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Dysbiosis of gut microbiota in children with functional constipation and damp-heat constitution: a cross-sectional multi-omics analysis

Haihong Zhao^{1,5}, Xiuxia Lu^{2,5}, Yanbin Ye², Weiwei Li¹, Hui Ye¹, Ziyu Long³, Xuezhi Zhang¹✉, Jieling Wu²✉ & Ji Wang⁴

Functional constipation (FC), a prevalent gastrointestinal disorder in early childhood associated with gut microbial dysbiosis, shows significant connections with specific constitutional types in Traditional Chinese Medicine (TCM). While TCM's nine constitutional classifications identify damp-heat constitution as a potential risk factor for FC, pediatric-specific evidence remains scarce regarding this correlation. Using the Constitutional Medicine Questionnaire, 49 children under 2 years were stratified into two groups: 18 children with damp-heat constitution who suffered from constipation (DHC) and 31 children with balanced constitution who did not suffer from constipation (BC). Clinical assessments (Bristol Stool Scale, symptom profiles), gut microbial profiling (16 S rRNA sequencing), and urinary metabolomics (UPLC-Q-TOF/MS) revealed marked intergroup differences. DHC subjects demonstrated reduced stool consistency scores, heightened symptom severity, distinct microbial communities, and altered sphingolipid-related metabolic pathways. A diagnostic panel combining six microbial markers and six metabolites achieved effective discrimination. This multi-omics investigation delineates the pathophysiological characteristics of FC in DHC children, proposing novel therapeutic targets through TCM constitution-based microbial-metabolite interactions.

Functional constipation (FC) is a functional bowel disorder clinically defined by persistent defecatory dysfunction (infrequent and/or incomplete evacuation) in the absence of organic pathology, posing significant morbidity and substantial healthcare burden due to its chronicity and treatment complexity¹. In pediatric populations, organic causes of constipation are exceedingly rare, with studies indicating that over 90% of cases are of functional origin^{2,3}. Globally, the prevalence of FC in children was reported to be 9.5% in 2018⁴, while in Asia, the prevalence reached up to 12% in 2024⁵. FC is the most common gastrointestinal disorder in children, with the majority of cases developing during infancy and toddlerhood^{6–8}. If childhood constipation remains undiagnosed or inadequately treated, approximately 25% of affected individuals may experience persistent symptoms into adulthood, significantly impacting their quality of life⁹.

According to Traditional Chinese Medicine Constitution (TCMC) theory, individuals are classified into nine constitutional types, each with unique causes and distinct manifestations. A balanced constitution represents a state of general health with minimal susceptibility to disease. Among the unbalanced constitutions, damp-heat constitution is one of the most common types, characterized by an excess of dampness and heat in the body. This condition is typically manifested by symptoms such as dry or sticky stools, deep yellowish urine, and a red tongue with a yellowish greasy coating¹⁰. In preliminary research, we observed that the gut microbiota and metabolomics of children with damp-heat constitution differ from those of healthy children¹¹. Our team conducted a statistical analysis of the constitutional distribution among 1069 children under the age of

¹Traditional Chinese Medicine and Integrative Medicine Department, Peking University First Hospital, Peking University, Beijing, China. ²Department of Children's Health Care, Guangdong Women and Children Hospital, Guangzhou, China. ³Traditional Chinese Medicine School, Faculty of Chinese Medicine Science Guangxi University of Chinese Medicine, Nanning, China. ⁴National Institute of Traditional Chinese Medicine Constitution and Preventive Treatment of Disease, Beijing University of Chinese Medicine, Beijing, China. ⁵Haihong Zhao and XiuxiaLu contributed equally to this work. ✉email: Zhang.xuezhi@263.net; jieling3861@163.com; doctorwang2009@126.com

2 in China. The results indicated that a balanced constitution accounted for 56.22%, followed by damp-heat constitution at 16.00%¹¹. These findings demonstrate that damp-heat constitution is highly prevalent among children. Individuals with a damp-heat constitution are predisposed to various diseases^{12,13}. For instance, this constitution is characterized by excessive dampness and heat in the body, which can impair intestinal peristalsis and delay fecal elimination, ultimately leading to constipation. Song¹⁴ utilized the chi-square test to analyze the relationship between functional constipation (FC) and TCMC, concluding that there is a statistically significant correlation between FC and damp-heat constitution. Furthermore, Wang¹⁵ investigated the distribution of constitutional types among children with FC and found that damp-heat constitution accounted for as high as 13.18%. Based on these insights, it is essential to conduct further research into the underlying mechanisms of damp-heat constitution and its association with FC in children, as this may provide valuable information for developing targeted interventions and improving clinical outcomes.

With the rapid advancement of modern research techniques, an increasing number of studies have explored the correlation between different constitutional types and intestinal flora^{16–19}. However, the majority of these investigations have focused on adult populations, with limited attention devoted to children under 2 years of age. The gut microbiota of infants and toddlers plays a critical role in shaping their health at various developmental stages and exerts long-term effects on the maturation of the immune and endocrine systems throughout life^{20,21}. In this study, we used a cross-sectional study with children aged 0–2 years and screened children with damp-heat constitution and functional constipation (DHC) as well as those with balanced constitution but without functional constipation (BC). Fecal and urinary samples were collected from these participants and subjected to 16 S rRNA gene sequencing and UPLC-Q-TOF/MS analysis, respectively. This approach was employed to investigate the characteristics of the intestinal flora in DHC children and elucidate the underlying metabolic mechanisms associated with this condition. Such insights may provide a foundation for developing targeted interventions and improving clinical outcomes in pediatric populations.

Materials and methods

Study design and participants

Children aged 0–2 years from Guangdong and Beijing, China, were recruited between November 2022 and April 2023. Participants were screened using Wang Qi's nine body types in Constitutional Medicine Questionnaire (Supplementary file 1). This questionnaire has been integrated into national public health management systems and is widely utilized in countries such as Japan, Korea, and the USA^{22–24}. The questionnaire comprises 43 items corresponding to nine constitutional types, and individuals are classified as a specific constitution based on their transformation scores meeting predefined criteria¹¹.

Participants were required to meet the following criteria: (1) infants aged 0–2 years old; (2) parents who had cared for the infants for a long time and had a detailed knowledge of the infant's basic condition; (3) infants with damp-heat constitution or balanced constitution diagnosed using the Constitutional Medicine Questionnaires. Participants meeting one or more of the following criteria were excluded: (1) infants who received antibiotics and probiotics within 3 months prior to sample collection; (2) infants with a mental illness or severe disease that were unable to participate in and complete clinical studies in a standardized manner; (3) infants living far from the hospital where stool and urine samples were not readily available; (4) infants with incomplete clinical data.

FC was diagnosed according to the Rome IV criteria⁸. Children classified as DHC (damp-heat constitution with functional constipation) and BC (balanced constitution without functional constipation) were included in the fecal flora and urinary metabolomics study. The study was approved by the Ethics Committee of Beijing University of Chinese Medicine (Approval No. 2020BZYL122). All guardians of the subjects were provided with detailed information regarding the trial procedures and provided written informed consent. The study flow chart was presented in Fig. 1.

Fecal sample collection, DNA extraction and 16 S rRNA sequencing

Fecal samples were collected and processed following standardized procedures, as previously described²⁵. Shortly, freshly produced stool samples that did not contain urine were collected every morning; > 3 g (two scoops) from the middle of the stool was scooped into a sterile stool cup. Initially, the child's guardian collected freshly produced, urine-free fecal samples at home and transported them to the hospital within one hour, maintaining low temperatures using an ice pack. Upon arrival, the samples were immediately flash-frozen in liquid nitrogen and subsequently stored at – 80 °C for long-term preservation. Genomic DNA was extracted from the fecal samples using the CTAB/SDS method. The quality and concentration of the extracted DNA were assessed via agarose gel electrophoresis. The hypervariable V3–V4 region of the 16 S rRNA gene was amplified by PCR using barcode indexed primers (341 F-806 R), and the resulting amplicons were purified to construct sequencing libraries. Finally, the libraries were sequenced on the Illumina MiSeq platform to generate high-throughput sequencing data for analysis.

