Determining the protective effects of Ma-Mu-Ran Antidiarrheal Capsules against acute DSS-induced enteritis using 16S rRNA gene sequencing and fecal metabolomics

Zheng Si-li¹, Zhang Dong-ning¹,³, Duan Yan-fen¹, Huang Fang², Han Lin-tao¹*, Mo Guo-yan¹*

¹ Pharmacy Faculty, Hubei University of Chinese Medicine, Wuhan, Hubei 430065, China; ² College of Basic Medical Sciences, Hubei University of Chinese Medicine, Wuhan, Hubei 430065, China; ³ Xinjiang Uygur Pharmaceutical Co., Ltd., Urumqi, Xinjiang 830026, China

[ABSTRACT] Ma-Mu-Ran Antidiarrheal Capsules (MMRAC) is traditional Chinese medicine that has been used to treat diarrhea caused by acute enteritis (AE) and bacillary dysentery in Xinjiang (China) for many years. However, the potential therapeutic mechanism of MMRAC for AE and its regulatory mechanism on host metabolism is unclear. This study used fecal metabolomics profiling with GC/MS and 16S rRNA gene sequencing analysis to explore the potential regulatory mechanisms of MMRAC on a dextran sulfate sodium salt (DSS)-induced mouse model of AE. Fecal metabolomics-based analyses were performed to detect the differentially expressed metabolites and metabolic pathways. The 16S rRNA gene sequencing analysis was used to assess the altered gut microbes at the genus level and for functional prediction. Moreover, Pearson correlation analysis was used to integrate differentially expressed metabolites and altered bacterial genera. The results revealed that six intestinal bacteria and seven metabolites mediated metabolic disorders (i.e., metabolism of amino acid, carbohydrate, cofactors and vitamins, and lipid) in AE mice. Besides, ten altered microbes mediated the differential expression of eight metabolites and regulated these metabolisms after MMRAC administration. Overall, these findings demonstrate that AE is associated with metabolic disorders and microbial dysbiosis. Further, we present that MMRAC exerts protective effects against AE by improving host metabolism through the intestinal flora.

[KEY WORDS] acute enteritis; Ma-Mu-Ran Antidiarrheal Capsules; 16S rRNA sequencing; fecal metabolomics; Gut microbiota

Introduction

Acute enteritis (AE) is a severe inflammatory disease of the digestive system caused by bacteria, fungi, parasites and viruses. It is characterized by bacterial translocation, ulceration, necrosis and mucosal bleeding. Besides acute diarrhea, nausea, vomiting, abdominal pain, fever, and functional disorder with cardiovascular or liver and kidney are also its main clinical manifestations. However, severe inflammation of the colon increases the likelihood of developing colorectal cancer. The gut microbiome promotes the occurrence of intestinal inflammation. Gut microbiota enhances barrier immunity to maintain homeostasis, thereby promoting human health. Mounting evidence has demonstrated that gut microbial dysbiosis can induce and aggravate some human diseases (gastrointestinal, metabolic, and immune-mediated diseases). Notably, modern studies have displayed that AE is related to perturbations of the intestinal microbiota and dyshomeostasis of the intestinal immune system. The intestinal flora is also associated with disordered regulation and metabolism in AE patients. Microbial dysbiosis can increase potentially harmful microorganisms, which affect the intestinal immune system and induces inflammatory processes. This disturbance destroys the intestinal mucosal barrier and absorption function, impairing the transportation and use of nutrients and drugs, thereby aggravating the disease deterioration.

Traditional Chinese medicine (TCM) plays a crucial role in treating and preventing diseases due to its unique guidelines, practical bases, and approaches. Several studies have confirmed that TCM interacts with intestinal bacteria, and the ingredient of TCM modulate the structure and metabolism of intestinal bacteria to achieve therapeutic effects. Besides, gut microbiota biotransforms or react

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[Corresponding author] hanlintao@hbtcm.edu.cn; guoyanmo@hbtcm.edu.cn

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with certain bioactive ingredients of TCM to promote medicine absorption [18, 19]. Ma-Mu-Ran Antidiarrheal Capsules (MMRAC), a compound preparation composed of 14 traditional Chinese medicines, including Coptidis Rhizoma (HL), Draconis Sanguis (XJ), Olibanum (RX), Turkish Galls (MSZ), Bambusae Concretio Silicea (TZH), Semen Plantaginis Psyllii (ZZCQZ), Chebulae Fructus Immaturus (XQG), Fructus Berberis (XBG), Fructus Coriandri (YSZ), Chebulae Fructus (HZR), Terminalis Bellicariae Fructus (MHZR), Flos Granati (SLH), Granati Pericarpium (SHP), Gummi Tragacanthaie (XHSJ). It is commonly used to clinically treat AE and bacillary dysentery in Uyghur medicine [20]. Ma-Mu-Ran formula granules are included in Uyghur Medicines Volume of the “Medicine Standards of the Ministry of Health of the People's Republic of China” (1999 edition) to treat diarrhea, vomiting, and indigestion. Previous studies have indicated that MMRAC can substantially alleviate intestinal infection of Shigella Freundii 2a and has a therapeutic and improving effect on ulcerative colitis (UC) [21, 22]. Meanwhile, mounting evidence shows that berberine hydrochloride and gallic acid, the main effective components of MMRAC, can suppress inflammation and exert a protective effect in colitis [23-25]. However, the potential mechanisms and interactions of MMRAC with intestinal bacteria re unknown. This study aimed to investigate the effects of MMRAC on fecal metabolites and gut microbiota of AE mice. Besides, metabolomics approach and 16S rRNA gene sequencing techniques were used to further explore the potential therapeutic mechanism of MMRAC.

