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An orally administered magnoloside A ameliorates functional dyspepsia by modulating brain-gut peptides and gut microbiota

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ABSTRACT

Aims: Functional dyspepsia (FD) is very common worldwide with a high prevalence of 10%–30%, and it becomes a heavy burden to patients because of its hard to be cured. In our previous study, phenylethanoid glycosides were found to exist in Houpo, a traditional Chinese medicine commonly used for the treatment of abdominal distention, pain and dyspepsia. In the present study, the effect of magnoloside A (MA), a main phenylethanoid glycoside in Houpo, on FD was firstly evaluated and its potential mechanism was concluded.
Materials and methods: MA was orally administered consequently for 3 weeks, and its effect on a FD rat model established through transient neonatal gastric irritation and mature alternate-day fasting was tested. Levels of brain-gut peptides and inflammatory factors in blood or tissues were determined by ELISA methods. Meanwhile, the gut microbiota was analyzed by 16S rRNA gene sequencing and short chain fat acids were determined by GC/MS.
Key findings: MA exhibited anti-FD activities by fastening the delayed gut emptying rate of FD rat and increasing the levels of gastrin, motilin, and calcitonin gene related protein; and decreasing the levels of 5-hydroxytryptamine, nitric oxide synthase, and vasoactive intestinal peptide. On the other hand, MA can modulate the composition of gut microbiota, resulting in the variation of the short chain fat acids.
Significance: MA ameliorated FD rats by modulating of the secretion of related brain-gut peptides and altering the composition of intestinal microbiota.

1. Introduction

Functional dyspepsia (FD) is a worldwide disease with a high prevalence rate of 10%–30% [1]. For many FD patients, the chronic symptoms markedly influenced their qualities of life and caused significant direct and indirect health costs [2]. As a typically physical and psychological disease, the pathogenesis of FD remains to be studied. Besides the hypothesis that it is attributed to be a combination of upper gastrointestinal motility and visceral hypersensitivity disorders combined by the enteric nerve system (ENS) [3], accumulating data suggest that factors involving brain-gut signaling disturbances and the dyspepsia of gut microbiota also play important roles in FD [4–6]. Gut microbiota is an important factor for the homeostasis of the host due to its participation in nutrient processing and essential compounds producing, such as short-chain fatty acids (SCFAs). Moreover, it also contributes to the development of the gastrointestinal system [7,8], for example, changes in microbiota composition were found to be related to functional and psychiatric disorders such as irritable bowel syndrome [4]. Brain-gut peptides (BGPs), including, but not limited to, gastrin (GAS), motilin (MTL), and calcitonin gene related protein (CGRP), were found in ENS, cerebral nervous system (CNS), and endocrine cells in gastrointestinal tract, they not only bridge the brain-gut signaling but also regulate gastrointestinal motility. The existence of a gut-brain-microbiota axis resulting from recent reports [9,10] suggested that the improvement of FD could be achieved through altering the intestinal microbiota and BGPs.

Owing the advantage of being low toxic, effective, green and accessible, medicinal plant-derived natural product is a common source for drug discoveries [11]. The bark of *Magnolia officinalis* Rehd. et Wils., a traditional Chinese medicine (TCM) known as Houpo in Chinese, is a