Gut microbiota analysis

Sequencing analysis was performed using the DATA2 workflow following the merging of paired-end reads and assignment to respective samples. Sequences with similarity $\geq 97\%$ were grouped into the same ASV. The α and β diversity of the microbial community were evaluated using QIIME2 2019.4 software²⁶. To identify differentially abundant microbial taxa, LEfSe analysis was conducted, with a threshold of LDA score > 2.0 ²⁷. Additionally, PICRUSt software (v 1.1.1) was employed to predict functional differences by analyzing gene composition based on the KEGG database^{28–31}.

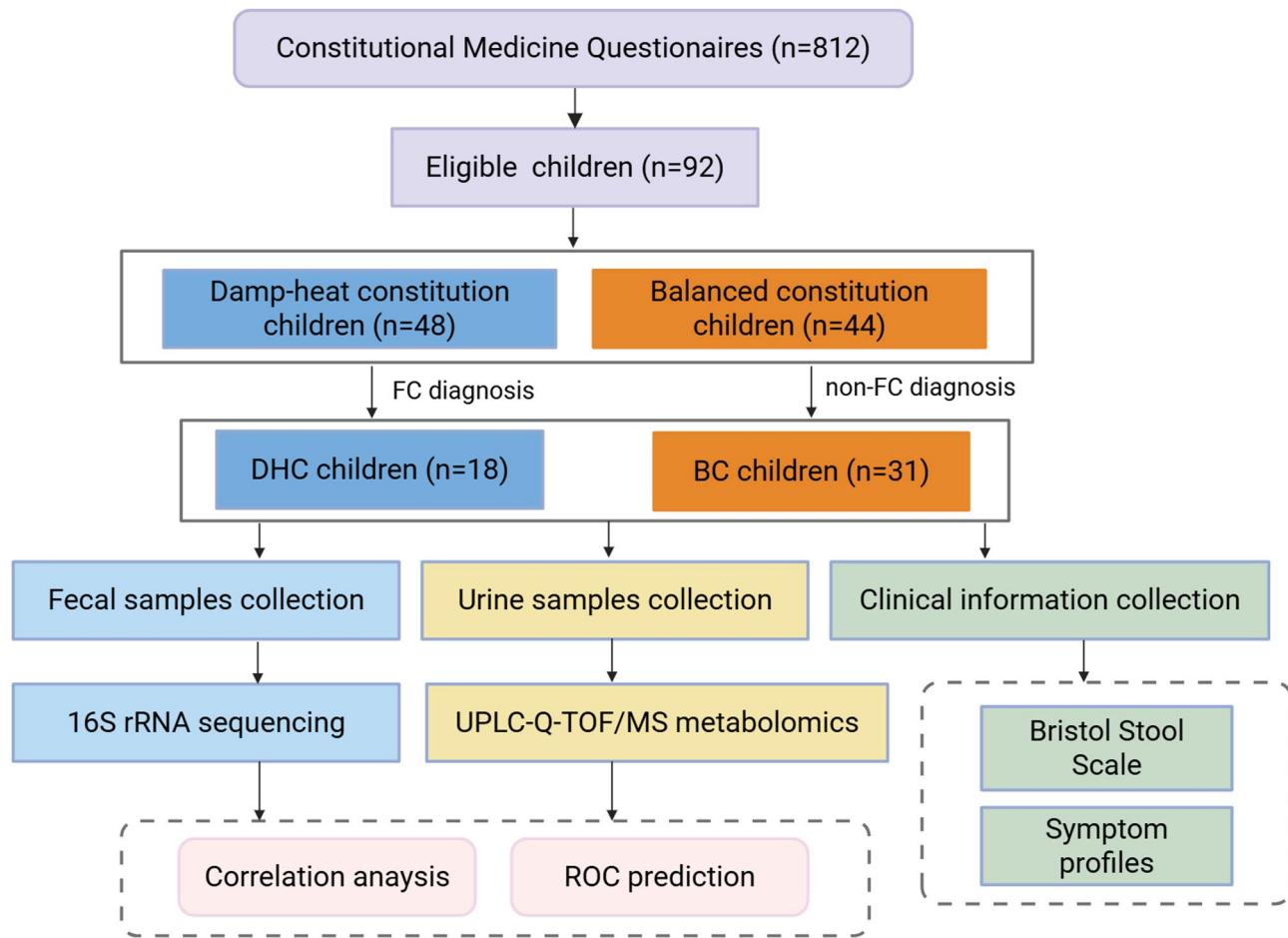


Fig. 1. The study flow chart. FC, functional constipation; DHC, damp-heat constitution with functional constipation; BC, balanced constitution without functional constipation.

Urine samples collection and UPLC-Q-TOF/MS processing

The middle portion of morning urine was collected at home by the child's guardian and transported to the hospital within 1 h, maintained at a low temperature using an ice pack. Upon arrival, the samples were centrifuged, and the supernatant was removed for rapid freezing and stored at -80°C . For metabolite extraction, the supernatant samples were shipped on dry ice to Shanghai Applied Protein Technology Co. Ltd. for further processing³². Briefly, the samples were thawed, mixed with methanol/acetonitrile solvent, sonicated, and centrifuged. The resulting supernatant was collected and dried, then reconstituted in acetonitrile/water solvent, vortexed, and centrifuged again. Finally, the last supernatant was analyzed using ultra-high-performance liquid chromatography (UHPLC) coupled with quadrupole time-of-flight mass spectrometry (Q-TOF MS) employing a TOF 5600 + system. This comprehensive workflow ensured accurate and reliable metabolomic profiling of the urine samples.

Metabolomics data analysis

For data analysis, the mzXML format was utilized for data processing³³. Metabolite characteristics were detected using the XCMS 3.10.1 software package (Scripps, La Jolla, CA, USA), with peak alignment performed via the obiwar algorithm³⁴. Subsequently, the data underwent metabolite structure identification and preprocessing, followed by quality evaluation of experimental data and final statistical analysis. Univariate analysis, exemplified by fold change analysis, was employed as one of the most commonly used statistical methods. Orthogonal partial least squares discriminant analysis (OPLS-DA) was conducted for multivariate analysis to identify global metabolic differences between the DHC and BC groups. The variable importance in projection (VIP) was used to determine characteristic metabolites in the two groups. MetaboAnalyst web software was utilized for metabolic pathway analysis, and Fisher's exact test was applied to evaluate the significance level of enriched pathways.

Correlation analysis, biomarker identification and statistical analysis

Spearman analysis and matrix heat map were utilized to show the correlation between differential flora and metabolites in the two groups by R and Cytoscape software. The area under a receiver operating characteristic curve (AUC) was adopted to mine markers of flora and metabolites in DHC children. For microbiome studies, multiple comparison corrections were performed as follows: box plots were generated using R scripts to visually illustrate α diversity differences between DHC and BC groups. Kruskal-Wallis rank-sum tests and Dunn's

Variables		DHC (n = 18)	BC (n = 31)	P-value
Age (months)		16.50 ± 5.33	19.06 ± 4.88	0.162
Gender (n, %)	Boys	7 (38.89%)	17 (54.84%)	0.282
	Girls	11 (61.11%)	14 (45.16%)	
Delivery mode (n, %)	Vaginal delivery	10 (55.56%)	17 (54.84%)	0.961
	Caesarean delivery	8 (44.44%)	14 (45.16%)	
Feeding pattern (n, %)	Breast feeding	9 (50.00%)	20 (64.52%)	0.319
	Non-breast feeding	9 (50.00%)	11 (35.48%)	

Table 1. Demographic characteristics of the DHC and BC groups.

BSS term	DHC group (score, %)	BC group (score, %)	P-value
Separate hard lumps, nuts-like	2 (3.17%)	0	–
Sausage-shaped but lumpy	4 (6.35%)	0	–
Sausage-shaped with cracks	24 (38.10%)	3 (1.99%)	–
Smooth snake	8 (12.70%)	40 (26.49%)	–
Soft blobs	0	65 (43.05%)	–
Fluffy pieces	18 (28.57%)	36 (23.84%)	–
Watery	7 (11.11%)	7 (4.64%)	–
Total score (mean ± SD)	3.5 ± 1.72	4.87 ± 0.88	0.006

Table 2. BBS score of the DHC and BC groups.

post-hoc tests were employed for multiple comparison correction to validate statistical significance. Inter-group differential analysis utilised PERMANOVA tests. For metabolomics, statistical analysis employed t-tests supplemented with FDR (False Discovery Rate) correction for multiple comparisons. Statistical analyses were performed using SPSS and GraphPad Prism software. $P < 0.05$ was taken as a significant difference.

