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Research article

Damp-heat constitution influences gut microbiota and urine metabolism of Chinese infants

Haihong Zhao [a], Yuhan Zong [a], Wenle Li [a], Yaqi Wang [b], Weibo Zhao [a], Xianghe Meng [c], Fan Yang [a], Jingwei Kong [d], Xiaoshan Zhao [a,e], Ji Wang [a,*]

[a] National Institute of Traditional Chinese Medicine Constitution and Preventive Treatment of Disease, Beijing University of Chinese Medicine, Beijing, 100029, China
[b] College of Basic Medical Sciences, Zhejiang Chinese Medical University, Hangzhou, 310053, Zhejiang, China
[c] Neurology Department, Xuanwu Hospital of Capital Medical University, Beijing, 100053, China
[d] Nutrition and Metabolism Research Division, Innovation Center, Heilongjiang Feihe Dairy Co., Ltd., Beijing, 100015, China
[e] School of Chinese Medicine, Southern Medical University, Guangzhou, 510515, Guangdong, China

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ABSTRACT

Background: As an increasingly popular complementary and alternative approach for early detection and treatment of disease, traditional Chinese medicine constitution (TCMC) divides human beings into those with balanced constitution (BC) and unbalanced constitution, where damp-heat constitution (DHC) is one of the most unbalanced constitutions. Many studies have been carried out on the microscopic mechanism of constitution classification; however, most of these studies were conducted in adults and rarely in infants. Many diseases are closely related to intestinal microbiota, and metabolites produced by the interaction between microbiota and the body may impact constitution classification. Herein, we investigated the overall constitution distribution in Chinese infants, and analyzed the profiles of gut microbiota and urine metabolites of DHC to further promote the understanding of infants constitution classification.

Methods: General information was collected and TCMC was evaluated by Constitutional Medicine Questionnaires. 1315 questionnaires were received in a cross-sectional study to investigate the constitution composition in Chinese infants. A total of 56 infants, including 30 DHC and 26 BC, were randomly selected to analyze gut microbiota by 16S rRNA sequencing and urine metabolites by UPLC-Q-TOF/MS method.

Results: BC was the most common constitution in Chinese infants, DHC was the second common constitution. The gut microbiota and urine metabolites in the DHC group showed different composition compared to the BC group. Four differential genera and twenty differential metabolites were identified. In addition, the combined marker composed of four metabolites may have the high potential to discriminate DHC from BC with an AUC of 0.765.

Conclusions: The study revealed the systematic differences in the gut microbiota and urine metabolites between infants with DHC and BC. Moreover, the differential microbiota and metabolites may offer objective evidences for constitution classification.

* Corresponding author.
E-mail address: doctorwang2009@126.com (J. Wang).

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1. Introduction

In China, traditional Chinese medicine (TCM) has been used to treat diseases and manage health for over thousands of years. Guided by TCM, traditional Chinese medicine constitution (TCMC), which was developed in the 1970s, divides healthy individuals into nine constitutions, each with its own biological features and disease risks [1, 2, 3]. Balanced constitution (BC) represents an overall healthy state and is not susceptible to illness; damp-heat constitution (DHC) indicates excessive dampness and heat in the body, which besides heredity factors, implies having too much sweet and greasy food and living in a humid environment for a long time. Some studies have found that those with DHC are prone to obesity, diabetes, hyperlipoidemia, inflammatory bowel disease and metabolic syndrome [4, 5]. Therefore, identifying DHC and intervening in advance is of critical importance for preventing these diseases.

Gut microbiota could modulate the host immune response and interfere with host state through metabolic regulation [6]. Since the use of blood samples is inconvenient, as they are susceptible to infection and unable to reflect the overall state of the body [3], we chose urine samples from infants for metabolomics. Many studies have shown that different adults constitution has its own characteristics reflected in intestinal microbiota and metabolites [7, 8, 9, 10]; however, less is known about this relationship in infants. Herein, for the first time, we investigated the constitution distribution in Chinese infants and analyzed the profiles of gut microbiota and urine metabolites of DHC, in the hope of understanding constitution classification, identifying the biomarkers of DHC and preventing DHC-mediated diseases.

2. Materials and methods

2.1. Study design and ethical approval

Infants aged 0–2 years from Beijing and Guangdong, China, were recruited through poster posting and social media distribution of Constitutional Medicine Questionnaires from November 2020 to October 2021 (Supplemental file 1). With the guidance of TCMC, Constitutional Medicine Questionnaire was established as the measurement tool for constitution [11]. The questionnaire was developed in China by academician Wang and colleagues, and has been translated and used in Canada, Japan, Korea, and the USA, among other countries [12]. As shown in supplemental file 1, the questionnaire consisted of 43 items with 9 subscales, each measuring one constitution. Specifically, BC corresponded to 1–3 items; qi-deficiency constitution (QDC) corresponded to 4–9 items; yang-deficiency constitution (YADC) corresponded to 10–13 items; yin-deficiency constitution (YIDC) corresponded to 14–20 items; phlegm-dampness constitution (PDC) corresponded to 21–24 items; DHC corresponded to 25–29 items; blood stasis constitution (BSC) corresponded to 30–33 items; qi stagnation constitution (QSC) corresponded to 34–37 items; inherited special constitution (ISC) corresponded to 38–42 items; BC also corresponded to the last item. These items were scored on a 5-point Likert scale, with higher scores indicating a higher likelihood of having the given constitution. The scores for each constitution were calculated as follows: raw score = the sum of the scores of each item, conversion score = [(raw score - number of items)/(number of items $\times$ 4)] $\times$ 100. To ensure the reliability of the results, BC individuals were considered as those with $\geq$60 points on the BC conversion score, while unbalanced constitution conversion scores were defined as $<$30 points; DHC individuals were considered as those with $\geq$40 on the DHC conversion score. A total of 1315 questionnaires were received, and 1069 valid questionnaires were finally screened after excluding invalid and repeated data.

