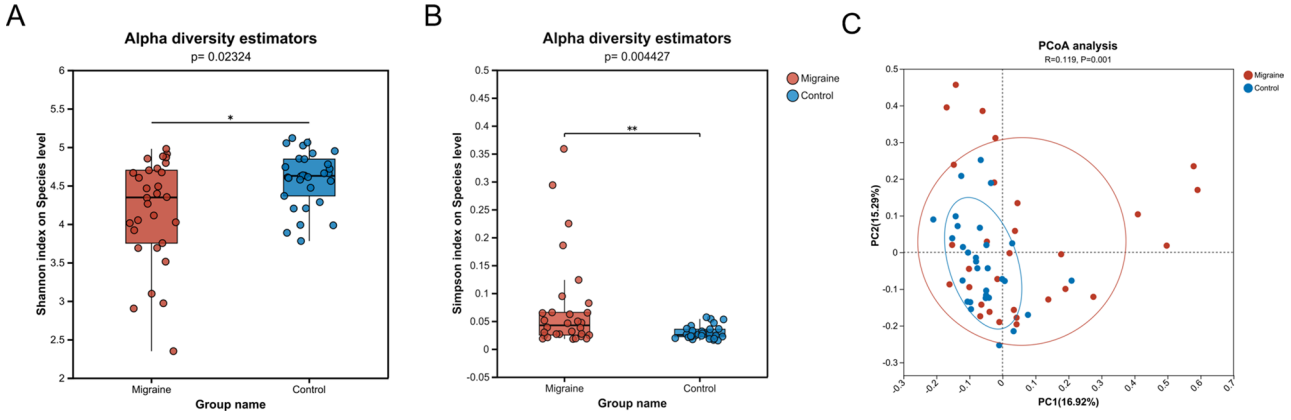


Fig. 2 Diversity analysis between migraine group and healthy control group. **A–B** α-diversity of the gut microbiota in the migraine group and healthy controls: **A**: Shannon index (mean ± S.D.: Migraine, 4.17 ± 0.68; Healthy controls, 4.58 ± 0.36); **B**: Simpson index (mean ± S.D.: Migraine, 0.07 ± 0.08; Healthy controls, 0.03 ± 0.01). **C**: Principal coordinate analysis (PCoA) of β-diversity based on Bray– Curtis distance, illustrating significant structural differences in microbial communities between the migraine and control groups. PC1 and PC2 denote the first and second principal coordinates, respectively, with PC1 explaining the maximum proportion of variance, and PC2 accounting for the largest share of the remaining variance

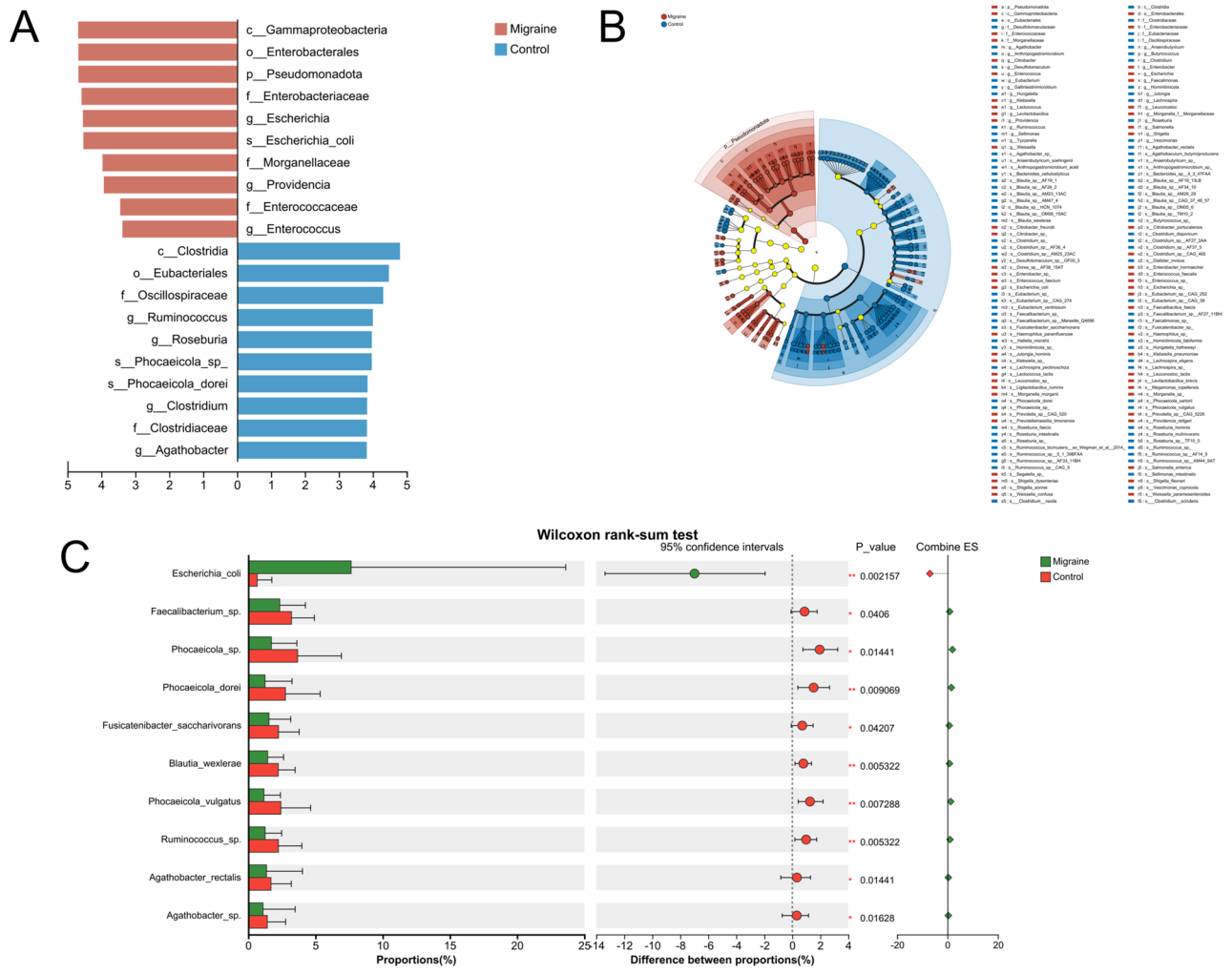


Fig. 3 Differences in species composition of gut microbes between migraine group and healthy control group. **A–B**: Results of LEfSe analysis comparing differential bacterial abundance between the migraine group and the control group. **C**: Bar plot comparing species abundance between the migraine group and the control group. The y-axis represents species names at different taxonomic levels, the x-axis represents the percentage abundance of each species within the sample set, and different colors indicate different groups. Significance levels are denoted as follows: *$0.01 < P \leq 0.05$, **$0.001 < P \leq 0.01$, ***$P \leq 0.001$