Results

General information of participants

A total of 812 questionnaires were received, 31 BC children and 18 DHC children were enrolled in the study after data screening, constitution identification and FC diagnosis. It is widely believed that age^{35,36}, gender^{37,38}, delivery mode^{39–41}, and feeding pattern^{42–44} can influence the gut bacterial composition; therefore, in this study, the above variables were statistically analyzed in the DHC and BC groups and the results showed no significant differences (Table 1).

DHC children exhibited changes in stool shape and frequency

Given the young age of the participants, who may not be able to articulate their perceptions, parents utilized the Bristol Stool Scale (BSS), a widely recognized and validated tool that combines fecal images with descriptive terms, to evaluate stool consistency and assess constipation. For BC children, the predominant stool shapes were soft blobs, followed by smooth snake-like forms and fluffy pieces. These characteristics may be attributed to the young age and the typically thinner diameter of feces in this population. In contrast, DHC children predominantly exhibited sausage-shaped stools with cracks, with a smaller proportion showing fluffy pieces. This pattern was influenced not only by age-related characteristics but also by the presence of FC. Compared to BC children, the BSS scores were significantly lower in the DHC group ($P = 0.0006$), indicating more pronounced constipation in DHC children (Table 2).

In addition to BSS assessments, the prevalence of various FC symptoms was also evaluated. The results demonstrated that nearly half of the DHC children had a history of excessive stool retention (41.46%), followed by painful or hard bowel movements (29.27%) and fewer than two defecations per week (19.51%). By comparison, only 12.9% of BC children experienced one or two constipation-related symptoms (Table 3). These findings underscore the distinct gastrointestinal manifestations associated with DHC and highlight the importance of considering constitutional differences in pediatric constipation management.

DHC children changed the diversity of gut flora

Fecal samples from the DHC and BC groups were processed using the DADA2 method, which included dereplication, size filtering, denoising, merging, and chimera removal. The DADA2 algorithm performs exact sequence matching without clustering, effectively corresponding to 100% similarity clustering. Each sequence generated after DADA2 quality control is referred to as an amplicon sequence variant (ASV), representing a precise microbial sequence. This approach is more accurate than the traditional 97% similarity-based operational taxonomic unit (ASV) clustering⁴⁵. The DHC and BC groups contained 27,962 and 15,101 specific ASVs, respectively, with 2041 overlapping ASVs identified between the two groups (Fig. 2A). These results provide a high-resolution view of the microbial community structure in both groups.

FC symptom	DHC group (n, %)	BC group (n, %)	P-value
Two or fewer defecations per week	8 (19.51%)	1 (3.23%)	–
History of excessive stool retention	17 (41.46%)	0	–
History of painful or hard bowel movements	12 (29.27%)	1 (3.23%)	–
History of large-diameter stools	2 (4.88%)	2 (6.45%)	–
Presence of a large fecal mass in the rectum	2 (4.88%)	0	–
Total number of people with symptoms	41 (100%)	4 (12.9%)	0.034

Table 3. Number of participants with FC symptom in two groups. Some participants had more than two FC symptoms. BSS, Bristol Stool Scale; FC, functional constipation.

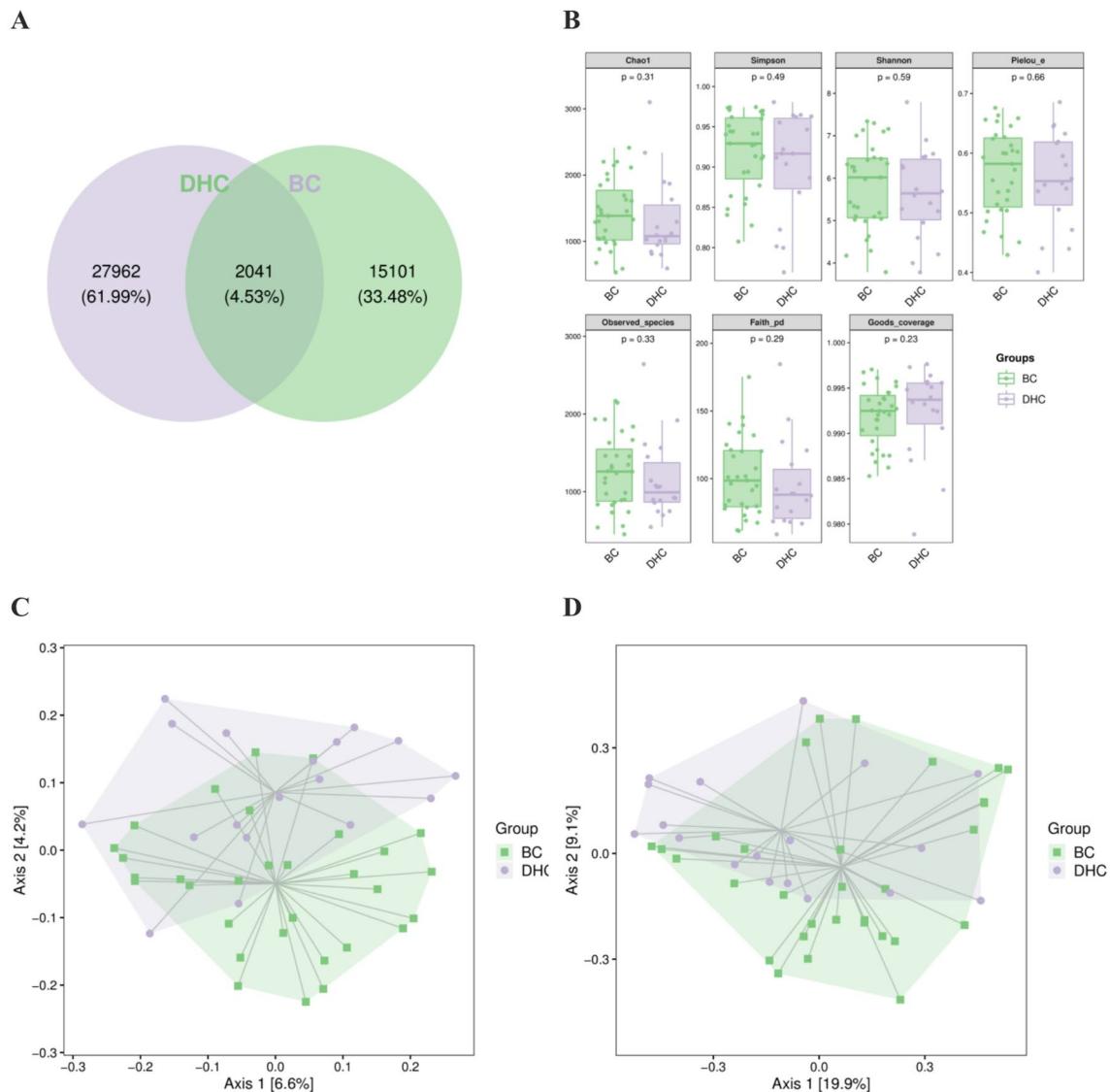


Fig. 2. DHC children changed the gut microbial diversity. (A) Venn diagram of ASVs in two groups. (B) α diversity of microbiota. (C) β diversity of microbiota based on unweighted unifrac distance. (D) β diversity of microbiota based on weighted unifrac distance. DHC, damp-heat constitution with functional constipation; BC, balanced constitution without functional constipation.

To comprehensively evaluate bacterial diversity within the samples, α diversity analysis was conducted in this study. Metrics such as Chao 1 and Observed species were used to assess species richness, Shannon and Simpson indices for species diversity, Faith's PD for phylogenetic diversity, Pielou's evenness for species evenness, and

Good's coverage for sampling adequacy. No statistically significant differences were observed between the DHC and BC groups for any of these metrics ($P > 0.05$) (Fig. 2B).