**MATERIALS AND METHODS**

**Chemicals and Reagents**

Ma-Mu-Ran Antidiarrheal Capsules (MMRAC) was kindly provided by Xinjiang Uygur Pharmaceutical Co., Ltd. (Xinjiang, China). Dextran sulfate (DSS) was obtained from Shanghai Aladdin Biochemical Technology Co., LTD (Shanghai, China). The GC grade methoxyamine hydrochloride, chlorotrimethylsilane (TMCS), and N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) were purchased from Sigma-Aldrich; Merck KGaA. DNeasy PowerSoil Kit, QuantiT PicoGreen dsDNA Assay Kit, and MiSeq Reagent Kit v3 were acquired from QIAGEN, Inc. (Netherlands), Invitrogen (Carlsbad, CA, USA), and Shanghai Personal Biotechnology Co., Ltd. (Shanghai, China), respectively. Agencourt AMPure Beads were manufactured by Beckman Coulter (Indianapolis, IN). Other chemicals were of analytical grade.

**Animal treatment**

Specific pathogen-free (SPF) C57BL/6 male mice (weight: 20 ± 2 g) were sourced from Liaoning Changsheng Biotechnology Co. Ltd. (Liaoning, China). They were housed for six days under the following conditions: 12 h photoperiod, 24-26 °C temperature, relative humidity of 50-70%, and provided with food and water ad libitum. The mice were randomly divided into a control group (CON) and two dextran sulfate sodium (DSS)-induced groups, i.e., AE model group (MOD) and MMRAC treatment group (MMRAC) (10 mice per group). The mice in the CON group were provided with drinking water ad libitum with phosphate-buffered solution (PBS). However, the other groups were given PBS with 4% DSS solution through drinking water to build an AE model as previously described [27-29]. The mice in the MMRAC group were given MMRAC solution (daily dose of 0.351 g/kg) for two consecutive weeks, similar to the modeling method of MOD group. Similarly, mice in CON and MOD groups were treated with sterile saline via the same administration method in the MMRAC group.

**DAI Measurement**

The activity and fecal indications of the mice were observed during the experiment. Moreover, the food/fluid consumption of each group and the body weight of each animal were recorded every day. A disease activity index (DAI) score was obtained based on weight loss, stool consistency, and intestinal bleeding and used to evaluate colitis severity [30]. Scoring criteria was as follows: weight loss: 0 ( < 2%); 1 (2-5%); 2 (5-10%); 3 (10-15%); and 4 (> 15%); stool consistency: 0 (normal), 1 (softer/sticky stool), 2 (moderate diarrhea/unformed stool) and 3 (diarrhea/watery stool); and bleeding: 0 (no blood), 1 (weak hemoccult positive), 2 (strongly hemoccult positive and visual bloody stool), and 3 (gross bleeding) [31].

**Sample Collection and Preparation**

Fecal samples were collected in a sterile EP tube and stored in a -80 °C refrigerator for fecal metabolomics analysis. All mice in each group were then euthanized, and the entire colon and intestinal contents were collected. The intestinal contents were stored at -80 °C for subsequent gut microbiota analysis. Meanwhile, the colonic tissue was stored in a tube with 4% paraformaldehyde at 4 °C overnight for histopathological analysis.

**H&E staining**

The colonic samples with 4.5 μm sections were embedded in paraffin wax, then stained with hematoxylin/eosin (HE) and imaged. A previous histological score system [32] was modified and shown as follows: crypt architecture; degree of inflammatory cell infiltration; muscle thickening; goblet cell depletion; crypt abscess (0, normal or absent; 1, mild or present; 2, moderate; 3, severe).

**Profiling of chemical composition in MMRAC**

The 100 mg powder in MMRAC was suspended in 70% methanol aqueous solution and thoroughly vortexed until fully dissolved. The supernatant obtained after centrifugation (12,000 rpm, 10 min) was filtered through a 0.22 μm membrane before UPLC-MS/MS analysis.