Participants with DHC and BC were included in the study of fecal microbiota and urine metabolism. Participants with serious illness, aged $>$2 years old, ethnic minorities, wrong phone numbers left by their parents, and residents far from the hospital were excluded. We also made sure that included participants did not use antibiotics or probiotics during the previous 3 months. Some factors affecting intestinal microbiota, such as age, sex, delivery mode and feeding pattern, were also considered.

All the guardians of infants, usually their parents, understood the trial process and signed the written informed consent. This research was approved by the Ethics Committee of Beijing University of Chinese Medicine (No. 2020BZYLL122). All information, including basic personal information and the content of the questionnaire, were kept strictly confidential to the extent permitted by law.

2.2. Fecal sample collection and sequencing

The guardians collected their children's fecal samples ($>$3 g) that were not contaminated with urine through sterile plastic cups at home. The stools were stored at 4 °C and delivered to the hospital within 1 h, after which they were transferred into liquid nitrogen and frozen at -80 °C until DNA extraction. Bacterial DNA was extracted using the cetyltrimethylammonium bromide/sodium dodecyl sulfate method. 16S rRNA (V3–V4) genes were amplified with barcode-indexed primers (341 F-806 R), and amplicons were mixed and purified with the AxyPrepDNA Gel Extraction Kit. Then, amplicon libraries were generated using the NEB Next®Ultra™ DNA Library Prep Kit for Illumina, and their quality was assessed. Finally, the library was sequenced on an Illumina Miseq platform, and 250/300 bp paired-end reads were generated.

2.3. Gut microbiota analysis

The paired-end reads were merged using FLASH and assigned to each sample according to unique barcodes. The UPARSE-OTU and UPARSE-OTUref algorithms were used to perform sequences analysis with UPARSE software package. Sequences with $\geq$97% similarity were classified as the same OTUs. Alpha diversity was computed using Chao 1, ACE, Shannon and Simpson. Comparisons of these indices were analyzed by student's t-test for normally distributed variables and Wilcoxon test for non-normally distributed variables.

Unweighted unifrac distance was used for principal coordinate analysis (PCoA) to compare the microbial community structure by QIIME software package as beta diversity. The linear discriminant analysis (LDA) of effect size (LEfSe) was conducted to detect taxa with significant differences in the sample division. Only taxa with LDA>2.0 and $P < 0.05$ were selected.

2.4. Urine sample collection and profiling

Midstream urine samples were collected with a sterile urine cup, stored at 4 °C, and transported to the hospital within 1 h. The urine was centrifuged at 2000×*g* for 30 min under 4 °C and urine supernatant was obtained and shifted to a frozen pipe. Then, the pipe was transferred into liquid nitrogen to be frozen and then placed at -80 °C until used in ultrahigh-performance liquid chromatographt-quadrupole time of flight/mass spectrometry (UPLC-Q-TOF/MS) analysis. The supernatant was thawed at 4 °C, and each sample (100 μL aliquots) was mixed with 400 μL of pre-cooled methanol/acetonitrile solvent (1:1, vol/vol). The mixture was vortex mixed, detected by low-temperature ultrasound for 30 min, maintained at -20 °C for 60 min, and centrifuged for 20 min (14000×*g*, 4 °C). The supernatant was collected and dried, after which the supernate was re-dissolved in 100 μL acetonitrile/water solvent (1:1, vol/vol), vortex mixed for 60 s, and centrifuged again for 15 min (14000×*g*, 4 °C). The resulting supernatant was collected and analyzed. Quality control samples were equally mixed from all samples and analyzed with other samples to monitor the instrument's stability. After preparation, urine samples were separated using an UHPLC system for chromatographic analysis. For subsequent Q-TOF mass analysis, the samples were operated using a triple time-of-flight (TOF) 5600 + system. Particular detection was performed by Shanghai Applied Protein Technology Co. Ltd [13].

2.5. Metabonomics data analysis

The raw data were converted into the mzXML format using ProteoWizard [14], and then peak alignment, retention time correction, and peak area extraction were performed by XCMS software [15]. Metabolite structure identification was performed by searching the self-built database of the laboratory through accurate mass matching (<5 ppm) and secondary spectrum matching. One set of ion peak data missing more than 50% of data was deleted. Multidimensional statistical analysis was performed after the data was preprocessed by Pareto scaling. The variable importance in the projection (VIP) value of each variable in the OPLS-DA model was used as the criterion for selecting potential biomarkers. Univariate analysis included Student's t-test and fold change analysis. Metabolic pathway analysis was performed with the MetaboAnalyst web software. Fisher's Exact Test was used to analyze and calculate the importance level of the enrichment pathway.

2.6. Correlation analysis, biomarkers identification and statistical analysis

Spearman correlation analysis was performed to calculate the correlation coefficient between identified microbiota and metabolites, and matrix heat map was carried out with R version 3.4.2 and Cytoscape version 3.5.1. The receiver operating characteristic (ROC) curve analyses were performed to identify markers with a high area under the curve (AUC) using MedClac version 20.1.4. Statistical analysis was performed with SPSS version 26.0 and GraphPad Prism version 7. Results were expressed by mean ± standard deviation ($\bar{x} \pm s$). *P* value < 0.05 indicated a statistically significant difference.

3. Results

3.1. General information

The infants constitution types were assessed, revealing that BC was the predominant type and DHC was the second type (Table 1). As it is well-known that the gut bacteria are affected by age [16, 17], gender [18, 19], delivery mode [20, 21, 22], and feeding pattern [4, 5, 23], statistical analysis was performed to exclude the influence of the above factors between the DHC group and the BC group. Finally, 30 DHC and 26 BC subjects were included in the study (Table 2).

Table 1
Distribution of Chinese infants constitutions.