To investigate the differences in microbial community composition between groups, β diversity analysis was conducted. PCoA, based on unweighted and weighted unifrac distance, was used to examine the clustering patterns of microbial communities in the DHC and BC groups. The results demonstrated distinct separations between the microbial profiles of two groups (PERMANOVA $P = 0.003$, $P = 0.012$), suggesting significant differences in bacterial composition between the DHC and BC groups (Fig. 2C,D).

DHC children changed the composition of gut flora

The gut flora composition was analyzed at five taxonomic levels, namely, phylum, class, order, family, and genus, and the top 20 flora were listed at each level (Fig. 3A–E). Compared with that in the BC group, the relative abundance of phylum Actinobacteria and its genus *Bifidobacteria* increased in the DHC group, whereas the relative abundance of phylum Firmicutes decreased, which was consistent with the results on constipation by Wang et al.⁴⁶ LEfSe analyses allowed simultaneous analysis of differences at all taxonomic levels to be presented via the cladogram (Fig. 3F), and placed considerable emphasis on finding robust biomarkers across groups (Fig. 3G). A total of 38 characteristic flora were found, of which 15 were detected in the DHC group and 23 in the BC group. In the DHC group, the characteristic flora at the phylum level were Actinobacteria and Synergistetes, and at the genus level were *Bifidobacterium*, *Oscillospira*, *Eggerthella*, *Alistipes*, *Robinsoniella*, and *Desulfovibrio*. In the BC group, the characteristic phyla were Firmicutes and Cyanobacteria, and the characteristic genera were *Veillonella*, *Megamonas*, *Lachnospira*, *Campylobacter*, *Gemella*, *Selenomonas*, *Haemophilus*, and *Turicibacter*. Using the PICRUSt2 software, KEGG and MetaCyc pathways were used to predict the functional potential of DHC. The results consistently indicated that the function of DHC is primarily concentrated in three areas: carbohydrate, amino acid, cofactor and vitamin metabolism/biosynthesis (Fig. 3H–I).

DHC children altered the metabolomics profile

Intestinal bacteria play a direct role in the development of gastrointestinal disorders, and their metabolites can significantly influence the body's physiological and pathological states. Urine, as the end product of human metabolism, contains a rich array of metabolites that reflect the body's biochemical metabolic status. It is also non-invasive, non-infectious, and highly suitable for analyzing metabolic changes in young children. In this study, urine samples were collected from 31 BC children and 16 DHC children for untargeted metabolomic analysis.

The total ion flow plots of the quality control (QC) samples showed substantial overlap, indicating that the response intensities and retention times of the various chromatographic peaks were essentially identical. This suggests that variation caused by instrument error was minimal throughout the entire experimental process (Supplementary file 2). Differential analysis of all metabolites was performed using univariate statistical analysis and visualized through volcano plots (Fig. 4A, B). OPLS-DA revealed an overall trend of separation between DHC and BC samples (Fig. 4C,D), suggesting that the OPLS-DA model effectively differentiated the two groups. These findings indicate that the metabolite composition in DHC children underwent significant alterations compared to BC children.

Differential metabolite screening between two groups

The differences in metabolite expression profiles between groups can be quantified and analyzed using variable importance in projection (VIP) values derived from OPLS-DA, allowing for the identification of significantly altered metabolites. In this study, a stringent screening criterion of $VIP > 1.0$ and $P < 0.05$ was applied to select differential metabolites. As illustrated in Fig. 5A,B, a total of 59 metabolites were identified in positive ion mode and 9 in negative ion mode. The horizontal coordinates revealed distinct expression trends of metabolites across the two groups, indicating significant differences in their metabolic profiles. From the vertical coordinates, certain metabolites clustered together, suggesting potential functional similarities or involvement in shared biological processes. Detailed parameters of each differential metabolite are provided in Supplementary file 3.

Metabolic pathway annotation in DHC children

To further explore the metabolic mechanisms of DHC, the differential metabolites in urine were mapped to relevant physiological pathways. Analysis of the differential abundance scores indicated that the DHC group was associated with alterations in sphingolipid signaling pathways, sphingolipid metabolism, and apoptosis-related pathways (Fig. 5C). These findings provide insights into the potential metabolic alterations and biological processes involved in DHC.

Correlation analysis between the differential flora and metabolites

To investigate the potential relationship between metabolic changes and gut microbiota, Spearman correlation analysis was performed. A total of 119 significant correlations were identified between microbial taxa and metabolites, with 6 pairs exhibiting strong correlations (absolute correlation coefficients > 0.6). Specifically, *Oscillospira* showed strong positive correlations with indoxyl sulfate and coniferyl aldehyde. *Selenomonas* demonstrated negative correlations with fingolimod, palmitic acid, and octadecanoic acid. Additionally, *Veillonella* exhibited a negative association with indoxyl sulfate (Fig. 6). These findings suggest that specific gut microbes may play a role in regulating the levels of certain metabolites, potentially influencing the metabolic profile observed in DHC individuals.

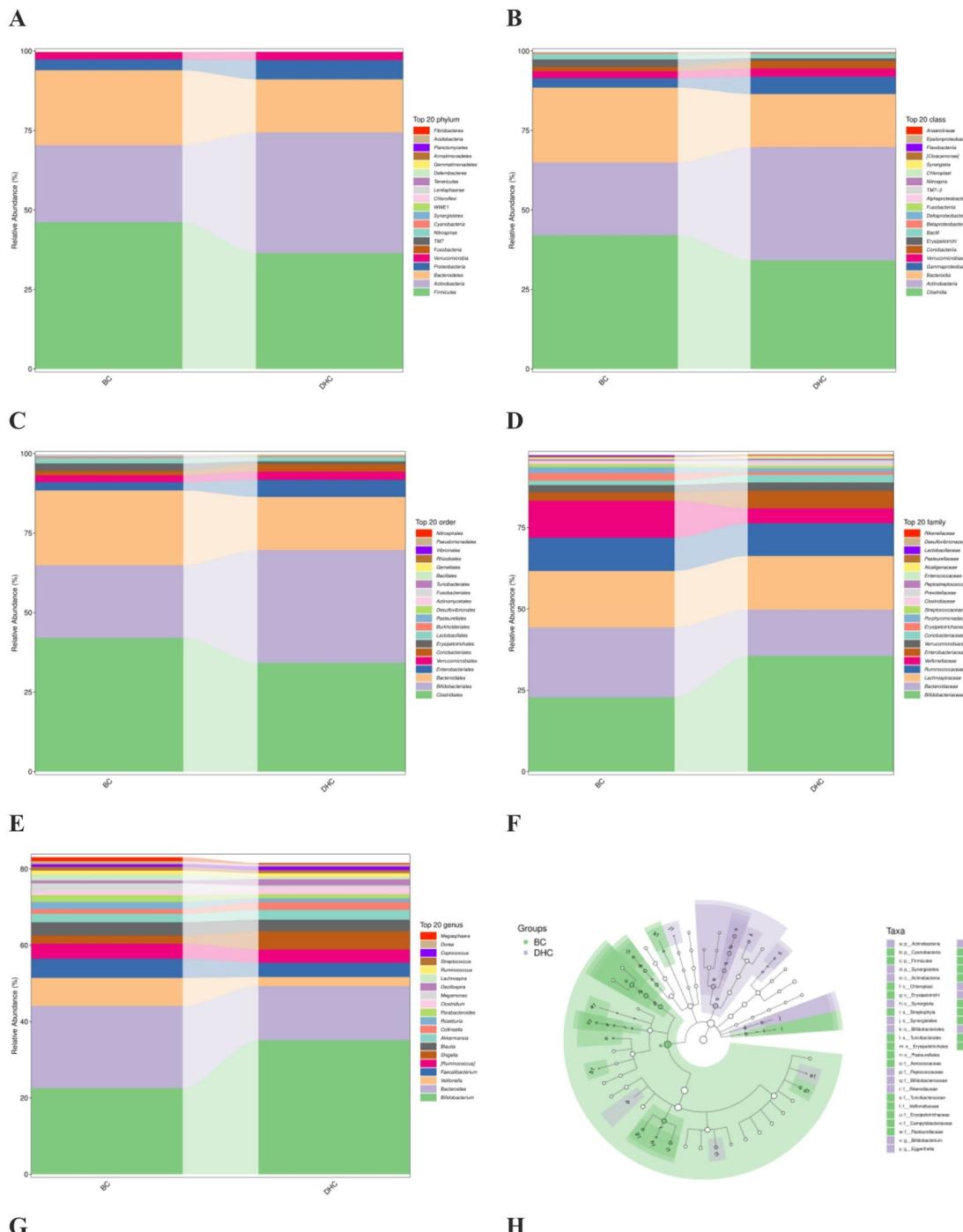


Fig. 3. DHC children changed the gut microbial composition. (A–E) Histogram showing the relative abundance of the top 20 taxa in phyla, class, orders, family, genera, respectively. (F) Cladogram of the phylogenetic distribution at all taxonomic levels. (G) LEfSe analysis showing gut flora with an LDA ($\log_{10}>2$). The length of the bar graph representing the size of the LDA. (H) KEGG pathway. (I) MetaCyc pathway. DHC, damp-heat constitution with functional constipation; BC, balanced constitution without functional constipation.