AB4500 Q-TRAP-UPLC/MS/MS system with an Agilent SB-C18 column (2.1 mm × 100 mm, 1.8 μm) was used to assess the samples at 40 °C. The solvent system contained of A (water+0.1% formic acid) and B (acetonitrile+0.1% formic acid) (flow rate: 0.35 mL/min). The gradient program was as follows: 5-95% B within 9 min; 95% B for 1 min; 95-5% B
within 1 min; 5% B for 3 min. The triple quadrupole-linear ion trap (Q-TRAP) mass spectrometer was used at an ion spray voltage of 5.5 kV in positive mode and -4.5 kV in negative mode. The ESI source temperature, ion source gas and curtain gas were 550 °C, 50 and 25.0 psi, respectively. Metware database (MWDB), a self-established database at Wuhan MetWare Biotechnology Co., Ltd., was used for the identification of spectral data [31]. The interferences of the repetitive signals were eliminated under the multiple reaction monitoring (MRM) modes. Quantification was conducted using area integration of chromatographic peaks.

**Fecal metabolomics**

Feces (30 mg) were weighed with 0.1 mg accuracy and extracted with 1 mL methanol after thawing at room temperature. The samples were blended, vortexed, and ultrasound in ice bath, then centrifuged at 13000 rpm, 4 °C for 10 min. Subsequently, 200 μL supernatant was transferred and evaporated in a brown glass vial via centrifugation at 35 °C for 2 h. Derivation and trimethylsilylation were performed by adding 40 μL methoxyamine pyridine solution (40 mg/mL) for 90 min at 30 °C and 80 μL MSTFA+1%TMCS at 37 °C for 60 min. Samples were then prepared in a 250-μL vial for further analysis.

A 7890B GC system (Agilent Technologies, Inc.) with Agilent DB-5ms capillary column (30 m × 0.25 mm × 0.25 μm) was used to obtain a better separation as follows: oven temperature program, 60 °C for 1 min, 60 °C to 250 °C (10 °C/min), 250 °C for 10 min; injector temperature, 250 °C; injection volume, 1 μL; carrier gas, helium (1.1 mL/min). The temperatures of the ion source and quadrupole rods were 230 °C and 150 °C, respectively. Mass spectra were obtained at a scan of m/z=50.0-600.0 using a positive ionization mode.

Raw GC-MS data were processed using the Agilent Masshunter software (Agilent Technologies, Inc.) to integrate the obtained signal and subsequently exported in the Automated Mass Spectral Deconvolution and Identification System (AMDIS) tool. Metabolites were identified in the National Institute of Standards and Technology (NIST) and RTL System (AMDIS) tool. Metabolites were identified in the NIST and RTL Library [38, 39]. Multivariate statistical analyses, including principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA), were conducted using MetaboAnalyst 4.0 online tool (http://www.metaboanalyst.ca/) [38] after normalization and scaling of metabolite data sets. R²X and Q² values, representing the total explained variance and the model predictability, respectively, were obtained. Metabolites with p-value < 0.05 and VIP > 1.0 from the PLS-DA model were considered as potential biomarkers. Impact-value > 0.05 was taken as screening criteria to find potential pathways.

**16S rRNA gene sequencing analysis**

Microbial genomic DNA was extracted from intestinal fecal samples of five randomly selected mice from each group to further microbial community analysis. PCR amplification was performed using primer 520F (5′-barcode+ACTCCTACGGGAGGCAGCA-3′) and 802R (5′-GGACTACHVGGGTWTCTAAT-3′) in the V3 and V4 region of the 16S rRNA genes. PCR amplified product was confirmed by 2% agarose gel electrophoresis and purified using Agencourt AMPure Beads, quantified by Quant-iT PicoGreen dsDNA Assay Kit, and sequenced using the Illumina MiSeq platform. MiSeq sequencing data were processed with slight modification according to the official tutorials of the QiIME2 2019.4 (https://docs.qiime2.org/2019.4/tutorials/) [37]. Specific steps were as follows: (1) primers cutting with cutadapt plugin [38]; (2) quality filtering using the DADA2 plugin [39]; (3) clustering amplicon sequence variants (ASVs) with mafft [40]; (4) construction of phylogeny using fasttree2 [41]. Taxonomy-based analyses, the species abundance table and community structure plot, were performed on this basis with R packages (v3.2.0).

Alpha diversity of samples was described from richness (Chao1, Observed species) diversity (Shannon, Simpson), evenness (Pielou’s evenness), and coverage (Good’s coverage), etc. Beta diversity analysis (PCoA, UPGMA) was conducted using the Bray-Curtis distance algorithm. Differential abundant taxa across groups were detected via the Linear discriminant analysis effect size (LEfSe) [42]. In addition, microbial functions were predicted by the PICRUSt2 [43] based on KEGG databases, and the potential pathway of microbial metabolism was visualized by the STAMP software.