Constitution	Case	Percentage (%)
Balanced constitution	601	56.22
Damp-heat constitution	171	16.00
Qi stagnation constitution	103	9.64
Yin-deficiency constitution	93	8.70
Inherited special constitution	51	4.77
Qi-deficiency constitution	17	1.59
Yang-deficiency constitution	24	2.25
Phlegm-dampness constitution	6	0.56
Blood stasis constitution	3	0.28

Table 2
Influencing factors of the DHC and BC groups.

Influencing factors	DHC group	BC group	*P* value
Age (months)	7.67 ± 4.69	9.15 ± 6.11	0.308
Gender			0.129
Boys	22	14	
Girls	8	12	
Delivery Mode			0.643
Vaginal Delivery	18	14	
Caesarean Delivery	12	12	
Feeding Pattern			0.145
Breast Feeding	15	18	
Non-Breast Feeding	15	8	

3.2. Microbial community diversity analysis

Fecal samples were collected and 16S rRNA sequencing was performed to analyze the bacterial composition and test whether there was a connection between intestinal microbiota and DHC in infants. We totally obtained 5,955,937 reads, including 3,056,643 (51.32%) reads in the DHC group, 2,899,294 (48.68%) reads in the BC group. These reads could be classified into 5,357 OTUs, with 1,076 and 1,119 unique to DHC and BC, respectively.

The microbiota showed no differences in ACE, Chao 1, Shannon, and Simpson indexes between the DHC and BC groups (all $P > 0.05$), which suggested that the richness and evenness of intestinal microbiota were similar between the two groups (Figure 1A). Principal coordinate analysis (PCoA) based on unweighted unifrac distance matrix showed inconsistent clusters in the overall structure of the two groups where BC samples were more concentrated whereas DHC samples were more dispersed ($P < 0.01$), indicating that the composition of DHC-related microbiota considerably differed from that of the BC-related microbiota (Figure 1B).

3.3. Composition of gut microbiota

Linear discriminant analysis (LDA) of effect size (LEfSe) was performed to identify the taxonomic features with the greatest differences between DHC and BC cohorts (LDA>2.0, $P < 0.05$). As shown in Figure 2A, at the phylum level, the DHC and BC groups were composed of Actinobacteria, Firmicutes, Proteobacteria, and Bacteroidetes, which accounted for the overwhelming majority of the microbiota. Based on LEfSe analysis, at the family level, the relative abundance of Enterococcaceae and Pasteurellaceae largely differed between the two groups (all $P < 0.05$); at the genus level, the DHC group had distinctly depleted in *Subdoligranulum* ($P = 0.0066$), *Eubacterium_hallii_group* ($P = 0.0057$), *Haemophilus* ($P = 0.0498$) but was significantly enriched in *Enterococcus* ($P = 0.0468$) (Figure 2B).

3.4. Comparison of urine metabolites profiling

The metabolites produced by gut microbiota have a remarkable impact on host physiology. Urine samples were assessed by untargeted metabonomics to describe the effect of DHC on metabolism. The orthogonal to partial least squares-discriminate analysis (OPLS-DA) showed that samples from the DHC group were distinctly separated from the samples of the BC group in positive and negative ion modes, indicating that the urine metabolic profile was obviously different in infants from the two groups (Figure 3A).

3.5. Composition of differential metabolites

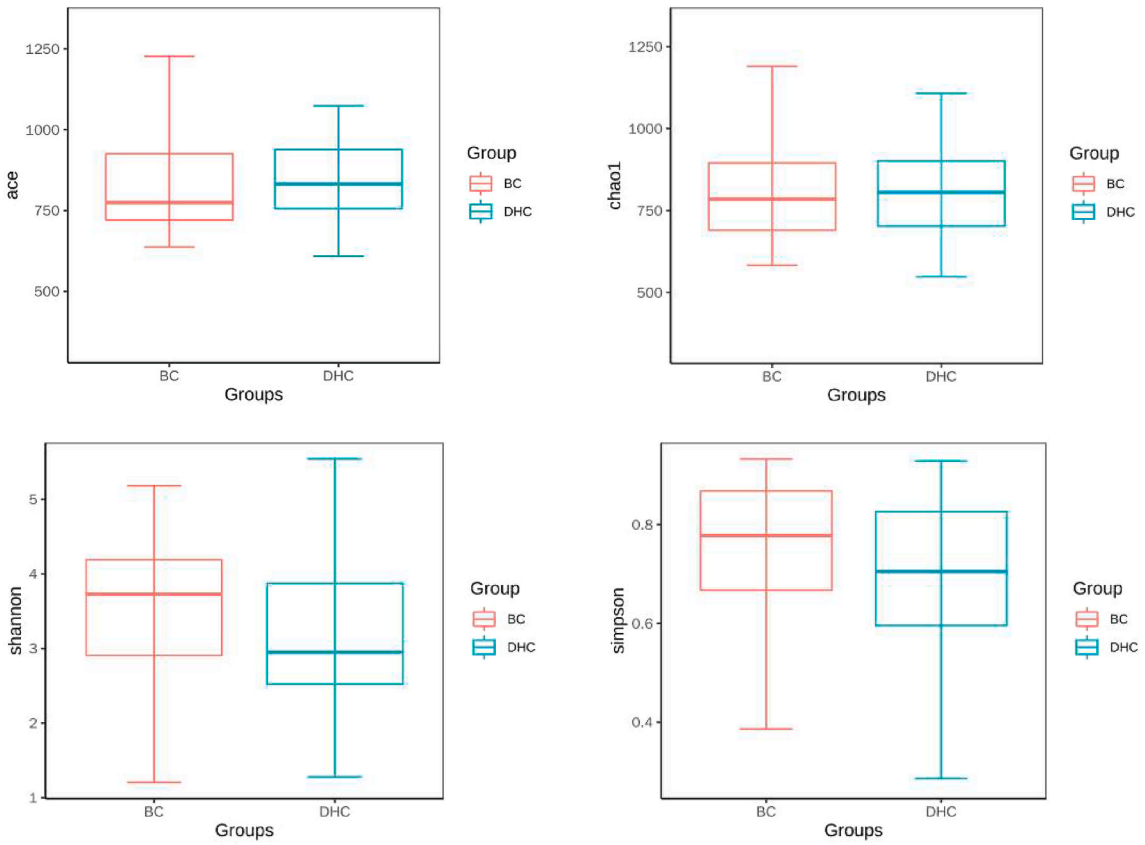
The up-regulated and down-regulated metabolites in two ion modes were detected in both groups with fold change standards (FC > 1.5 or FC < 0.67, $P < 0.05$) (Figure 3B). Differential metabolites were screened according to the thresholds of VIP>1.0 and $P < 0.05$. Finally, 20 metabolites were obtained, including 12 in positive ion mode and 8 in negative ion mode (Supplemental file 2).