cellular energy homeostasis and biosynthetic processes. Additionally, pathways associated with microbial environmental adaptation and resource utilization were up-regulated, including Microbial Metabolism in Diverse Environments, Pentose and Glucuronate Interconversions, Glyoxylate and Dicarboxylate Metabolism, and Lipopolysaccharide Biosynthesis. Furthermore, the Bacterial Secretion System pathway, which has been implicated in microbe–host interactions, exhibited significant up-regulation in the migraine cohort ($P < 0.05$). In contrast, the Amino Sugar and Nucleotide Sugar Metabolism pathway showed a relatively stable abundance pattern in the healthy control group ($P < 0.05$).

Further analysis of KEGG Orthology (KO) pathway abundance revealed four significantly up-regulated pathways in the migraine group: Biosynthesis of Secondary Metabolites (ko01110), Folate Biosynthesis (ko00790), Propanoate Metabolism (ko00640), and Arginine and Proline Metabolism (ko00330). Six pathways were significantly down-regulated: Porphyrin Metabolism (ko00860), Thiamine Metabolism (ko00730), Amino Acid Biosynthesis (ko01230), 2-Oxocarboxylic Acid Metabolism (ko01210), Arginine Biosynthesis (ko00220), and Valine, Leucine, and Isoleucine Biosynthesis (ko00290) (Fig. 4A, B).

Differences in gut microbial species and functional composition among pediatric migraine patients with different headache frequencies

We further stratified participants by attack frequency to compare microbial functional pathways and species composition and performed exploratory trend tests across frequency categories. The pathways of metabolite biosynthesis, including the biosynthesis of secondary

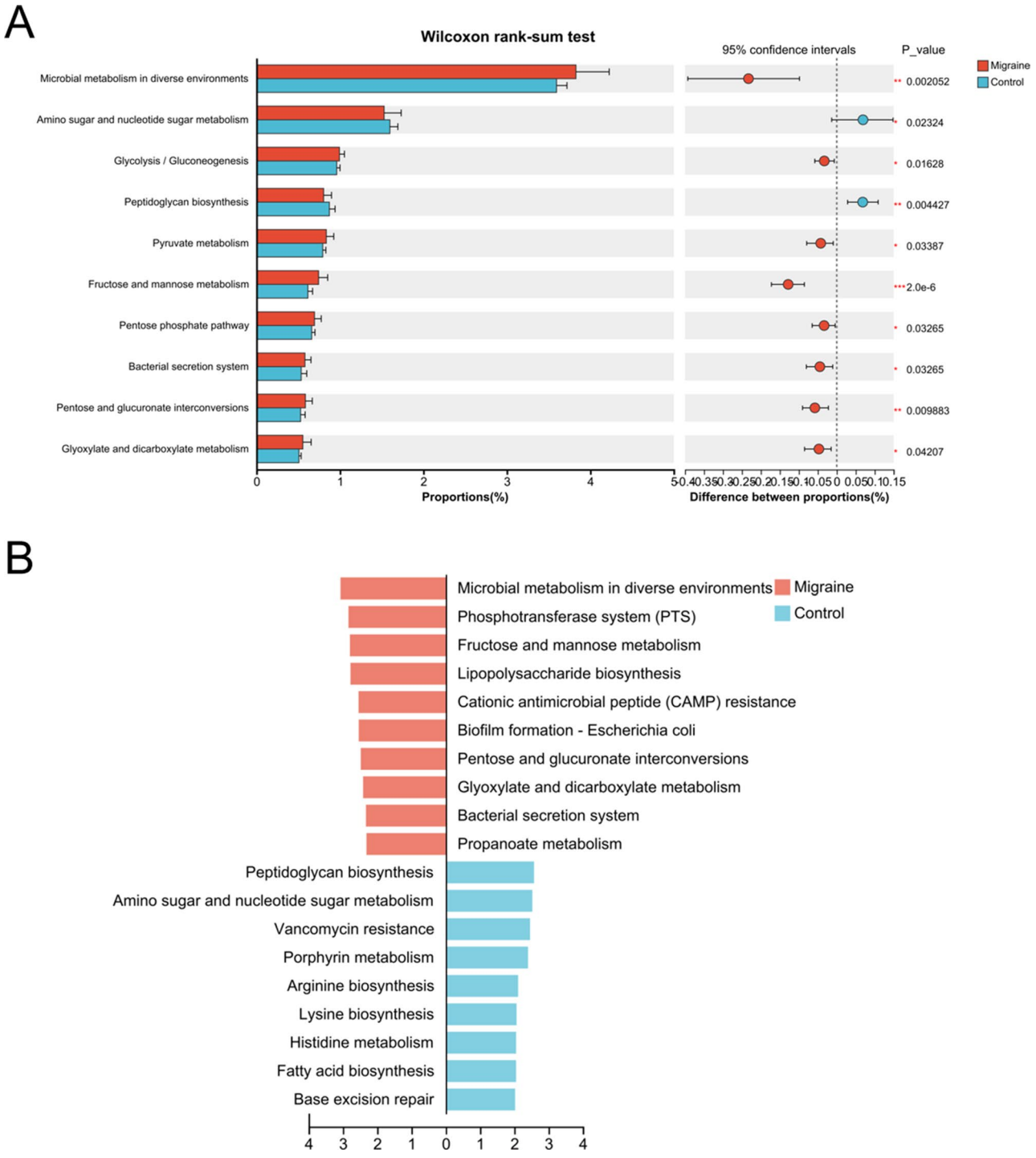


Fig. 4 Differences in functional composition of intestinal microorganisms between migraine group and healthy control group. **A**: The y-axis indicates the functional pathways at different classification levels, the x-axis represents the percentage abundance of each pathway within the samples, and different colors correspond to the different groups. Significance levels are denoted as follows: *$0.01 < P \leq 0.05$, **$0.001 < P \leq 0.01$, ***$P \leq 0.001$. **B**: Histogram of LDA scores for functional pathways that contribute significantly to group differences. The LDA score, derived from linear discriminant analysis, reflects the effect size of each pathway; a higher score indicates a greater contribution to the observed differential abundance between groups

metabolites and tryptophan biosynthesis, showed decreasing gradients, whereas carbohydrate digestion and absorption showed a non-monotonic pattern. However, none of these differences remained statistically significant after FDR correction. At the species level, *Segatella copri* showed a downward trend with increasing migraine frequency, whereas *Enterococcus sp.* and *Bifidobacterium breve* showed upward trends. After FDR

correction, none of these differences reached statistical significance (Fig. 5).