**Correlation profiling between the metabolites and intestinal microbiotas**

Pearson correlation analysis was used to evaluate the relationship between significantly different metabolites and altered bacterial genera. Strong correlations were displayed as heat maps (**P < 0.05, *P < 0.01**) based on correlation coefficients (|r| > 0.5).

**Statistical analysis**

Statistical comparisons between groups were analyzed by unpaired t-test using GraphPad Prism 8.0 (GraphPad Software, USA). P < 0.05 was considered statistically significant.

**Results**

**MMRAC attenuates DSS-induced AE**

Mice in DSS-induced groups gradually manifested diarrhea, bloody stools, weight loss, and gross blood on the anus in severe cases. Besides, mice in the MOD group had a significantly increase in body weight and DAI (Fig. 1A, B). Also, histopathological changes (Fig. 1C), including cellular infiltration, mucosal necrosis, goblet cell depletion and transmural inflammation, occurred in the colon tissues of the DSS-induced groups. The MOD group had a substantially damaged crypt architecture, with significant cellular infiltration, goblet cell depletion, resulting in a severe colonic inflammation compared with the MMRAC group. Meanwhile, the histological scores (Fig. 1D) significantly decreased in the MMRAC group. Therefore, MMRAC attenuate colon inflammation and has a protective effect on the colon of DSS-induced AE mice.
Chemical component analysis

The UPLC-MS/MS technique was used to assess the chemical composition of MMRAC. The total ion flow maps for quantitative analyses were shown in Fig. S1A-B with the MRM modes. A total of 577 substances, including 55 tannins, 201 flavonoids, 175 phenolic acids, 38 alkaloids, 27 terpenoids, 31 lignans and coumarins, five quinones, and 45 others were detected and categorized into seven classes (Fig. S1C and Table S1).

Multivariate analysis of fecal metabolites and potential metabolic pathways

The optimal GC-MS conditions were used to obtain representative chromatograms (Fig. 2), with each group indicating differentially expressed fecal metabolites. Multivariate analysis methods, such as PCA and PLS-DA, were used to analyze the subtle changes in complex MS data among different groups. The PCA score plots demonstrated a visible separation between different groups (Fig. 3A). In addition, PLS-DA analysis (Fig. 3B-C) showed obviously segregation in CON vs. MOD, and MOD vs. MMRAC groups, suggesting that the model was well established. The cross-validation parameters ($R^2_Y$ and $Q^2$) between the MOD and CON groups, and MMRAC and MOD groups were greater than 0.7, indicating that the PLS-DA model and data quality were reliable. Fifteen potential metabolic markers (Table 1) were identified in AE mice treated with MMRAC based on the VIP value ($VIP > 1.0$) and T-test ($P < 0.05$). The markers were mainly involved in amino acid metabolism (pyrrole-2-carboxylic acid, 2-ketoisocaproic acid, L-isoleucine, and urea), carbohydrate metabolism (N-acetyl-D-mannosamine, succinic acid, L-lactic acid), lipid metabolism (beta-sitosterol, cholesterol, 1-hexadecanol), and metabolism of cofactors and vitamins (L-tyrosine, Alpha-Tocopherol).

Metabolic pathway analysis was performed to further explore the underlying meaning of the metabolites in Table 1. A pathway was considered to be a potential target when it satisfied the impact-value $> 0.05$ (Fig. 3D). The circles in different colors and sizes represent the different levels of significance and pathway impact values, respectively. Pathway analysis revealed that the mechanism of MMRAC in treating AE mice was related to phenylalanine, tyrosine and tryptophan biosynthesis, tyrosine metabolism, and primary bile acid biosynthesis (Table 2).

Alpha and beta diversities of gut microbiota
Species annotation analysis (Fig. 4A and B) displayed microbiota of the top ten relative abundance at phylum and genus levels. Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria were the main components of intestinal microbiota at the phylum level. Bacteroidetes, Actinobacteria and Proteobacteria decreased, and Firmicutes increased in the MOD group compared with the CON group. However, the composition showed the opposite trend after MMRAC treatment. Ten genera were identified in intestinal flora of mice, of which Allobaculum, Lactobacillus, Bifidobacterium, Bacteroides, and Oscillospira changed obviously in relative abundance among the three groups. Box plots of Alpha diversity indices (Fig. S2) and Rarefaction curves (Fig. S3) indicated that the sequencing data in each sample was suitable for further study. Beta diversity analysis, the principal coordinate analysis (PCoA), and unweighted pair-group method with arithmetic means (UPGMA) hierarchical clustering showed distinct separations among CON, MOD and MMRAC groups (Fig. 4C, D). Hierarchical clustering analysis can be used to determine the similarity in species composition based on the length of branches between samples.