3.6. Metabolic pathway analysis of altered profiles

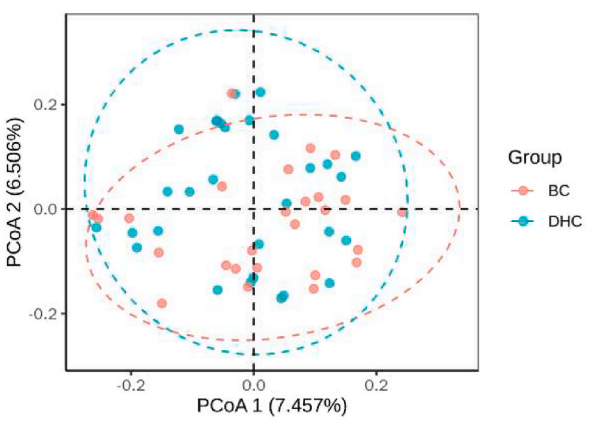
The metabolic pathways of infants with DHC included sphingolipid metabolism, prolactin signaling pathway, D-Glutamine and D-glutamate metabolism, and ubiquinone and other terpenoid-quinone biosynthesis (Figure 3C).

3.7. Correlation between gut microbiota and urine metabolites

Spearman correlation analysis was conducted to investigate whether metabolic changes were associated with gut microbiota community (Figure 4). *Eubacterium_hallii_group* had the strongest relationship with metabolites, and was specifically negatively correlated with 1-methylxanthine, octadecanoic acid and Pro-Trp. *Enterococcus* were negatively correlated with stachydrine and positively correlated with P-coumaric acid.

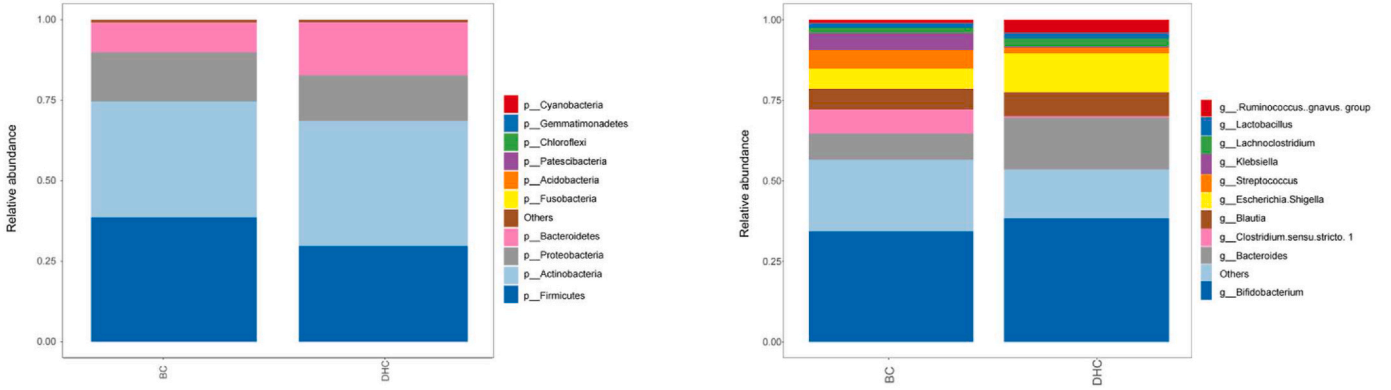


A



B

Figure 1. Biodiversity and phylogenetic analysis between the DHC group and the BC group. (A) Alpha diversity analysis for ACE, Chao 1, Shannon and Simpson metrics. (B) Beta diversity analysis represented by PCoA graphs of unweighted unifrac distance.



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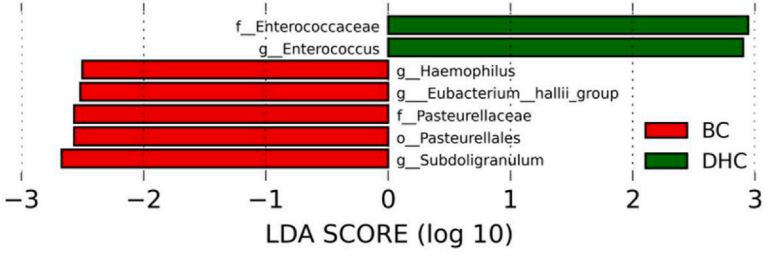
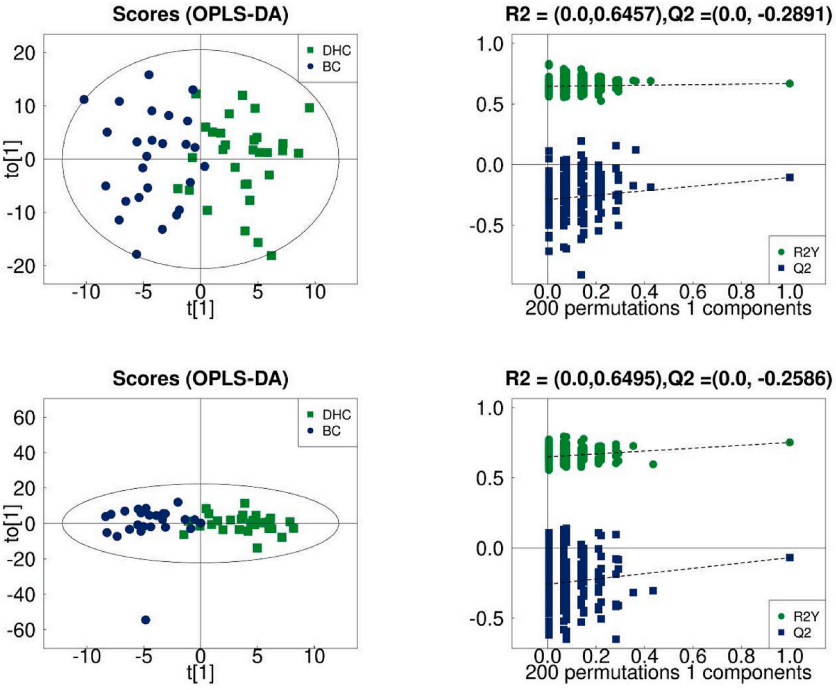