Potential biomarker of DHC children identification

To identify potential biomarkers for DHC children, receiver operating characteristic (ROC) analysis was employed. This method is widely recognized for its ability to evaluate the predictive accuracy of models by plotting sensitivity against 1-specificity across various thresholds.

The top six metabolites identified as potential biomarkers were oleamide, palmitamide, sphingosine, linoleoylglycine, 2-4-6-tri-tert-butylaniline, and 4-5-epoxy-7Z-10Z-13Z-16Z-19Z-docosapentaenoic acid

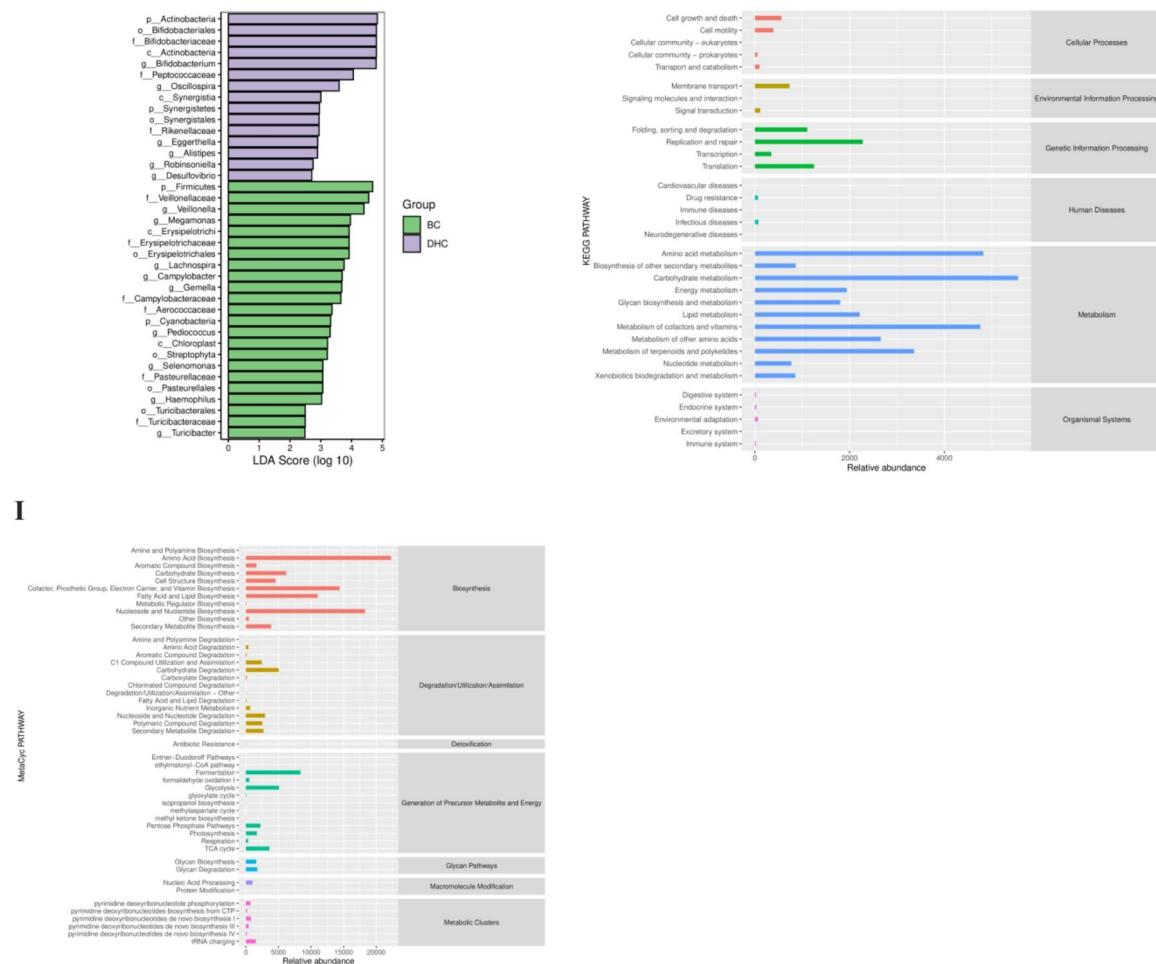


Fig. 3. (continued)

methyl ester. Additionally, the top six microbial taxa included *Bifidobacterium*, *Eggerthella*, *Megamonas*, *Oscillospira*, *Selenomonas*, and *Turicibacter*.

When combining these metabolites and microbial taxa, the resulting model demonstrated excellent discriminatory power, with an area under the curve (AUC) of 0.975. This indicates a highly accurate classification of DHC children. Furthermore, the sensitivity of this potential biomarker was 88.89%, while the specificity reached 100%, suggesting that the model not only effectively identifies DHC children but also minimizes false positives (Fig. 7). These results highlight the potential utility of this combined metabolite-microbiota panel as a robust biomarker for DHC children. Such findings underscore the importance of integrating both metabolic and microbial profiles in identifying constitution-specific biomarkers, which could have significant implications for personalized medicine and early intervention strategies.

Discussion

Academician Wang Qi of the Chinese Academy of Engineering and founder of TCMC theory proposed that human can be systematically categorized according to age stages, including childhood, youth, middle age, menopause, and old age. Each age group exhibits distinct constitutional characteristics, reflecting unique morphological structures, physiological functions, and psychological states. Interestingly, different constitutions demonstrate varying susceptibilities and predispositions to specific diseases⁴⁷.

The first 1000 days from conception to 2 years of age is a critical period for early childhood growth and development, during which the gastrointestinal microbial community plays a critical role in immune, endocrine, metabolic and other host developmental pathways. And the emerging view of human developmental biology suggests that trillions of microorganisms and their genes are formed and stabilized for survival in the human body during the first 2 years of life⁴⁸. Therefore, we focused on the gut microbiota of children with damp-heat constitution aged 0–2 years. China's earliest surviving pediatric monograph, *Lu Xin Jing*, records that children under 3 years of age have vigorous Yang Qi, corresponding mainly to the damp-heat constitution of nine constitutions, which is consistent with the results of our cross-sectional survey of children's constitution in China¹¹. The individuals with damp-heat constitution are more susceptible to functional constipation due to excessive dampness and heat in the intestines, which impairs their conduction function.