Identification and function prediction of significantly altered microbiota

The linear discriminant analysis effect size (LEfSe) was carried out and visualized as a cladogram to detect differentially abundant taxa across these groups (Fig. 4E). Each individual circle represents microbial communities at each level, and small circles with different diameter represent intestinal flora with different relative abundance. A higher LDA score indicated a significant difference in the taxa. The top 60 differences in gut bacteria occurred at different levels with an LDA score > 3.0. Notably, 16 microbes, Bifidobacterium,
Fig. 3  Results of multivariate analysis among three groups. A: PCA plots of CON, MOD and MMRAC group. B, C: PLS-DA score plot of CON vs MOD ($R^2_Y = 0.994$, $Q^2 = 0.770$), and MOD vs MMRAC group ($R^2_Y = 0.998$, $Q^2 = 0.872$). D: Pathway analysis of fecal samples using MetaboAnalyst. The key pathways include: (a) phenylalanine, tyrosine and tryptophan biosynthesis, (b) tyrosine metabolism and (c) primary bile acid biosynthesis.

Table 1  Statistical analysis results of the main metabolite in feces

<table>
<thead>
<tr>
<th>NO.</th>
<th>RT (min)</th>
<th>Metabolite</th>
<th>MMRAC/MOD</th>
<th>VIP</th>
<th>Related pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7.36</td>
<td>L-Lactic acid</td>
<td>↓**</td>
<td>1.654</td>
<td>Pyruvate metabolism</td>
</tr>
<tr>
<td>2</td>
<td>8.22</td>
<td>Acetohydroxamic acid</td>
<td>↓**</td>
<td>2.354</td>
<td>Metabolic pathways</td>
</tr>
<tr>
<td>3</td>
<td>9.56</td>
<td>2-Ketoisocaproic acid</td>
<td>↑*</td>
<td>1.193</td>
<td>Valine, leucine and isoleucine degradation</td>
</tr>
<tr>
<td>4</td>
<td>10.04</td>
<td>Urea</td>
<td>↓**</td>
<td>2.713</td>
<td>Arginine and proline metabolism</td>
</tr>
<tr>
<td>5</td>
<td>10.79</td>
<td>L-Isoleucine</td>
<td>↑*</td>
<td>1.253</td>
<td>Valine, leucine and isoleucine biosynthesis</td>
</tr>
<tr>
<td>6</td>
<td>11.09</td>
<td>Succinie acid</td>
<td>↓**</td>
<td>1.731</td>
<td>Citrate cycle</td>
</tr>
<tr>
<td>7</td>
<td>12.04</td>
<td>Pyrrole-2-carboxylic acid</td>
<td>↑**</td>
<td>2.378</td>
<td>Arginine and proline metabolism</td>
</tr>
<tr>
<td>8</td>
<td>18.43</td>
<td>L-Tyrosine</td>
<td>↓**</td>
<td>1.430</td>
<td>Phenylalanine, tyrosine and tryptophan biosynthesis</td>
</tr>
<tr>
<td>9</td>
<td>18.66</td>
<td>1-Hexadecanol</td>
<td>↑*</td>
<td>1.379</td>
<td>Fatty acid degradation</td>
</tr>
<tr>
<td>10</td>
<td>19.93</td>
<td>N-Acetyl-D-mannosamine</td>
<td>↓**</td>
<td>1.398</td>
<td>Amino sugar and nucleotide sugar metabolism</td>
</tr>
<tr>
<td>11</td>
<td>21.82</td>
<td>Heptacosane</td>
<td>↑**</td>
<td>1.659</td>
<td>Metabolic pathways</td>
</tr>
<tr>
<td>12</td>
<td>25.47</td>
<td>Monostearin</td>
<td>↓**</td>
<td>1.710</td>
<td>Metabolic pathways</td>
</tr>
<tr>
<td>13</td>
<td>27.94</td>
<td>Alpha-Tocopherol</td>
<td>↓**</td>
<td>1.418</td>
<td>Ubiquinone and other terpenoid-quinone biosynthesis</td>
</tr>
<tr>
<td>14</td>
<td>28.10</td>
<td>Cholesterol</td>
<td>↑*</td>
<td>1.464</td>
<td>Primary bile acid biosynthesis</td>
</tr>
<tr>
<td>15</td>
<td>29.47</td>
<td>Beta-Sitosterol</td>
<td>↓**</td>
<td>1.651</td>
<td>Steroid biosynthesis</td>
</tr>
</tbody>
</table>

MMRAC/MOD: MMRAC group compared to MOD group; ↑: up-regulated, ↓: down-regulated; *P < 0.05, **P < 0.01.
Fig. 4  Relative abundance of gut microbiota at the phylum (A) and genus (B) levels in three groups revealed by 16S rRNA sequencing (different colors represent different bacteria at phylum or genus levels). (C) Principal coordinate analysis score (PCoA) analysis among CON, MOD and MMRAC groups. (D) Hierarchical clustering analysis of UPGMA in three groups. Differences in microbiota composition between three groups with Linear discriminant analysis Effect Size (LEfSe), visualized by Cladogram (E).
Olsenella, Odoribacter, Alistipes, Rikenella, Mucispirillum, Atopococcus, Turicibacter, Christensenella, Coprococcus, Ruminococcus, Coprobacillus, Sutterella, Desulfovibrio, Acinetobacter, Akkermansia, were identified at the genus level. Microbial function prediction analysis indicated that the main changes occurred in the amino acid metabolism, carbohydrate metabolism, and lipid metabolism pathways (Fig. 5).