B

Figure 2. Comparisons of fecal microbiota. (A) Stacked bar charts of microbiota composition of the DHC and BC groups at the phylum (left) and genus (right) levels. The top ten bacteria were shown for each level. (B) LDA bar graph of differential microbiota between DHC and BC groups.



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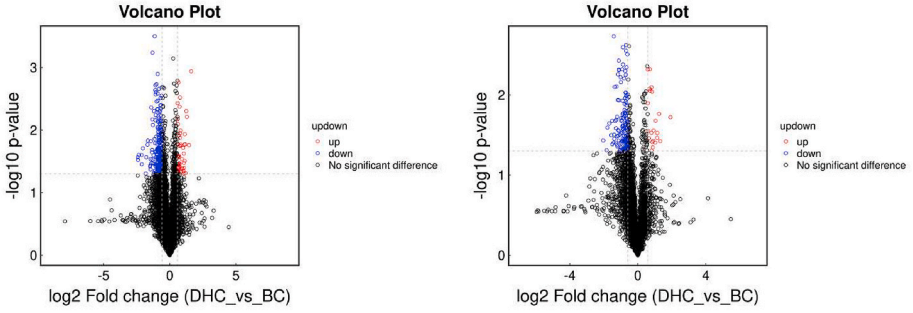

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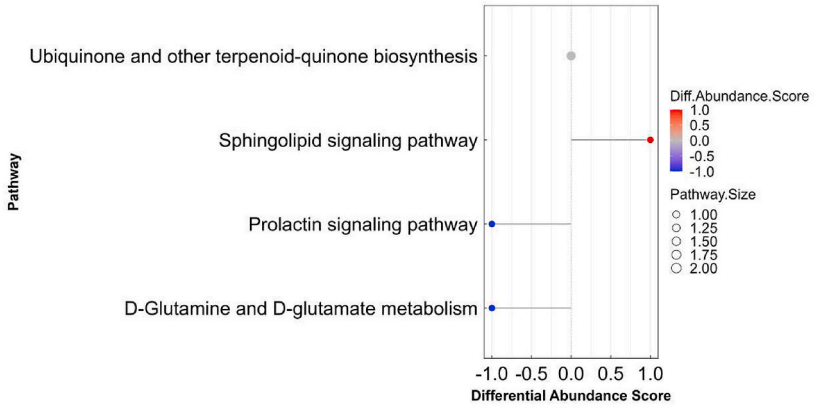

C

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Figure 3. Urine metabolism detected by untargeted metabolomics. (A) OPLS-DA score plot of the DHC and BC groups in positive (above) and negative (below) ion modes. (B) Volcano map of differential metabolites in positive (left) and negative (right) modes. (C) Metabolic pathways of infants with DHC.