Association analysis of species and function of gut microbes in children with migraine and healthy children and combined receiver operating characteristic (ROC) analysis

Based on the relative abundance of species and functional pathways in children with migraine and healthy controls, we conducted a correlation analysis between species abundance and functional abundance for significantly differential pathways (considering the top 15 species by total abundance at the species level, and the top 15 functions at Pathway Level 3).The results indicated that *Bacteroides sp.* showed the strongest association with fatty acid metabolism and fatty acid biosynthesis, while *Anaerostipes sp.* showed the highest relative contribution to the propanoate metabolism pathway (Fig. 6A). Within the butanoate metabolism pathway, *Escherichia coli* had the highest relative functional contribution in the migraine group. In contrast, in the healthy control group, *Escherichia coli* exhibited the lowest relative contribution, whereas *Bacteroides sp.* demonstrated the highest association (Fig. 6B).

For the arginine and proline metabolism pathways and the biosynthesis of secondary metabolites, *Escherichia coli* had the highest relative functional contribution in the migraine group but the lowest in controls. In the latter group, *Blautia sp.* was identified as the primary contributor. Additionally, *Bifidobacterium sp.* was most strongly associated with the arginine biosynthesis pathway (Fig. 6C).

We hypothesized that the combined application of functional factors and metabolite factors of the intestinal microbial community may be more conducive to the diagnosis of migraine in children, so we constructed a classifier for combined ROC analysis. We found that Histidine metabolism (AUC = 0.68 (95% CI : 0.54–0.82)) ; Pyruvate metabolism (AUC = 0.57(95% CI: 0.42–0.72)); Faty acid metabolism (AUC = 0.58(95% CI: 0.43–0.72)); Glycolysis Gluconeogenesis (AUC = 0.65(95% CI: 0.5–0.8));Propanoate metabolism (AUC = 0.55(95% CI: 0.4–0.71));Secondary bile acid biosynthesis (AUC = 0.68(95% CI: 0.55–0.82)); GENESET Ongin(AUC = 0.54(95% CI: 0.39–0.7)); Tryptophan metabolism (AUC = 0.61(95% CI: 0.45–0.76)); Butanoate metabolism (AUC = 0.54(95% CI:0.39–0.69)); Arginine and proline metabolism (AUC = 0.59(95% CI: 0.43–0.74)); Primary bile acid biosynthesis (AUC = 0.65 (95% CI : 0.51–0.8)) showed promising diagnostic performance for distinguishing children with migraine from healthy controls, with lipopolysaccharide (LPS) biosynthesis (AUC = 0.75, 95% CI: 0.63–0.87) achieving the highest classification accuracy. (Fig. 6D).

Differences in intestinal metabolites between children with migraine and healthy children and association analysis between metabolomics and metagenomics

To rapidly characterize the composition and differences in metabolites between the migraine and healthy control groups, a comparative analysis was performed. The results revealed 937 differentially abundant metabolites ($P < 0.05$, OPLS-DA: VIP > 1) in the migraine group compared to the healthy controls, among which 155

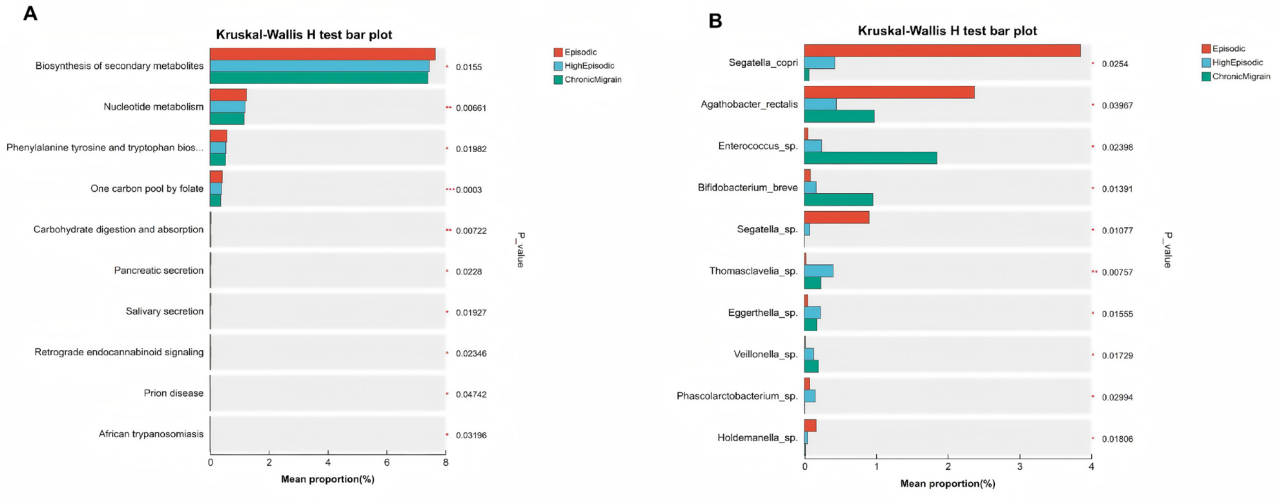


Fig. 5 Differences in species and functional profiles among pediatric migraine groups. **A**: Bar plot of functional differences among pediatric migraine patients with different frequencies. **B**: Bar plot of species differences among pediatric migraine patients with different frequencies. The y-axis indicates the names of species/functions at different taxonomic levels, and the x-axis indicates the percentage value of a given species/function in the sample. Different colors represent different groups. *$0.01 < P \le 0.05$, **$0.001 < P \le 0.01$, ***$P \le 0.001$