**Correlation analysis for gut bacteria and fecal metabolites**

Correlation heat maps were established based on Pearson’s correlation analysis to evaluate metabolite-microbial relationships. Altered fecal metabolites were significantly associated with perturbed gut microbiota (Fig. 6). The correlation analysis between the intestinal microbiota (B5, B6, B10, B14, B16) and metabolites showed an opposite trend between MOD and CON groups. The significantly correlated intestinal microbiota and metabolites were selected from heat maps in each group for further analysis.

The interaction network diagram was established by integrating information about metabolites and microbes in correlation, metabolic pathway, and microbial function prediction analyses. As shown in Fig. 7, the significantly correlated metabolites and intestinal microbiota were involved in four primary metabolic pathways, including the metabolism of amino acid, carbohydrate, cofactors and vitamins, and lipid. Six intestinal bacteria (Acinetobacter, Ruminococcus, Desulfovibrio, Olsenella, Rikenella, Coprococcus) and seven related metabolites (L-lactic acid, acetoxyhydroxamic acid, L-tyrosine, N-acetyl-D-mannosamine, pyrrole-2-carboxylic acid, beta-sitosterol, alpha-tocopherol) were associated with occurrence of AE. However, ten microbes (Christensenella, Ruminococcus, Akkermansia, Alistipes, Odoribacter, Turicibacter, Coprococcus, Rikenella, Desulfovibrio, Bifidobacterium) and eight related metabolites (L-lactic acid, acetoxyhydroxamic acid, succinic acid, N-acetyl-D-mannosamine, 2-ketoisocaproic acid, L-isoleucine, cholesterol, alpha-tocopherol) were detected after MMRAC administration.

**Discussion**

A healthy intestinal environment maintains human health by regulating the balance of gut microbiota, metabolites, and the host’s immune system [44]. However, changes in microbial populations can disrupt the beneficial interactions between microbiota and host, thereby inducing diseases [45]. Numerous studies have indicated that TCMs can modulate the composition and functional structure of intestinal microbiota, thus...
improving metabolic disorders \[46\]. In this study, the 16S rRNA sequencing analysis was used to investigate microbial disorder in AE mice and microbial regulation after MMRAC treatment. In addition, the fecal samples can directly contain host-gut cometabolites and provide a functional readout of microbial activity. Herein, fecal metabolomic analysis based on GC-MS was used to determine significant changes in fecal metabolites in AE mice after MMRAC administration. From correlation analysis, the significant related differential fecal metabolites and altered intestinal microbiota were closely related to amino acid, carbohydrate, cofactors and vitamins, and lipid metabolism.

**Amino acid metabolism**

Five fecal metabolites were related to amino acid metabolism, of which L-tyrosine was correlated with two potential metabolic pathways (tyrosine metabolism, biosynthesis of phenylalanine, tyrosine and tryptophan). As shown in Table 1, L-isoleucine and 2-ketoisocaproic acid were associated

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**Fig. 6** Heat map summarizing the correlation values between significantly altered fecal metabolites and perturbed gut microbiota. M1-M15 corresponds to fifteen differential metabolites, namely L-lactic acid; Acetohydroxamic acid; L-isoleucine; 2-Ketoisocaproic acid; Succinic acid; L-Tyrosine; 1-Hexadecanol; N-Acetyl-D-mannosamine; Heptacosane; Monostearin; Alpha-Tocopherol; Cholesterol; Beta-Sitosterol; Urea; Pyrrole-2-carboxylic acid. B1-B16 represents sixteen different bacteria, including Bifidobacterium; Akkermansia; Alistipes; Odoribacter; Rikenella; Ruminococcus; Mucispirillum; Olsenella; Flexispira; Desulfovibrio; Christensenella; Sutterella; Coprobacillus; Acinetobacter; Turicibacter; Coprococcus, respectively. *P < 0.05, **P < 0.01.