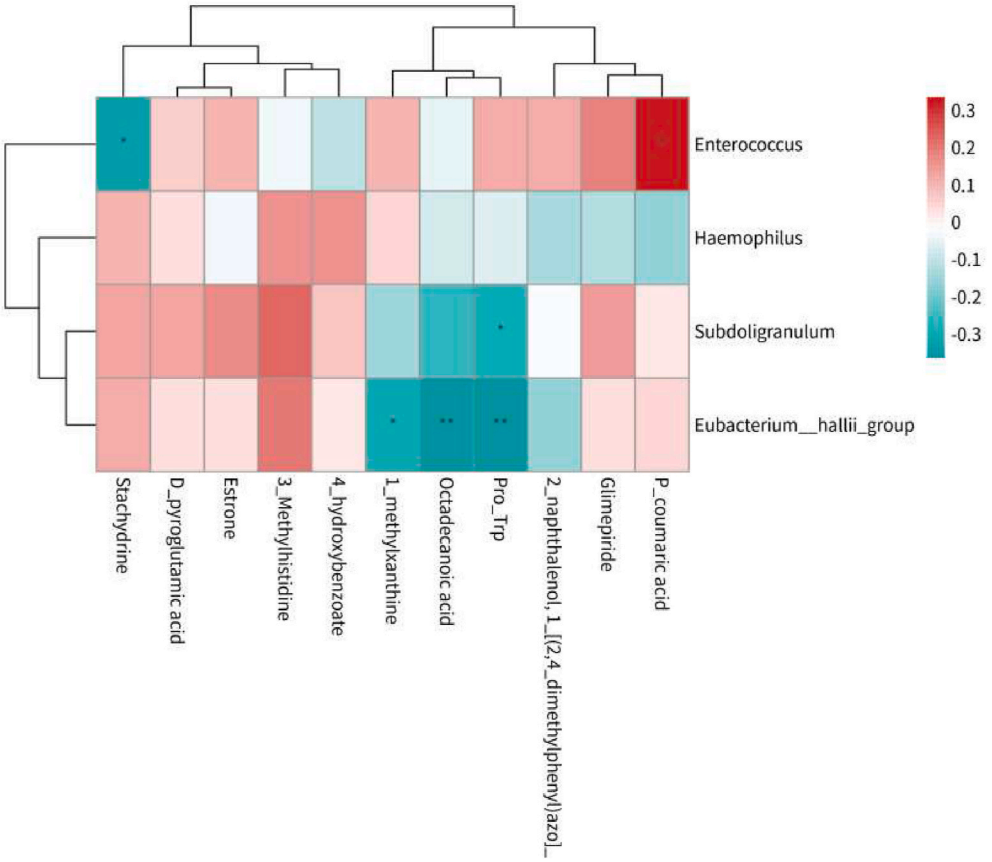


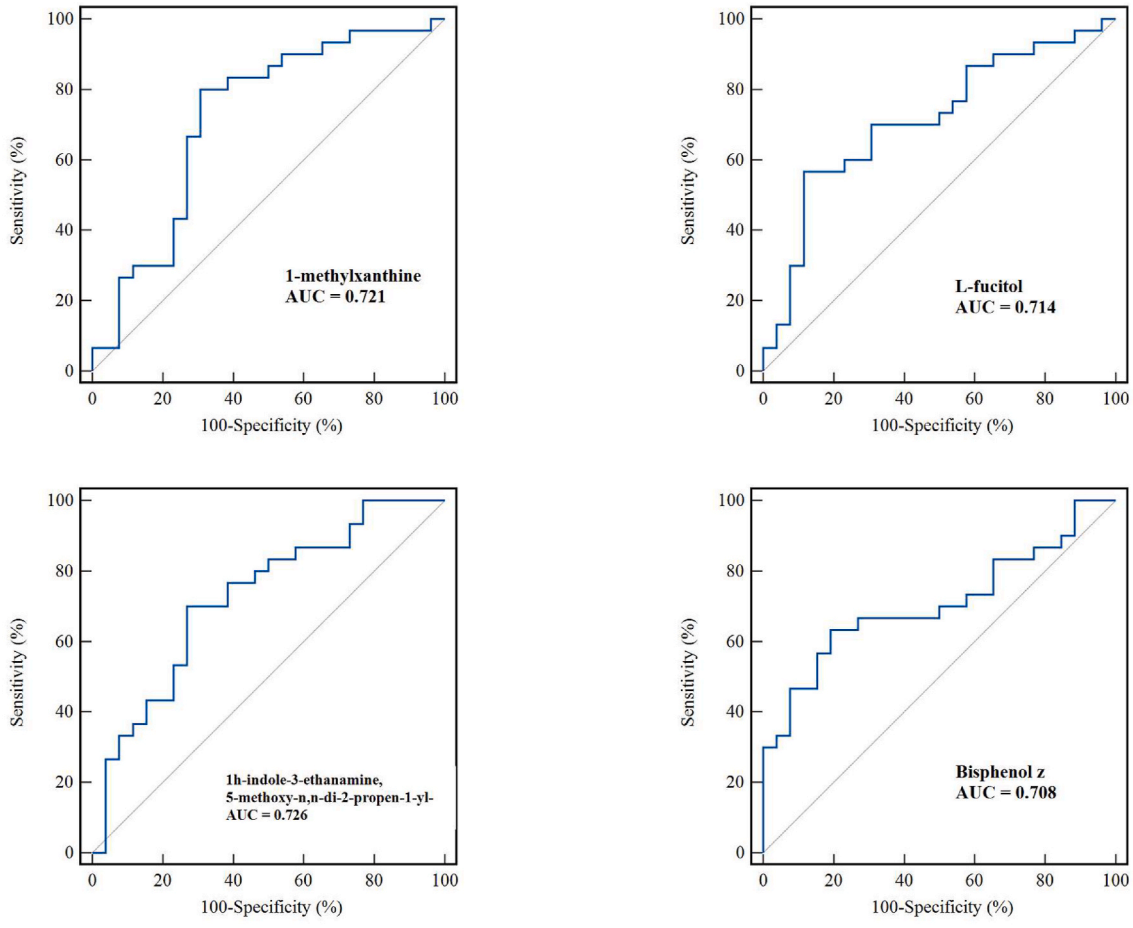
Figure 4. Correlation heatmap between identified metabolites and microbiota. Significant difference indicated by $^{*}P < 0.05$, $^{**}P < 0.01$.

3.8. Biomarkers of DHC with high accuracy

Receiver operating characteristic (ROC) analysis was performed to identify highly accurate markers among all differential genera and metabolites to discriminate DHC from BC. Of all results, four metabolites (1-methylxanthine, bisphenol_z, L-fucitol, and 1h-indole-3-ethanamine, 5-methoxy-n,n-di-2-propen-1-yl-) were screened with AUC>0.7 (all $P < 0.05$) (Figure 5A). Moreover, a combination AUC of the above four markers was 0.765, showing a higher potential than that of single metabolic marker (Figure 5B). The sensitivity and specificity of the combined marker were 70.0% and 80.7%, respectively.