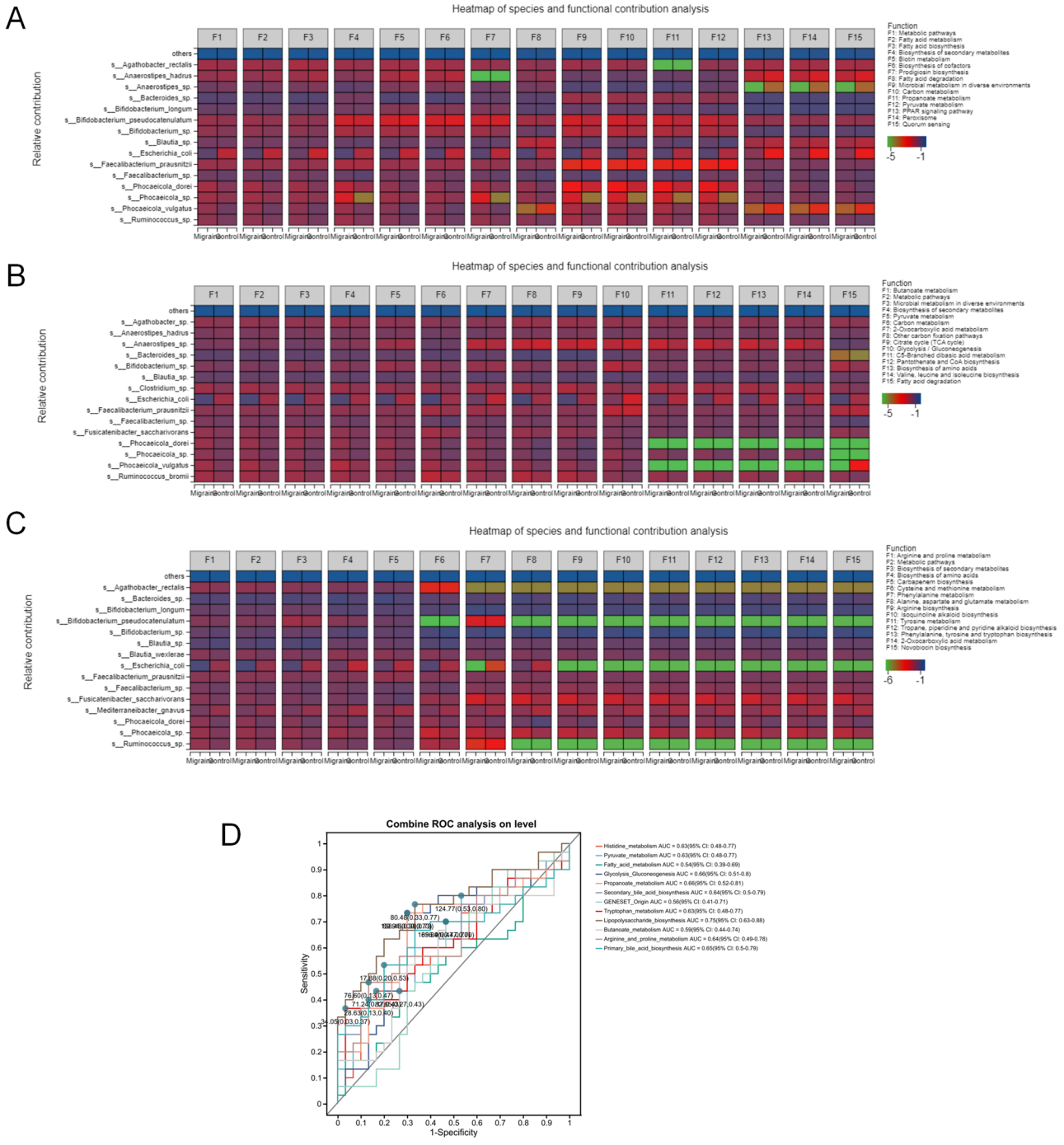


Fig. 6 Association analysis of species and function of gut microbes in migraine group and healthy control group and combined ROC analysis. **A**: Heatmap showing the contribution of microbial species to fatty acid metabolism-related functions. **B**: Heatmap of species contribution to butanoate metabolism-related functions. **C**: Heatmap of species contribution to arginine and proline metabolism-related functions. **D**: ROC curve: The x-axis represents 1–Specificity (range 0–1), and the y-axis represents Sensitivity (range 0–1). The point marked on the curve indicates the optimal cutoff value, with corresponding Sensitivity and Specificity values provided in parentheses. The AUC is shown for each functional pathway

metabolites were up-regulated and 782 were down-regulated (Fig. 7A, B).

To better visualize the importance and expression trends of these differential metabolites, an OPLS-DA model was applied to calculate variable importance in projection (VIP) values. Among the top 30 metabolites ranked by VIP score, 20 showed decreased expression, with DHEA exhibiting the most pronounced reduction (Fig. 7C, Supplementary Table 1).