**Fig. 7** Interaction network diagram of the metabolites-microbes-pathways based on correlation analysis. The results of metabolic pathways and 16S function prediction is referred to Table 1 and Fig. 5. The red (blue) lines indicate a positive (negative correlation) between the metabolites and microbes.
with valine, leucine, and isoleucine biosynthesis/degradation, while pyrrole-2-carboxylic acid and urea were involved in the metabolism of arginine and proline. Studies have shown that tryptophan is a precursor of the biosynthesis of numerous microbial metabolites \cite{67}. Furthermore, the gut microbiome can influence the systemic circulation of branched-chain amino acids (valine, leucine, and isoleucine) \cite{49}. The 16S functional prediction analysis also showed that the intestinal microbes were significantly correlated with amino acid metabolism. Specifically, the altered intestinal microbiota of AE mice was related to the metabolism of alanine, aspartate and glutamate, the degradation of valine, leucine and isoleucine, and cysteine and methionine metabolism. Besides, the changes in flora after MMRAC intervention were related to the first two metabolic pathways mentioned above.

Gut microbiota can regulate digestion and absorption of amino acids \cite{49}. Moreover, malnutrition of amino acids induces intestinal inflammation and diarrhea \cite{69}. L-tyrosine was significantly positively correlated with Acinetobacter in MOD group, while pyrrole-2-carboxylic acid was significantly negatively associated with Rikenella and Coprococcus. Similarly, researches have shown that the fecal samples of the patients with the intestinal disease have increased abundance of Coprococcus and decreased abundances of Acinetobacter and Rikenella \cite{51-53}. Additionally, bacteria can produce amino acids, and intestinal flora can produce many metabolites using amino acids as substrates \cite{54}. Therefore, the metabolism of arginine and proline mediated by Rikenella and Coprococcus was disturbed in the MOD group. However, L-isoleucine was significantly negatively related to Turicibacter in the MMRAC group. Also, Turicibacter was significantly increased in the MMRAC group compared with the MOD group. Previous research showed that the abundance of Turicibacter is negatively associated with colitis development \cite{55, 65}. Therefore, MMRAC induces a regulatory effect on valine, leucine, and isoleucine biosynthesis/degradation in AE mice via Turicibacter.

**Carbohydrate metabolism**

L-lactic acid and N-acetyl-D-mannosamine were significantly changed in the MOD group among the three metabolites involved in carbohydrate metabolism (Table 1). Lactic acid, a common human metabolism compound, is essential in multiple cellular processes, including energy regulation, immune tolerance, memory formation, wound healing, ischemic tissue injury, and cancer growth and metastasis \cite{37, 58}. Gut microbiota can produce lactate (racemic mixture), of which traces of L-lactate can be found in the feces since it is metabolized in the gut and elsewhere with a high turnover \cite{59}. Researchers found that lactate can downregulate proinflammatory responses and antagonise intestinal pathogens in a colitis mouse model \cite{60, 61}. Notably, L-lactic acid content was significantly increased in the feces of the MOD group. Its metabolism was affected in DSS-induced AE. In addition, N-acetyl-D-mannosamine (ManNAc) and its derivatives restore the orexin neurons to regulate critical brain activities, thereby affecting sleep, eating, emotions, and metabolism \cite{62}. A study showed that ManNAc can affect amino sugar and nucleotide sugar metabolism by inhibiting the phosphorylation of D-glucose \cite{66}. The correlation analysis results revealed that L-lactic acid and N-acetyl-L-mannosamine was significantly positively correlated with Acinetobacter, Desulfovibrio and Ruminococcus in the MOD group. Desulfovibrio was significantly increased, promoting inflammation promotion and thus can be used as biomarkers to predict gastric cancer \cite{64}. These also confirm that a disorder of carbohydrate metabolism occurred in AE mice. Similarly, metabolites and significantly related microbes were associated with carbohydrate metabolism in the MMRAC group.

Besides L-lactic acid and ManNAc, succinic acid also significantly changed after MMRAC treatment. Studies have shown that body cells can metabolize succinic acid. Succinic acid participates in the tricarboxylic acid cycle (TCA) essential in basal cell metabolism \cite{69}. Connors et al. indicated that succinate could activate immune cells and enhance inflammation. Besides, elevated succinate levels in the intestinal lumen are associated with microbiome dysbiosis \cite{69}. Correlation analysis revealed that these three metabolites were significantly positively correlated with many intestinal microbes. Therefore, metabolites or microbes related to carbohydrate metabolism were regulated after MMRAC administration, exerting a therapeutic effect.