4. Discussion

Constitution is a relatively stable inherent characteristic that is formed based on the innate endowment and acquired characteristics, integrated with the morphological structure, physiological function, and psychological state throughout life [24]. People with a good innate endowment that is well-regulated in life usually have a balanced constitution (BC). Individuals with BC are well-proportioned, energetic, cheerful, adaptable to the natural environment and social surroundings, and rarely get sick. Except for BC, the remaining eight constitutions are considered unbalanced constitutions, which are accompanied by various diseases. Individuals with qi-deficiency constitution (QDC) lack qi (vital energy), often speak in a low voice, tend to fatigue, sweat, and easily catch colds. In addition, because the spleen and stomach function are weakened, and the nutrition supply is insufficient, these individuals may eat less and have a yellowish complexion. Individuals with yang-deficiency constitution (YADC) lack yang qi, which is needed to warm the body. They tend to have cold hands and feet, easily get diarrhea after eating cold food and have a pale complexion. At night, the level of yang qi is lower than in the daytime, which prevents the water flow in the body and water is discharged directly, making these people urinate a lot. Individuals with yin-deficiency constitution (YIDC) lack yin fluid (such as water) and usually have dry skin, lips, and dry stools. As their fluid levels are too low to restrain the fire, these individuals also show some heat syndromes, such



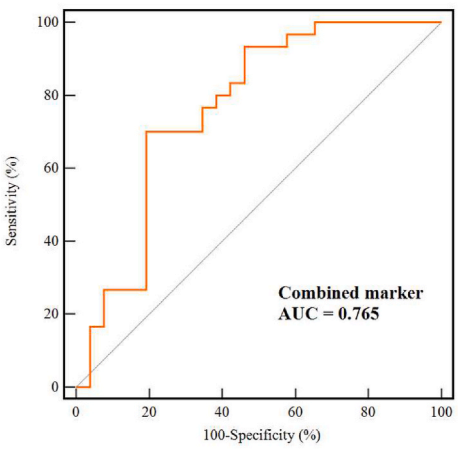


Figure 5. ROC curves for metabolites of DHC infants. (A) Separate metabolic panels. (B) Combined marker panel including four metabolic markers.

as hot palms or soles, night sweats, and uneven distribution of tongue coating. Individuals with phlegm-dampness constitution (PDC) have a large amount of pathogenic dampness, often leading to being overweight, inactivity, and thick tongue coating. Damp-heat constitution (DHC) implies superabundant dampness and heat. Excessive heat results in yellow and smelly urine, while excessive damp-heat causes people to have red eyes or eye excrement, sticky stools. Blood stasis constitution (BSC) has the tendency of poor blood circulation or the state of blood stagnation. BSC individuals usually have a dull complexion, darker skin and lips, and sometimes black or purple bruising appears on their skin for no apparent reason. Qi stagnation constitution (QSC) is greatly influenced by heredity and formed due to long-term emotional disorders and the stagnation of qi circulation. It is mainly characterized by shyness, introversion, excessive startle response, and irritability. Individuals with inherited special constitution (ISC) often manifest various allergic reactions, such as nasal congestion, runny nose, itchy skin, sneezing, coughing, and wheezing.

It is generally believed that the first 1000 days from conception to 2 years of age represents a critical window of early childhood growth and development during which the gastrointestinal microbial community plays a critical role in immune, endocrine, metabolic and other host developmental pathways. An emerging perspective of human developmental biology suggests the trillions of microbes (microbiota) and their genes (microbiome), which reside within the human body, assemble and stabilize during the first 2 years of life [25]. Herein, this study focused on the changes in the microbiota of infants with different constitutions aged 0–2 years. We first investigated the constitution types in Chinese infants from 0-2 years old, revealing the following constitution distribution: BC (56.22%) > DHC (16.00%) > QSC (9.64%) > YIDC (8.70%) > ISC (4.77%) > YADC (2.25%) > QDC (1.59%) > PDC (0.56%) > BSC (0.28%). These results showed that BC was the most common constitution of infants and DHC was the most common unbalanced constitution. In an epidemiological survey of Chinese adults [26], the proportion of BC was 28.80%, indicating that the general health state of infants is better than that of adults; the proportion of DHC was 10.23%, which is lower than what was observed in infants, reflecting the vigorous yang of infants and conforming to their physiological characteristics.

Different constitutions have specific manifestations and susceptibility to certain diseases. As DHC implies an excess of dampness and heat in the body, these infants are more likely to have sweaty hands and feet, yellow urine, sticky stool and may get eczema. Previous studies have shown that those with DHC are more prone to obesity, diabetes, hyperlipoidemia and metabolic syndrome [4, 5]. In the present study, our results demonstrated that the infants with DHC exhibited different characteristics of gut bacteria related to metabolic diseases. At the phylum level, the abundance of Bacteroidetes was largely different between the DHC group and the BC group and was greatly enriched in the DHC group. It is generally considered that Bacteroidetes is associated with human metabolic diseases [27, 28]. At the genus level, the abundance of *Enterococcus* in the DHC group was increased compared with the BC group. An extensive number of studies have reported that *Enterococcus* was observed in patients with diabetes mellitus, obesity and other metabolic syndrome [29, 30]. In addition, its species *Enterococcus faecalis* was found to cause hypertension and renal injury in rats by disturbing the lipid metabolism [31]. *Subdoligranulum* and *Eubacterium_hallii_group* were decreased in DHC infants, which are hardly conducive to the occurrence of DHC-mediated metabolic diseases. The abundance of *Subdoligranulum* was negatively correlated with fat mass, adipocyte diameter, levels of leptin, and other parameters related to metabolic risks such as C-reactive protein, insulin [32]. Prebiotics oligofructose increased the levels of *Subdoligranulum* in obese and diabetic mice [33], and the anti-diabetic drugs metformin and acarbose increased the levels of *Subdoligranulum* in diabetic patients [32]. *Eubacterium_hallii_group* serves as a potential probiotics because it helps improve insulin sensitivity and regulate metabolic pathways that increase energy expenditure and reduce energy intake [34, 35]. Another genus *Haemophilus* appears in large numbers in the first 6 months of life, but its role is controversial. One study reported it as a marker for adults patients with type 2 diabetes [36], but several other studies showed that it was associated to low metabolic syndrome risk score and significantly decreased after a year of intensive weight loss lifestyle intervention [37, 38]. In our study, *Haemophilus* was less abundant in DHC infants, showing that it may be negatively associated with metabolic diseases.