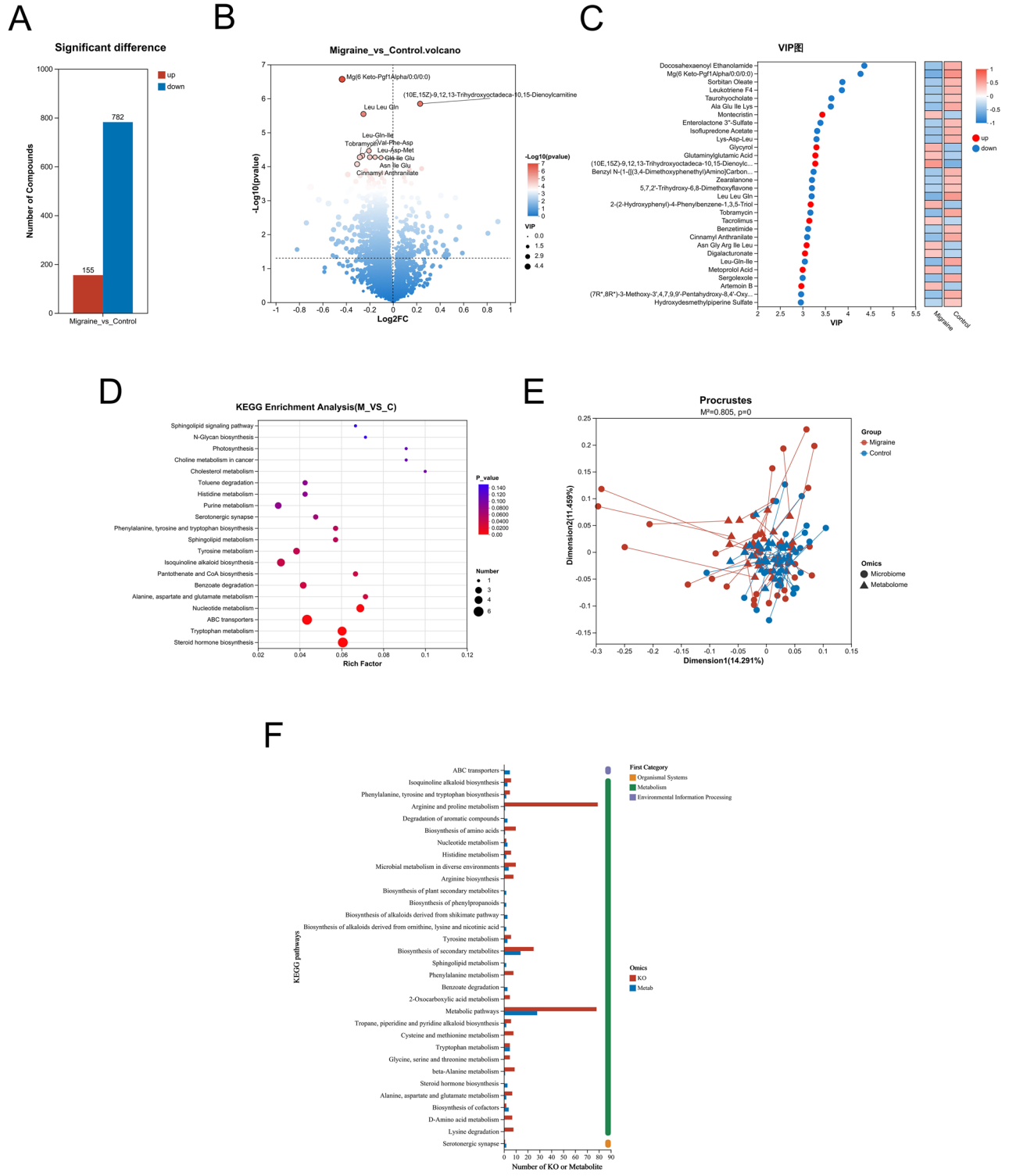


Fig. 7 (See legend on next page.)

Enrichment analysis of KEGG pathways revealed that four pathways—Steroid hormone biosynthesis, Tryptophan metabolism, ABC transporters, and Nucleotide metabolism—exhibited the smallest p-values ($P < 0.005$) and highest impact values. Additionally, kynurenic acid [28] within the tryptophan metabolism pathway was found to be significantly down-regulated (Supplementary Table 2).

Integration of metagenomic and metabolomic analyses revealed significant correlations between gut microbial

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Fig. 7 Differences in intestinal metabolites between children with migraine and healthy children and association analysis between metabolomics and metagenomics. **A**: The x-axis indicates comparison groups; the y-axis shows the number of metabolites. Red bars represent up-regulated differential metabolites, and blue bars represent down-regulated differential metabolites. **B**: Volcano plot of differential metabolites: the x-axis represents the $\log_2$(fold change) in metabolite expression between groups; the y-axis shows the -log^{10}(p-value), with higher values indicating more significant differences. Points represent metabolites, with point size corresponding to the VIP value. Red points denote significantly up-regulated metabolites, blue points significantly down-regulated metabolites, and gray points non-significant metabolites. Points on the left indicate down-regulated metabolites; points on the right indicate up-regulated metabolites. Points located farther toward the sides and top correspond to more significant differential expression. **C**: VIP bubble chart: the y-axis lists metabolites ranked by VIP value (top to bottom); the x-axis shows the VIP value. The heatmap on the right displays relative metabolite expression across samples, with each column representing a sample and each row a metabolite. The color gradient indicates expression levels, as shown in the color scale. **D**: KEGG pathway enrichment bubble plot: the x-axis represents the enrichment ratio (num_in_study/ num_in_pop); the y-axis shows KEGG pathways. Bubble size indicates the number of metabolites enriched in the pathway; bubble color corresponds to the significance of enrichment (*P*-value). **E**: Procrustes analysis: lines connect microbiome (solid circle) and metabolome (solid triangle) data from the same sample. Shorter lines indicate higher concordance between the two datasets. Significance was assessed using Monte Carlo label permutation tests: $P < 0.01$ indicates highly significant consistency in trends between microbial abundance and metabolite expression; $P < 0.05$ indicates significant consistency; $P \geq 0.05$ indicates no significant consistency. **F**: KEGG pathway annotation analysis

communities and metabolite profiles, suggesting a close association between the gut microbiota and intestinal metabolites in the two groups. Moreover, the results showed substantial alterations in the gut microbiota of children with migraine (Mantel test: $r = 0.35$, $P = 0.001$; $M^2 = 0.805$, $P < 0.01$; Fig. 7E). Pathway annotation analysis of the differential metabolite set indicated that the identified differential species and metabolites were enriched in the arginine and proline metabolism pathway and the tryptophan metabolism pathway (Fig. 7F).

Discussion

In this study, we performed a multi-omics analysis integrating gut metagenomics and untargeted metabolomics to investigate alterations in the gut microbiota of children with migraine in Hebei, China. Although previous studies from various regions have reported associations between gut microbial diversity and pediatric migraine [29–30], most relied on 16 S rRNA taxonomic profiling and plasma metabolomics. To our knowledge, this is the first comprehensive investigation of both gut microbiota and fecal metabolites in children with migraine, suggesting a potential association between migraine and gut microbiota dysbiosis in the pediatric population. These findings are essential for developing non-invasive diagnostic approaches and providing insights into the metabolic features of childhood migraine.