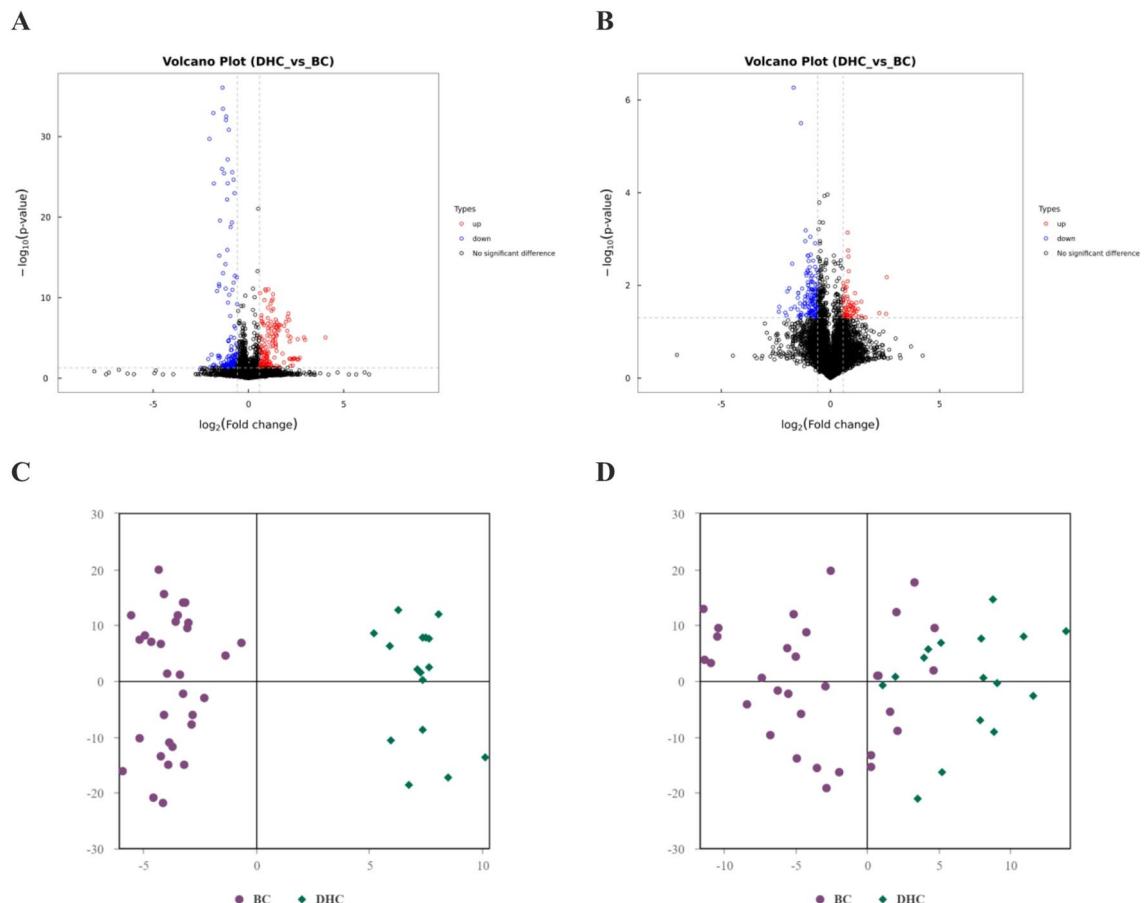


Fig. 4. DHC children changed the metabolome. **(A)** Volcano plots of positive ion mode. **(B)** Volcano plots of negative ion mode. Rose represented differential metabolites with Fold change > 1.5 , suggesting up-regulation of their expression in the DHC group. Blue represented differential metabolites with Fold change < 0.67 , suggesting down-regulation of their expression in the BC group. Black represented metabolites with no statistically significant difference. **(C)** OPLS-DA score graph for positive ion mode. **(D)** OPLS-DA score graph for negative ion mode. DHC, damp-heat constitution with functional constipation; BC, balanced constitution without functional constipation.

A survey investigating the TCMC distribution in FC-affected children revealed that damp-heat constitution constituted 5.26% of all constipated children⁴⁹. Dysbiosis of the intestinal flora is increasingly recognized as a critical etiological factor in pediatric FC. Emerging evidence from metagenomic studies highlights the significance of bacterial composition and metabolic capacity in the pathogenesis and progression of FC^{50,51}. Based on these findings, we conducted the investigation into the intestinal flora and urinary metabolomics of children aged 0–2 years with damp-heat constitution who suffer from FC. This study aimed to elucidate the microbial community characteristics of this specific population in a targeted manner.

The study highlights significant differences in the gut microbiota composition between the DHC (damp-heat constitution with functional constipation) group and the BC (balanced constitution without functional constipation) group. Specifically, two phyla and six genera exhibited significantly higher relative abundances in the DHC group compared to the BC group. Notably, the phylum Actinobacteria and its associated genus *Bifidobacterium* showed increased relative abundance in the DHC group, while the phylum Firmicutes demonstrated a decrease. This finding aligns with previous studies on constipation, such as the work by Wang et al.⁴⁷ which emphasized the role of gut microbiota in the pathogenesis of functional constipation.

Bifidobacterium, a well-known beneficial bacterium, contributes to alleviating constipation through mechanisms such as modulating gastrointestinal regulatory peptides and stimulating the production of short-chain fatty acids in the intestinal tract. Additionally, *Bifidobacterium* promotes lactic acid production, leading to a lower intestinal pH, which enhances intestinal peristalsis^{52,53}. However, in this study, the level of *Bifidobacterium* was significantly higher in the constipation group. It is important to note that the therapeutic effects of *Bifidobacterium* on constipation are strain-dependent. For instance, the presence of the *abfA* cluster in *Bifidobacterium longum* has been shown to influence its efficacy in treating constipation⁵⁴.

In contrast, the phylum Synergistetes, often considered an opportunistic pathogen, exhibited low relative abundance in individuals with damp-heat constitution in prior studies¹¹, which was elevated in the DHC group in this investigation. This suggests a strong association between Synergistetes and constipation, corroborated