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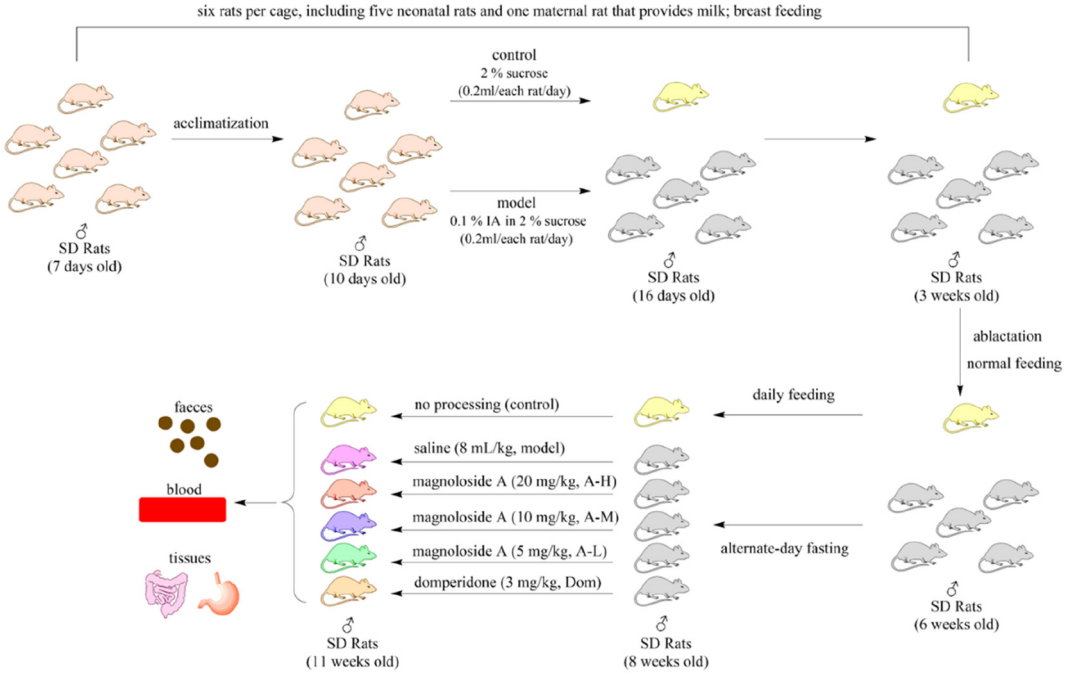

Fig. 1. Schematic diagram of animal experimental trial.

main ingredient in > 200 types of Chinese formulae commonly used in clinics, from which 71% were used for the treatment of abdominal distention, pain and dyspepsia [12]. Because Houpo is clinically used as an aqueous decoction, water-soluble compounds were studied in our previous work, and phenylethanoid glycosides (PhGs) were newly found to exist in Houpo and showed anti-spasmodic activity on isolated colon of rat [13–15]. However, no study has investigated the potent effect of PhGs on FD. Therefore, in the present work, the pharmacological effect of magnoloside A (MA), a main PhG with a content of 1%–2% in Houpo, on FD model rat was assessed and the potential basis of the effect was discussed through altering BGPs and the gut microbiota.

2. Materials and methods

2.1. Animal experiments

All animal care and experimental procedures were approved by the Laboratory Animal Ethics Committee of the Institute of Basic Theory of Traditional Chinese Medicine, China Academy of Chinese Medical Sciences (Beijing, China).

A FD rat model was established through transient neonatal gastric irritation [16] and mature alternate-day fasting (ADF). Sprague-Dawley male neonatal rats (7 days old; specific pathogen-free grade, SCXK 2012-0004, Academy of Military Medical Sciences, Beijing, China) were housed in individually ventilated cages (six animals per cage, including five neonatal rats and one maternal rat that provides milk) at the SPF animal facility of China Academy of Chinese Medical Sciences under controlled environmental conditions (temperature 21 ± 1 °C; relative humidity 60–70%). Maternal rats were free access to standard laboratory chow (Beijing Keaoxieli Feed Co. Ltd.; Beijing, China) and tap water, and maintained at a regular 12/12 h light/dark cycle. Rats were acclimatized to their environment for 3 days before the experiments. Neonatal rats were randomly allocated into two groups, the control group and the model group. Neonatal rats in the model group were fed 0.2 mL of 0.1% iodoacetamide (IA) (Sigma-Aldrich, Shanghai, China) in 2% sucrose (Sigma-Aldrich, Shanghai, China) daily for 6 days by oral gavages and the control group was fed 0.2 mL of 2% sucrose. The ablactation rats, 3 weeks old, were re-assigned among cages and free access to standard laboratory chow and tap water. At week 6, ADF was performed only on the model rats. At week 8, IA + ADF rats were randomly divided into five groups based on weight (body weight 280 ± 3 g, $n = 8$ per group) as follows: (1) normal saline group (8 mL·kg^{-1}, per day; named model group); (2) positive control group (domperidone, Xian Janssen Pharmaceutical Ltd., Xian, China, 3 mg·kg^{-1}, per day; named Dom group); (3–5) MA (purity ≥95%; extracted by ourselves [14] treated groups (5, 10 and 20 mg·kg^{-1}, per day, named A-L, A-M, and A-L group, respectively). The doses of MA were calculated based on Chinese Pharmacopeia (2015 version) [17]. The rats received saline, domperidone, or MA by oral gavage for 3 weeks. At the end of the trial, fresh stool samples were collected and stored immediately at −80 °C for subsequent analysis. After overnight fasting for 12 h, some rats were randomly chosen to test gastric emptying rate (see "Gastric emptying rate assessment"), others received 3% D-xylose (Solarbio, Beijing, China) (1 mL·100 g^{-1}) by oral gavage. After 50 min (for xylose-treated rats) or 3 h (for rats testing gastric emptying rate), all the rats were narcotized by 1% pelltobarbitalum natricum (Sigma, USA) and blood was collected from the abdominal aorta, and serum was isolated by centrifugation at 3240 r·min^{-1} (15 min at 4 °C) (GTR16-2, Beijing Era Beili Centrifuge Co., Ltd.). Subsequently, the supernatant was stored at −20 °C for later use. Spleen and colon were excised immediately and homogenized by scissor and Multi-sample freezing grinding machine at 70 Hz (10 min) (LANYI-DLM, Shanghai Lanyi Industrial Co., Ltd) in ice-cold normal saline (0.9%). The homogenate was centrifuged at 2500 r·min^{-1} (15 min at 4 °C) (TGL-16, Changsha Xiangyi Centrifuge Co., Ltd) and stored at −20 °C for further analysis.