The decrease in the Shannon index and increase in the Simpson index highlight the distinct gut microbial profile in children with migraine, suggesting that reduced microbial diversity may be linked to disease-specific pathological mechanisms. Evidence indicates that migraine is a disorder of the central nervous system involving dysregulation of the neuro–immune–endocrine network [31]. Gut dysbiosis may be involved in the release of pro-inflammatory factors [32], potentially contributing to neuroinflammation and the occurrence of headache attacks [33]. Although the total number of species showed only minor differences between the migraine and control groups in this study, the significant changes in alpha-diversity indices point toward a qualitative shift in the gut microbiota structure of children with migraine.

For instance, *Pseudomonadota*—significantly enriched in children with migraine—includes numerous pathogenic bacteria such as *Escherichia coli*, *Salmonella*, *Vibrio*, and *Legionella*. These findings highlight distinct functional differences in microbial metabolism between children with migraine and healthy controls and suggest that Escherichia coli may represent a major species associated with the gut microbiota of children with migraine, rather than establishing a causal role. Among these, *Providencia* and *Escherichia coli* appear to play pivotal roles and may serve as keystone species in the gut microbiota of these patients. The LPS derived from E. coli can activate the Toll-like receptor 4 (TLR4) signaling pathway, potentially leading to a pro-inflammatory response and the release of cytokines [34–35]. These inflammatory mediators have been suggested to interact with the central nervous system, potentially contributing to the exacerbation of neuroinflammation and migraine attacks. Notably, LPS is widely used to establish experimental models of neuroinflammation [36]. Consistently, Vuralli et al. reported elevated serum LPS levels in adult patients with chronic migraine [37].

Providencia may also increase exposure to gut-derived LPS and accelerate the release of pro-inflammatory factors. The increased abundance of *Escherichia coli* and the significant enrichment of Providencia in pediatric migraine patients are consistent with the upregulation of the lipopolysaccharide biosynthesis pathway in their gut metabolites. A reduction in beneficial bacteria may weaken the antagonistic effect of short-chain fatty acids against pathogenic bacteria, disrupt the intestinal barrier, and enable pathogenic bacteria to amplify central sensitization via the gut–brain axis mechanisms.

In addition, we also observed a significant enrichment of Enterococcus in pediatric migraine patients. The cell wall of Enterococcus contains large amounts of peptidoglycan [38], and fragments generated from its degradation can be recognized by the host's immune receptors

and trigger inflammatory responses [39]. Upregulation of the peptidoglycan biosynthesis pathway may further enhance this immune activation mechanism. Increased peptidoglycan synthesis not only reflects bacterial growth and proliferation [40], but also activates the host immune system [41]. These findings suggest that exposure to gut cell wall–related molecules and the immune receptors they engage (e.g., NOD-like receptors [42]) may be in a more readily activatable state, thereby exacerbating neuroinflammation. Therefore, we hypothesize that the upregulation of the LPS and peptidoglycan biosynthesis pathways jointly promotes neuroinflammatory responses in pediatric migraine. We propose that this mechanism may represent a potential pathway contributing to the vicious cycle perpetuating the pathological process of migraine in children. However, our cross-sectional data do not allow us to determine causality, and this putative self-reinforcing circuit requires confirmation in longitudinal and mechanistic studies.

Notably, in the frequency-stratified analysis, at the species level, *Enterococcus sp.* showed an increasing trend with increasing attack frequency. Although this trend did not reach significance after FDR correction, its direction is consistent with the enrichment of *Enterococcus* observed in the overall comparison and our inference regarding peptidoglycan-related immune activation, suggesting that higher attack frequency may be associated with a greater likelihood of sustained gut peptidoglycan exposure and inflammation amplification [43].

On the other hand, our metagenomic and KEGG pathway enrichment analyses revealed significant functional differences between children with migraine and healthy controls. This study found that thiamine metabolism was downregulated in pediatric migraine patients, accompanied by upregulation of glycolysis/gluconeogenesis, folate (vitamin B9) biosynthesis, pyruvate metabolism, and the pentose phosphate pathway. These metabolic alterations may jointly reflect a metabolic reprogramming process centered on mitochondrial energy metabolism dysfunction. Thiamine (vitamin B1) is a key coenzyme of the pyruvate dehydrogenase complex [44], participates in glycolysis and the tricarboxylic acid cycle in vivo [45], and its most important function is to promote cellular energy metabolism and provide energy for neurons [46]. In pediatric migraine patients, downregulated thiamine metabolism may not be an isolated event, but rather a marker of mitochondrial dysfunction. This phenomenon is accompanied by upregulation of glycolysis/gluconeogenesis, pyruvate metabolism, and the pentose phosphate pathway. We speculate that these changes together form a feedback loop: downregulated thiamine metabolism leads to decreased activity of the pyruvate dehydrogenase complex [47], limiting the conversion of pyruvate to acetyl-CoA, thereby impairing oxidative phosphorylation [48]. Under constrained oxidative phosphorylation, neurons are forced to rely on glycolysis to meet energy demands [49]. Meanwhile, compensatory upregulation of pyruvate metabolism and gluconeogenesis pathways may alleviate the metabolic stress caused by pyruvate accumulation [50].

Increased fructose metabolism is usually associated with higher ATP consumption [51] and lactate accumulation [52], and alterations in mannose metabolism may affect the gut microbiota [53]. Therefore, upregulation of fructose and mannose metabolism may exacerbate this metabolic process, further affecting host metabolic homeostasis and aggravating neuroinflammation. These metabolic alterations may induce intracellular energy imbalance and oxidative stress in neurons, thereby triggering cortical spreading depression (CSD) and neuroinflammatory responses, ultimately leading to clinical migraine attacks. In addition, activation of the pentose phosphate pathway suggests elevated oxidative stress levels [54], which also points to mitochondrial dysfunction and may indirectly promote migraine attacks. On the other hand, the gut microbiota may be attempting to strengthen the one-carbon metabolism cycle [54] by upregulating folate synthesis, in order to eliminate homocysteine (Hcy) [55] and oxidative stress products accumulated due to mitochondrial dysfunction. These findings suggest that the microbial community in migraine patients exhibits relatively high metabolic activity, enhanced environmental adaptability, and potential pathogenicity, in contrast to the microbiota of healthy children, which appears more consistent with microbial homeostasis and structural integrity. This observation is consistent with the noted enrichment of *Pseudomonadota* [56].