**Metabolism of cofactors and vitamins**

Alpha-tocopherol, a compound with the highest vitamin E activity, is a peroxyl radical scavenger and has anti-inflammatory effects both \textit{in vitro} and \textit{in vivo} \cite{65, 66}. α-tocopherol can suppress the proliferation of gastric mucosal cells \cite{70}. Moreover, vitamin E can treat mild and moderate ulcerative colitis due to its anti-inflammatory and anti-oxidative properties \cite{71}. Alpha-tocopherol was significantly decreased in the MOD group and positively correlated with Rikenella and Coprococcus. However, Rikenella and Coprococcus were significantly decreased in the MOD group. Also, 16S functional prediction showed that the metabolism of cofactors and vitamins was significantly associated with AE. Alpha-tocopherol did not significantly increase in the MMRAC group, but it was significantly correlated with Desulfovibrio. Desulfovibrio is involved in the sulfate reduction in human gut microbiota \cite{52}, and sulfide production promotes bowel inflammation such as ulcerative colitis \cite{73}. These findings indicate that metabolism of cofactors and vitamins is perturbed in MOD group, and MMRAC can improve this metabolism to some extent by regulating Desulfovibrio.

**Lipid metabolism**

Metabolic pathway analysis showed that some metabolites (acetohydroxamic acid, beta-sitosterol, and cholesterol) were associated with lipid metabolism, including steroid biosynthesis, primary bile acid biosynthesis, and urea cycle. Notably, acetohydroxamic acid was significantly increased in the MOD group while significantly decreased in the MMRAC group. Acetohydroxamic acid decreases urinary ammonia.
levels and suppresses bacterial urease activity, thus ameliorating kidney stones and reducing infection in the urinary tract [34, 35]. Urea in the gastrointestinal tract is converted into ammonia by urease enzymes, and ammonia can be synthesized microbial protein [79]. Acetohydroxyacidic acid has a significant anti-H. pylori effect in an in vivo gerbil model [77]. Ryvchin et al. [78] revealed that Ruminococcus, a urease producer, produces acetate in non-IBD controls and most IBD patients. Similarly, acetohydroxyacidic acid was significantly negatively associated with Acinetobacter and Ruminococcus in the MOD group. Therefore, AE could be related to a disorder of the urea cycle mediated by Acinetobacter and Ruminococcus.

Besides, beta-sitosterol, related to steroid biosynthesis, was significantly decreased in the MOD group. Beta-sitosterol, a common sterol in herbal medicines, exerts anti-inflammatory effects by inhibiting the expression of proinflammatory cytokines (TNF-α, IL-1β, and IL-6) and inflammatory enzymes (cyclooxygenase) [79]. Previous studies have suggested that beta-sitosterol significantly increases the production of antimicrobial peptides and reduces levels of intracellular Salmonella typhimurium in intestinal epithelial cells, thus ameliorating colitis in mice [80]. Feng et al. also indicated that dietary stigmasterol and beta-sitosterol could improve colitis in mice [81]. Notably, beta-sitosterol was significantly positively correlated with Olsenella in the MOD group. Besides, its relative abundance increased after MMRAC administration. Zhang et al. [82] observed a significant increase in Olsenella in mice with colitis after fecal microbiota transplantation intervention.

Meanwhile, cholesterol, involved in primary bile acid biosynthesis, was significantly decreased in MMRAC group. Previous studies have suggested that cholesterol promotes malignant transformation and proliferation. Also, cholesterol and bile acid in the serum significantly increased in DSS-induced colitis mice [83, 84]. Correlation analysis revealed that cholesterol was not significantly associated with microbes in the MOD group. In contrast, it was significantly correlated with microbes in the other two groups. Cholesterol was significantly positively related to Christensenella and Bifidobacteria in the MMRAC group. Christensenella exerts the cholesterol-lowering function [85]. Marras et al. [86] showed that Bifidobacteria might have a therapeutic effect on ulcerative colitis, colorectal cancer, cholesterol reduction and metabolic disorders. Overall, these results illustrate that MMRAC can improve AE by regulating lipid metabolism via Olsenella, Bifidobacteria and Christensenella.

Collectively, given our study initially confirmed that AE in mice closely links to the perturbations of intestinal microbiota, and MMRAC can improve the disturbance to alleviate AE. However, further studies are needed for more comprehensive and reliable results. Although GC-MS is widely used for metabolomics analysis of various physiological fluids, it has limited analytical coverage and long sample preparation requirement. Therefore, a serum metabolomic study using LC-MS technique was conducted to further analyze the endogenous metabolites and explore biological pathways. Besides, future studies should assess the optimal dosage of MMRAC for AE treatment.

Conclusion

This study reveals fifteen differential fecal metabolites and sixteen significantly altered bacterial genera in DSS-induced AE mice after MMRAC treatment based on fecal metabolomics and 16S rRNA gene sequencing analyses. The significantly correlated microbes and metabolites are mainly involved in metabolism of amino acids, carbohydrates, cofactors and vitamins, and lipids. Furthermore, six intestinal bacteria and seven related metabolites mediated disorders of metabolisms in AE mice, while MMRAC affected ten microbes and eight related metabolites, thereby modulating the metabolic disorders. Overall, the findings preliminary demonstrate that AE occurrence is associated with metabolic disorders and microbial dysbiosis, and MMRAC exerts protective effects against AE by improving host metabolism through the intestinal flora.

References


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