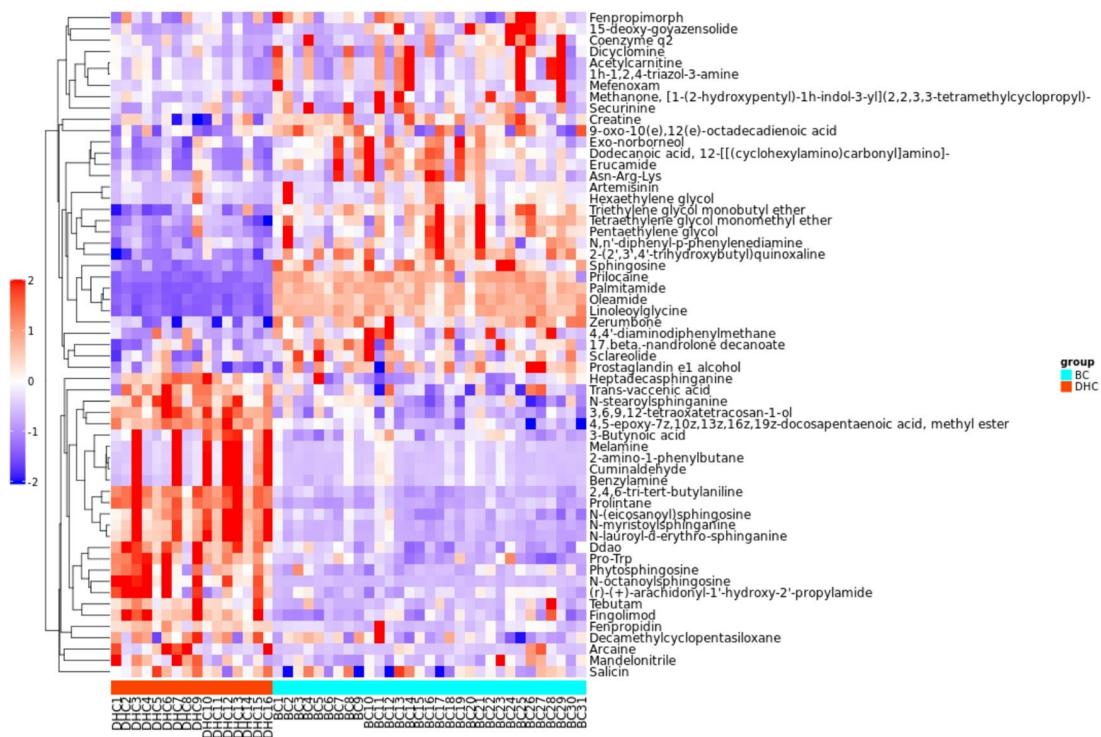
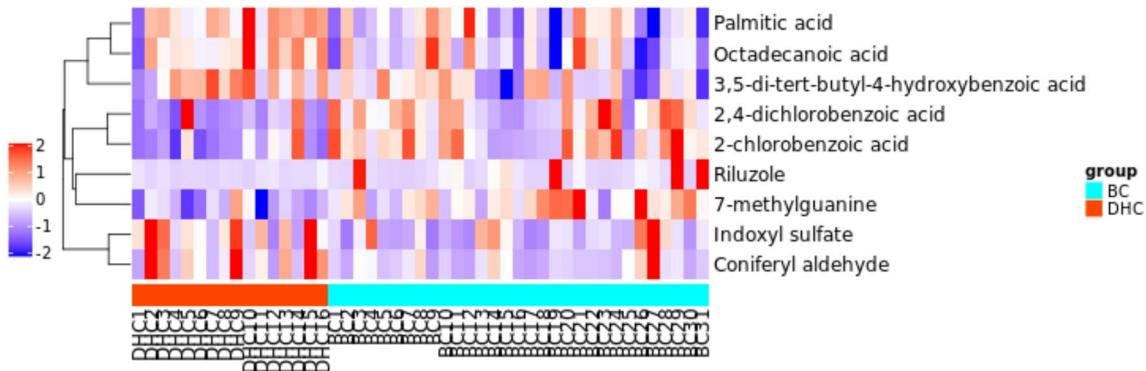
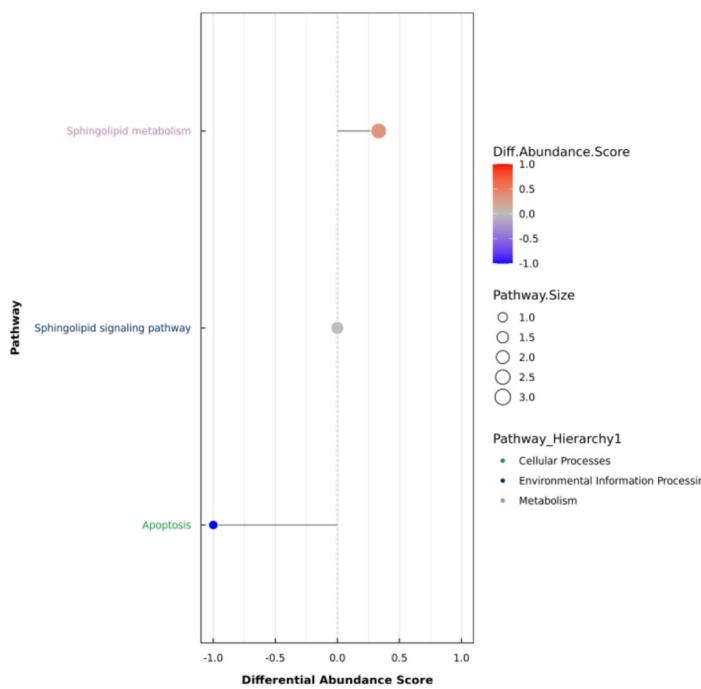
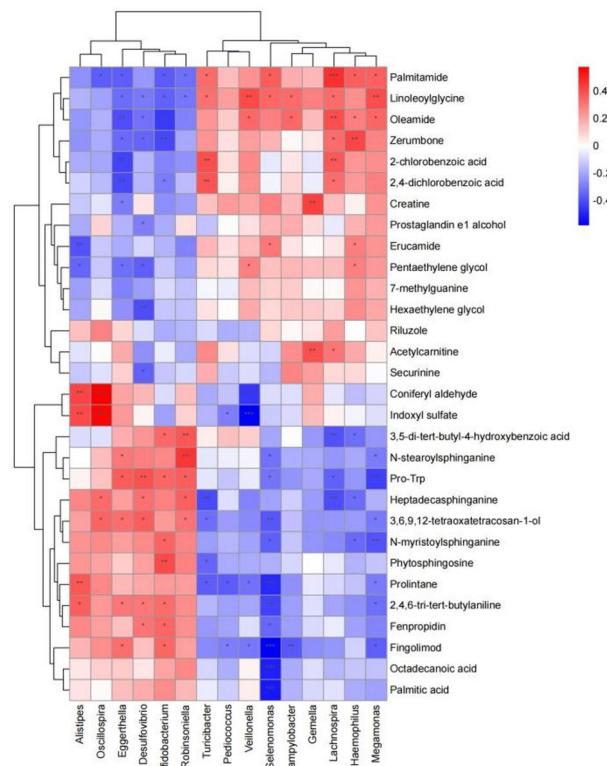
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Fig. 5. Differential metabolite analysis. **(A)** Hierarchical clustering heatmap of differential metabolites in positive ion mode. **(B)** Hierarchical clustering heatmap of differential metabolites in negative ion mode. The horizontal axis represents groups, the vertical axis represents metabolites. **(C)** Differential abundance score plot of differential metabolites. DHC, damp-heat constitution with functional constipation; BC, balanced constitution without functional constipation.

by findings in studies examining antipsychotic drug-induced constipation, where an increase in *Synergistetes* abundance was observed⁵⁵.

Another potentially pathogenic bacterium, *Eggerthella*, was found to be significantly more abundant in patients with Rett syndrome who experienced constipation. Although the altered intestinal flora in these patients was not entirely dependent on their constipation status, the dysbiotic flora produced a distinct SCFA profile, possibly linked to the abundance of *Eggerthella*⁵⁶. Furthermore, *Eggerthella* has been implicated in inducing intestinal inflammation and leakage, which are closely associated with gastrointestinal symptoms such as bleeding and bloating⁵⁷.

The relationship between fecal concentration and microbial composition is also noteworthy. For example, rapid colonic transit time selects for fast-growing microorganisms, whereas slow transit times associated

**Fig. 5.** (continued)**Fig. 6.** Correlation analysis between differential flora and metabolites. The horizontal axis represents microorganisms, and the vertical axis represents metabolites. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

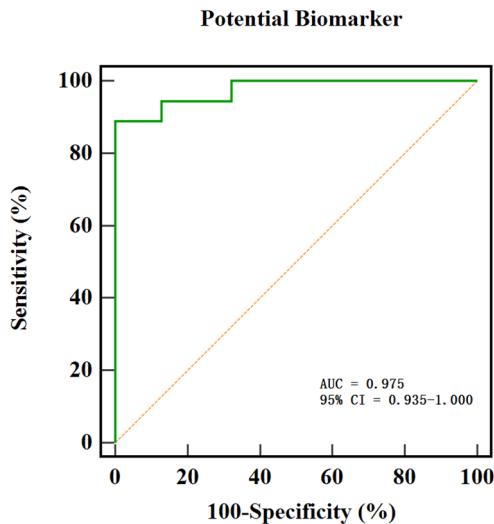


Fig. 7. ROC curve for potential biomarker for combined microbiota and metabolites of DHC children.

with constipation allow slow-replicating microorganisms to persist in the intestinal lumen. In the context of constipation, the genus *Oscillospira* tends to exhibit high abundance^{58,59}, a finding consistent with the results of this study. This observation underscores the importance of transit time in shaping the gut microbiota profile and its potential implications for constipation management.

Alistipes is one of the abundant members of the gut microbiome, mainly indole-positive, and is able to metabolize tryptophan. Tryptophan has two metabolic pathways: One is converted into 5-hydroxytryptophan, which is then converted into serotonin, stimulating intestinal motility. The other is the kynurenine pathway, a major pathway in which the synthesis of kynurenine reduces the amount of tryptophan available for the synthesis of 5-hydroxytryptophan. Reduced levels of 5-hydroxytryptophan have been demonstrated in a number of patients with constipation who are slow transporters. Sugitani et al. indicated that the mucosa-associated microbiome of FC was characterized by elevated levels of *Alistipes*. In addition, the consumption of *Bifidobacteria* showed inter-species differences in relieving constipation, and that several species of *Bifidobacteria* could improve constipation by increasing acetic acid concentration and the relative abundance of *Lactobacillus* and by decreasing the levels of *Alistipes* and others. These findings are consistent with the elevated abundance of *Alistipes* in the DHC group.