In frequency-stratified trend analyses, biosynthesis of secondary metabolites and tryptophan biosynthesis decreased with attack frequency, whereas carbohydrate digestion and absorption was non-monotonic. Although these trends were not significant after FDR correction, the directionality may reflect changes in gut microbial substrate utilization and energy acquisition strategies, which is directionally consistent with our discussion of energy metabolism abnormalities observed in the overall comparison.

Nitric oxide (NO) is widely implicated in migraine pathogenesis [57], and arginine serves as a direct precursor for NO synthesis [58]. Our results show that arginine and proline metabolic pathways are significantly up-regulated in children with migraine. We thus hypothesize that enhanced arginine metabolism in these children may contribute to excessive NO production, which could in turn be associated with cerebral vasodilation and activation of the trigeminovascular system. Concurrently, suppressed porphyrin metabolism may compromise

antioxidant capacity and contribute to mitochondrial dysfunction [59]. Together, these processes may contribute to oxidative stress and neurovascular inflammation, which could be involved in triggering migraine attacks in children.

In addition, branched-chain amino acids (BCAAs) are not only precursors of glutamate and γ-aminobutyric acid [60–61], but also play an important role in neuroprotection. In particular, leucine can promote neuroprotective functions through its interaction with the mTOR pathway. However, when the biosynthesis of these amino acids is downregulated, neurons may lack sufficient protection when facing oxidative stress [62], thereby exacerbating neuronal damage and headache attacks [63]. The early application of the BCAA transaminase inhibitor gabapentin in the treatment of chronic migraine [64] likewise confirms the close association between the BCAA metabolic pathway and the neurochemical basis of migraine. Moreover, in studies on serum amino acid changes in adult migraine patients, Domitrz also found alterations in BCAA levels, and patients with migraine without aura had lower valine and leucine levels [65].

It is worth noting that disruptions in intestinal microbiota in children with migraine are closely associated with the intestinal metabolic processes of the host. Our species–function association analysis further indicated that the microbiota contribution to key metabolic pathways differed between the two groups. In the butanoate metabolism pathway, *Escherichia coli* became the major contributing species in the migraine group, whereas the healthy control group was more dominated by *Bacteroides sp.* Evidence has shown that butyric acid can reduce intestinal inflammation and demonstrates anti-inflammatory potential [66]. The differences in species with high contributions to butyric acid metabolism between the two groups reflect disruptions in short-chain fatty acid metabolism and arginine and proline metabolism in the intestines of children with migraine in which *Escherichia coli.* may play an important contributory role. As another core component of short-chain fatty acids, propionate can not only exert anti-inflammatory effects by crossing the blood–brain barrier [67], but also serve as an alternative energy source for neurons by entering the tricarboxylic acid cycle [68]. We speculate that the upregulation of the propionate metabolism pathway may represent a compensatory response aimed at counteracting the metabolic dysregulation driven by *Escherichia coli*.

In contrast, the intestinal microbiota of healthy children is dominated by *Bacteroides sp.*, *Blautia sp.*, and *Bifidobacterium sp.*, which are generally considered to have beneficial effects, being associated with maintaining metabolic homeostasis, strengthening the intestinal barrier, and inhibiting inflammation [69]. At the species level, we further observed in the frequency-group comparisons that *Segatella copri* showed a decreasing trend with increasing attack frequency, whereas *Bifidobacterium breve* showed an increasing trend (both not significant after FDR correction). This suggests that microbial changes across different migraine attack frequencies may not follow a simple pattern of further decreases in beneficial bacteria or further increases in harmful bacteria, but may instead indicate that these species have more complex, context-dependent roles in the pediatric gut environment.

Therefore, this shift "from an SCFA-associated symbiont–dominated to an *Escherichia coli* dominated metabolic profile" may alter gut–brain axis signal transduction at multiple levels and influence migraine susceptibility. First, at the mucosal barrier level, impaired functions of SCFA-associated symbionts, such as those producing butyrate, may reduce the support of metabolites such as butyrate for epithelial energy supply [70] and mucosal homeostasis [71], thereby compromising intestinal barrier integrity and increasing the likelihood of translocation of endotoxins such as LPS across the barrier. Second, endotoxin exposure and barrier impairment can drive innate immune activation and induce the release of proinflammatory cytokines [72]. In migraine, a disease that relies heavily on neuro-immune interactions [73], the persistent presence of peripheral inflammatory signals may more readily trigger or maintain increased excitability of the trigeminovascular system [74] and central sensitization [75], thereby lowering the attack threshold and increasing susceptibility to pediatric migraine. However, this inference is currently based on cross-sectional associations, and future studies combining longitudinal follow-up and targeted quantification are needed to validate the causal direction and key mediating links.

Furthermore, the intestinal metabolites of children with migraine also demonstrated imbalances, and the neuroprotective mediators exhibited significant depletion. Among these metabolites, DHEA is an endogenous cannabinoid derived from omega-3 fatty acids [76] and has anti-neuroinflammatory properties [77]. Therefore, the depletion of DHEA may be implicated in migraine pathophysiology in children, potentially reducing endogenous anti-neuroinflammatory capacity, although this association does not establish a causal relationship.

More importantly, we found a significant decrease in kynurenic acid levels in intestinal metabolites, which is consistent with the significant decrease in plasma kynurenic acid levels found by Liu et al. [78]. Our study provides direct metabolic evidence for the hypothesis proposed by Doga Vuralli that the gut microbiota mediates dietary triggers through tryptophan metabolism [79], and additionally supports the hypothesis that tryptophan metabolites, especially kynurenic acid, may serve as potential biomarkers of pediatric migraine, although

further studies are needed to validate their diagnostic utility.

In this study, we explored the changes in intestinal microbiota in children with migraine. Metagenomics and non-targeted metabolomics jointly revealed the characteristics of "high energy consumption, high adaptability, and low synthesis" in the microbial community of children with migraine, while the microbial community of healthy children showed the characteristics of "stability, self-sufficiency, and extensive maintenance." High energy consumption is reflected by the upregulation of glycolysis, gluconeogenesis, and pyruvate metabolism, pathways that are closely associated with rapid ATP generation and carbon flux, reflecting a high metabolic demand. High adaptability is demonstrated by the concurrent upregulation of multiple metabolic pathways, suggesting that the microbiota or cells, when faced with limitations in oxidative phosphorylation, possess significant metabolic flexibility and can rapidly adjust their metabolic patterns. Low synthesis refers to the relatively low enrichment of pathways associated with long-term biosynthesis and structural maintenance, indicating that cells or microbiota are more inclined to meet short-term energy demands rather than relying on long-term synthesis and homeostasis maintenance. In contrast, the microbiota of healthy children exhibits characteristics of "stability, self-sufficiency, and maintenance," indicating that their microbiota has a stronger ability to maintain homeostasis and self-sufficiency. This characteristic differs from the metabolic pathway distribution in pediatric migraine patients, who do not show obvious high energy consumption and high adaptability traits, but instead concentrate more on stable metabolic pathways, such as fatty acid and amino acid synthesis, to maintain the long-term symbiotic relationship between the microbiota and the host.