Expect for bacteria, several pivotal metabolites are associated with metabolic diseases. Compared with the BC group, estrone was significantly reduced in the DHC group. Previous studies have reported that estrone can prevent obesity by regulating adipocyte function through estrogen receptors in adipose tissue [39, 40]. Besides, estrone regulates insulin and glucose homeostasis through estrogen receptor α, preventing insulin resistance and type 2 diabetes [41]. Compared with the BC group, the levels of 1-methylxanthine and P-coumaric acid were considerably increased while the level of stachydrine was decreased in the DHC group. Consistent with the changing trend, 1-methylxanthine is negatively correlated with leanness [42]. Some researches indicated that P-coumaric acid possesses anti-adiposity, antisteatotic, and hypolipidemic effects, which may be due to the decreased lipogenesis and/or increased fatty acid oxidation in the epididymal white adipose tissue and liver along with increased fecal lipid excretion [43, 44]; however, Nguyen et al. recently demonstrated that P-coumaric acid did not reduce body weight but displayed a significant reduction in fasting blood glucose and lower plasma insulin in high-fat diet induced obese mice [45], this inconsistency in observed results may be because of the different timing of P-coumaric acid treatment. Stachydrine promotes lipolysis and inhibits lipid accumulation in 3T3-L1 adipocytes, thus reducing weight gain and improving glucose tolerance and insulin resistance in a mouse model [46].

In the present study, the occurrence of DHC was found to be associated with sphingolipid metabolism, prolactin signaling pathway, D-Glutamine and D-glutamate metabolism, and ubiquinone and other terpenoid-quinone biosynthesis. Many studies have revealed altered levels of sphingolipid species, including glucosylceramides, sphingomyelins, and ganglioside GM3, in type 2 diabetes mellitus, obesity, and other metabolic diseases, suggesting that sphingolipid metabolites have emerged as important molecular players in metabolic diseases. The mechanisms by which sphingolipid metabolism is perturbed go beyond fatty acid oversupply, and may also involve oxidative stress, inflammatory signaling, endocrine signaling, dysbiosis, and other routes [47]. Prolactin is a major stimulus for the β-cell adaptation during gestation and guards postpartum women against gestational diabetes. Some studies indicated that prolactin had a role in whole-body insulin sensitivity by stimulating insulin release and regulating adipokine release. Moreover, the release of prolactin from subcutaneous adipose tissue was lower in obese compared to lean individuals, suggesting that adipose prolactin may be involved in obesity-related complications [48]. Glutamine was negatively correlated with the onset of type 2 diabetes

mellitus. The reduction of glutamate content reduces glutamine production by glutamine synthetase, leading to insufficient insulin secretion and the development of type 2 diabetes mellitus. The increase of glutamine/glutamate ratio can predict the risk of diabetes reduction, and these potential biomarkers are important for the early diagnosis and therapeutic evaluation of type 2 diabetes mellitus [49]. Another relevant study showed that red ginseng extract had beneficial effects on rats with type 2 diabetes mellitus, which may be mediated by improving metabolic disorders such as D-Glutamine and D-glutamate metabolism [50]. In our study, D-Glutamine and D-glutamate metabolism was decreased in the DHC group, consistent with the above study. In a metabolomic study of 210 day workers and 275 shift workers, shift workers were more likely to have weight gain and central obesity and were at higher risk for impaired lipid metabolism, accompanied by altered ubiquinone and other terpenoid-quinone biosynthesis [51]. It was found that the decreased expression of CoQ was observed in diabetic rats as well as obese mice [52, 53]. Accordingly, adding the reductive form of CoQ to food could significantly reduce body weight, inguinal white adipose tissue fat and inguinal white adipose tissue percentage of KKAy mice, which was a model of obesity and type 2 diabetes [54].

Interestingly, the correlation analysis between microbiota and metabolites showed that the level of 1-methylxanthine was negatively associated with the abundance of *Eubacterium_hallii_group*; the abundance of *Enterococcus* was positively related with the level of P-coumaric acid and negatively correlated with the level of stachydrine, which was fully consistent with the separate changing trend of bacteria and metabolites. Based on differential genera and metabolites, we identified four markers that had the ability to recognize DHC. Furthermore, the combined marker composed of the above makers had higher accuracy than single marker. These findings contribute to the early objective diagnosis of DHC, thus enabling practitioners to take action to ameliorate the unbalanced state before infants suffer from severe diseases, reflecting the concept of preventive treatment of disease in traditional Chinese medicine.

This study has several limitations: (1) the alpha diversity of intestinal microflora in the DHC group was similar to that of the BC group, possibly because of the large individual differences among infants. Further studies with larger samples are needed to validate this result; (2) some reports have shown inconsistency in specific bacteria associated with DHC-related diseases. Perhaps this is because the changes in bacteria can cause pathological changes in adults, which are not the same as in infants. Accordingly, longitudinal research from infancy to adulthood is necessary; (3) further study is needed to unravel the specific interaction between microbiota and metabolites in DHC, such as animal experimental model and human interventional research; (4) in order to minimize confounding effects on the gut microbiota in cross-sectional studies, a more precise age division will be considered in future studies.

5. Conclusions

In conclusion, our results showed that DHC was the most unbalanced constitution of infants and had unique characteristics of gut microbiota and urine metabolites. Some differential bacteria and metabolites were associated with DHC-mediated metabolic diseases. In addition, a combined marker was identified to distinguish DHC from BC with high accuracy. Our findings provided new insight into the diagnosis of DHC and may help to improve the health of individuals with DHC from infancy.

Declarations

Author contribution statement

Haihong Zhao: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Yuhan Zong, Wenle Li and Yaqi Wang: Performed the experiments; Analyzed and interpreted the data.

Weibo Zhao, Xianghe Meng, Fan Yang, Jingwei Kong and Xiaoshan Zhao: Contributed reagents, materials, analysis tools or data.

Ji Wang: Conceived and designed the experiments; Analyzed and interpreted the data.

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Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no competing interests.

Additional information

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