The gut microbiome plays a critical role in the pathophysiology of FC, and specific bacterial genera have been implicated in its development and alleviation. Among these, *Alistipes* is an abundant member of the gut microbiota, characterized by its ability to metabolize tryptophan through two primary pathways: the serotonin pathway and the kynurenine pathway. In the serotonin pathway, tryptophan is converted into 5-HTP, which is subsequently transformed into serotonin, a neurotransmitter that stimulates intestinal motility. Conversely, the kynurenine pathway reduces the availability of tryptophan for serotonin synthesis, potentially contributing to reduced intestinal motility⁶⁰. Studies have demonstrated that patients with constipation who exhibit slow colonic transit times often have lower levels of 5-HTP⁶¹. Elevated levels of *Alistipes* have been observed in the mucosa-associated microbiome of individuals with FC⁶², suggesting a potential link between this genus and impaired intestinal motility. Furthermore, certain species of *Bifidobacteria* have been shown to alleviate constipation by increasing acetic acid concentrations and the relative abundance of *Lactobacillus*, while simultaneously reducing levels of *Alistipes*⁶³. These findings align with the elevated abundance of *Alistipes* observed in the DHC group.

Another bacterial genus associated with gastrointestinal health is *Desulfovibrio*, a dominant sulfate-reducing bacterium in the human colonic flora. This genus has the ability to colonize the intestine via the mucus layer, and its metabolites may contribute to chronic gastrointestinal diseases. In studies involving constipated mice treated with varying concentrations of cellulose, *Desulfovibrio* levels were reduced in the intestinal tract, exhibiting a significant negative correlation with the total content of SCFAs⁶⁴. SCFAs, particularly acetate, propionate, and butyrate, play crucial roles in maintaining colonic health and promoting regular bowel movements.

Megamonas, belonging to the phylum Mycobacteriaceae and family Weilloniococcaceae, exhibits a strong degrading effect on cellulose. Its impact on the intestinal flora is marked by an increase in butyric acid levels, accompanied by a shift in the balance between acetic and lactic acids. Specifically, *Megamonas* leads to a decrease in acetic acid and an increase in lactic acid⁶⁵. Elevated lactic acid levels can increase intestinal osmotic pressure, resulting in higher water content in feces, which may not be conducive to alleviating constipation⁶⁶.

Lachnospira is another genus that differs significantly between constipated and non-constipated groups. Higher abundances of *Lachnospira* have been observed in non-constipated individuals, with levels being more than four times higher in healthy controls compared to constipated patients⁶⁷. Supplementation with plantain has been shown to significantly increase *Lachnospira* levels, with fecal water content positively correlated with acetic acid levels. Members of *Lachnospira* are capable of producing lactic and acetic acids, with lactic acid further metabolized into butyric or propionic acid. While low concentrations of butyric acid inhibit mucin secretion, leading to constipation, high concentrations of butyrate and acetic acid may exacerbate constipation

symptoms. These findings underscore the complex interplay between gut microbiota composition and functional constipation, highlighting the potential of targeted microbial interventions for managing this condition⁶⁸.

The liquid-quantity coupledomics technology employed in this study identified a distinct metabolite profile in children with DHC. A total of 68 differential metabolites were screened, along with three associated metabolic pathways. Notably, oleamide, an amide derivative of oleic acid and a member of the fatty acid amide family, emerged as a key modulator of enterodynamics. Oleamide has been shown to slow intestinal motility in mice by activating the cannabinoid receptor CB1⁶⁹. The traditional Chinese herbal formula MaZiRenWan was found to attenuate oleamide-induced intestinal motility slowness in mice, likely by enhancing the degradation of oleamide mediated by colonic fatty acid amide hydrolase, thereby improving intestinal motility in functional constipation⁷⁰.

Another significant finding pertains to the involvement of sphingolipid metabolism in DHC children. Sphingolipid metabolism generates several biologically active metabolites, including sphingosine-1-phosphate (S1P), phytosphingosine, and dihydrosphingosine. These metabolites play critical roles in regulating cardiovascular function, smooth muscle contraction, and acting as signaling molecules involved in cell survival, proliferation, migration, differentiation, and apoptosis^{71,72}. Specifically, S1P induces intestinal smooth muscle contraction via interstitial cells of Cajal (ICC), which are essential for gastrointestinal motility regulation^{73,74}. Low levels of S1P may disrupt normal ICC function, leading to decreased or inhibited colonic motility and contributing to constipation⁷⁵. Altered levels of dihydrosphingosine in aged mice have also been associated with gastric contractile dysfunction⁷⁶, while dietary supplementation with phytosphingosine and dihydrosphingosine has been shown to promote gastrointestinal motility⁷⁷. Therefore, decreased levels of these metabolites may result in reduced gastric motility.

Furthermore, variations in the levels of S1P, dihydrosphingosine, and phytosphingosine are linked to disturbances in lipid and glucose metabolism⁷⁸, potentially increasing susceptibility to metabolic disorders in individuals with damp-heat constitution. Previous studies have demonstrated that changes in sphingolipid distribution are associated with neurological disorders, diabetes, and cardiovascular diseases⁷⁹⁻⁸². This study is the first to identify a potential role for sphingolipid metabolism in DHC children.

Additionally, the combination of characteristic metabolites and gut flora provided an excellent discriminatory marker for DHC children, with an AUC of 0.975. This highlights the potential utility of metabolomic and microbiomic profiles in diagnosing and understanding the underlying mechanisms of functional constipation in this population. These findings underscore the importance of integrating metabolomics and microbiomics in elucidating the pathophysiology of functional constipation and identifying potential therapeutic targets.

This study presents several limitations that warrant consideration for future research. First, the study population consisted of Chinese infants and toddlers, which introduces potential confounding factors although factors such as mode of birth, feeding style, and age showed no significant differences between groups. To enhance the precision of the study, future investigations could focus on a narrower age range. Second, the study was limited by the number of questionnaires distributed and the relatively small sample size of the cohort. Cross-sectional designs cannot establish causal relationships. These constraints could potentially lead to sampling errors and reduce the external validity of the study. A larger, globally representative study with a broader geographic scope would be beneficial in addressing these limitations and ensuring more robust conclusions. Third, the phylogenetic resolution of 16 S rRNA sequencing used to analyze the gut flora was insufficient for identifying microbial species accurately. While this method is effective for profiling bacterial communities at higher taxonomic levels, its ability to resolve differences at the species level is limited. This limitation affects the precision of microbial identification, particularly for certain genera where finer distinctions are critical. Future studies could employ alternative techniques, such as shotgun metagenomic sequencing, to achieve higher-resolution analysis of the gut microbiota. Additionally, mechanistic insights could be gained through experimental approaches such as fecal microbiota transplantation and targeted drug interventions. These methods would allow researchers to explore causal relationships and validate the observed associations between specific microbial profiles and functional constipation. By addressing these limitations, future research can provide more comprehensive and actionable insights into the role of gut microbiota in DHC children.

Conclusions

This study represents a pioneering effort in elucidating the gut flora and metabolomic profiles of children with DHC. The findings offer critical insights into the pathogenesis of this condition by identifying specific differential flora and metabolites that influence key biological pathways, including sphingolipid signaling, sphingolipid metabolism, and apoptosis. It is noteworthy that specific combinations of microbiota and metabolites may serve as potential biomarkers, offering prospects for advancing precision interventions tailored to individual constitutions in managing constipation. Subsequent validation of identified microbial and metabolite biomarkers through larger-scale or longitudinal studies will be undertaken to deepen existing findings. This approach not only enhances our understanding of the underlying mechanisms but also opens new avenues for targeted interventions and personalized treatments in pediatric populations. Overall, the study highlights the potential of microbiome-based diagnostics and therapeutics in managing DHC and related conditions.

Data availability

The datasets generated and/or analysed during the current study are available in the National Center for Biotechnology Information repository. The Sequence Read Archive (SRA) ID is PRJNA1262707.

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Author contributions

H.Z.: designed the study and performed the microbiological analyses; X.L. and Y.Y.: some experiments, bioinformatic analysis; W.L. and H.Y.: analysis and interpretation of data; Z.L.: patients data collection; X.Z., J.W. and J.W. conceived and designed the study. all authors contributed to writing and revising the manuscript, have seen and approved the submission of the final version of the manuscript, and take full responsibility for the manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Ethics approval

This study protocol was reviewed and approved by the Ethics Committee of Beijing University of Chinese Medicine (No. 2020BZYL122) and was conducted in accordance with the 1964 Helsinki Declaration and its later amendments. Informed consent was obtained from children's parents to participate in the study.

Additional information

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1038/s41598-025-26439-6>.

Correspondence and requests for materials should be addressed to X.Z., J.W. or J.W.

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