In the gut microbiota of pediatric migraine patients, we observed a significant decrease in microbial diversity, characterized by an imbalance pattern with an enrichment of potential pro-inflammatory microbiota and a reduction of homeostasis-related microbiota. This result is consistent with the overall trend observed in recent adult migraine studies, which have revealed a "shift of microbiota imbalance towards an inflammatory phenotype." We found that *Bifidobacterium sp.*, *Ruminococcus*, and *Clostridia* were significantly enriched in healthy children, but were relatively reduced in the gut of migraine patients. This finding strongly supports the causal relationship between the dysregulation of *Bifidobacteriales* and migraine development, as proposed by He et al. [80]. On the functional level, the upregulation of LPS biosynthesis pathways in migraine patients not only corresponds to its diagnostic accuracy (AUC = 0.75), but also aligns with the high levels of LPS detected in serum from adult migraine patients [37]. Moreover, the upregulation of carbohydrate pathways observed in pediatric migraine patients shows some consistency with oral microbiota features found in adult migraine patients [81]. However, considering the differences in ecological niches and potential confounding factors, cross-site comparisons should still be interpreted with caution.

Interestingly, while Vuralli D et al. emphasized the pathogenic role of microbiota such as *Desulfovibrio* [37] adult chronic migraine cohorts with medication overuse, our study in the pediatric cohort found that *Escherichia coli* exhibited the largest abundance difference and contributed the most to metabolic functions as a potential pathogenic bacterium. This difference suggests that in early childhood, overexpression of opportunistic pathogens, represented by *Escherichia coli*, may constitute the primary driving force of migraine, while in adult patients, the microbiota structure may evolve further due to disease progression and pharmacological interventions. Pediatric and adult migraines share a core mechanism of "pro-inflammatory dysbiosis," but show age-specific differences in the key driving species and the underlying formation of this dysbiosis.

However, our study has some limitations. Diet is one of the major determinants of gut microbiota composition and metabolic output. Therefore, when interpreting our species–function–metabolite associations, it is important to consider the potential impact of not conducting a systematic dietary and lifestyle assessment. The lack of measurements for dietary structure and lifestyle variables may introduce residual confounding, which could partially influence the taxonomic, functional, and metabolomic differences observed, limiting causal interpretations. We attempted to reduce the interference from regional dietary and lifestyle differences by including children from the same geographic region; however, even so, unmeasured dietary and lifestyle exposures may still influence the microbiota–metabolome features observed in pediatric migraine. Future studies should incorporate standardized dietary tools (e.g., repeated 24-hour dietary recalls, validated food frequency questionnaires), collect key lifestyle variables (sleep, activity, stress), and include these covariates in multivariate and stratified models. By excluding children with current or prior use of migraine-specific preventive medications and enrolling medication-naïve patients at baseline, our findings may not generalize to treated pediatric migraine. In addition, analyses stratified by migraine frequency were exploratory and did not remain significant after FDR correction, possibly due to limited power and within-group heterogeneity. Larger, longitudinal cohorts incorporating standardized diet/lifestyle measures and targeted metabolomics are warranted to validate these trends and clarify directionality.

In summary, our study demonstrated the association of gut microbiota and gut metabolites with migraine in children, and suggested that gut microbiota may play a role in the pathophysiology of migraine in children, and provided key clues for understanding microbial community metabolism and related dysfunction in disease states. Notably, this effect may be closely related to functional alterations in *Pseudomonadota* and *Escherichia coli*, as well as to abnormal levels of metabolites derived from them. Moreover, our findings provide strong evidence supporting kynurenic acid as a potential diagnostic biomarker for pediatric migraine and a target for future metabolite-focused studies. The discovery that the lipopolysaccharide biosynthetic pathway may represent a potential mechanism-related biomarker candidate for migraine in children and may help to inform future efforts to move from purely symptom-based toward more mechanism-informed diagnostic approaches, although further validation is required.

Abbreviations

AUC	Area Under the Curve
HIT-6	Headache Impact Test-6
CD-HIT	Cluster Database at High Identity with Tolerance
LC–MS	Liquid Chromatography–Mass Spectrometry
KEGG	Kyoto Encyclopedia of Genes and Genomes
DALYs	Disability-adjusted life-years
SCFAs	Short-chain fatty acids
ICHD-3	International Classification of Headache Disorders, 3rd edition
VAS	Visual Analogue Scale
Ped MIDAS	Pediatric Migraine Disability Assessment Score
PE	Paired-end
FDR	False discovery rate
PCoA	Principal coordinate analysis
ANOSIM	Analysis of similarities
VIP	Variable importance in projection
KO	KEGG Orthology
ROC	Receiver operating characteristic
LPS	Lipopolysaccharide
TLR4	Toll-like receptor 4
NO	Nitric oxide
DHEA	Docosahexaenoyl Ethanolamide

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

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Author contributions

NT and ML contributed equally to this work.NT and ML contributed to the design of the study and drafting and revising the manuscript. NT and ML collected the data NT carried out the statistical analysis. NT, ML, YZ, MJ and YFL contributed to the interpretation of data. MJ and FY supervised and revised the manuscript. FY completed the experimental design, funding sources, review-editing. All authors reviewed and approved the final manuscript.

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Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

The study was granted ethical approval by the Ethics Committee of Children's Hospital of Hebei province(Ethics approval number: 2024132). The study was carried out following the ethical standards of the responsible committee on human experimentation and with the Declaration of Helsinki.

Consent for publication

The collection of data were obtained from the written informed consent of the guardian of the child and the ethical approval of the institutional review committee.

Competing interests

The authors declare no competing interests.

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