The schematic diagram of animal experimental trial is shown as Fig. 1.

2.2. Gastric emptying rate assessment

After overnight fasting for 12 h, rats randomly chosen to test gastric emptying rate ($n = 4$ for each group) were housed individually and given food enough for 3 h (21 g/rat), then the amount of the remained food was recorded after 3 h ($W_{remained}$). Keeping for fasting including water for 3 h, the rat was narcotized by 1% pelltobarbitalum natricum and its stomach was picked out, cleaned, and weighed both for the total

weight (W_{total}) and the suttle weight (W_{suttle}), respectively. The gastric emptying rate was calculated according to formula (1)).

$$\text{Gastric emptying rate (\%)} = 100\% - (W_{total} - W_{suttle})/(21 - W_{remained}) \times 100\% \quad (1)$$

2.3. Histological examination

The stomach histopathology was evaluated by haematoxylin and eosin (H&E) staining. Briefly, after fixation, sections of gastric wall were dehydrated in graded ethanol, cleared in xylene and embedded in paraffin. The sections (5 μm) were mounted on glass slides, rehydrated to distilled water and stained with H&E, and then they were imaged via DMI6000-microscope (Leica, Wetzlar, Germany).

2.4. BGPs and inflammatory factors measurement

Serum levels of MTL, GAS, somatostatin (SS), CGRP, and vasoactive intestinal peptide (VIP), levels of interferon-γ (IFN-γ) and interleukin 4 (IL-4) in spleen, and 5-hydroxytryptamine (5-HT) in colon were measured using enzyme linked immunosorbent assay (ELISA) per the manufacturer's instruction (Deyi Diagnostics, Beijing, China). For 5-HT, kits from Cusabio (Wuhan, China) were used by Deyi Diagnostics (Beijing, China). Briefly, each sample (50 μL) was mixed with a series of solutions including antibody, wash buffer, stop solution, etc. according to the manufacturer's instructions, and then incubated for corresponding time (10 min, 20 min, 30 min, etc.) at corresponding temperature (25 °C, 37 °C, etc.). Finally, the optical density value of sample was determined at 450 nm, and the concentration of the tested BGP and inflammatory factor was determined according to standard curve. In addition, serum levels of nitric oxide synthase (NOS), lactic acid (LD) and amylase (AMS) were tested using colorimetery per the manufacturer's instruction (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China) by Deyi Diagnostics (Beijing, China).

2.5. Gut microbiota analysis

Fecal samples were used to characterize the gut microbiota (Novogene; Beijing, China). Total DNA was extracted using the TIANamp Soil DNA Kit (DP 140311, Tiangen Biotech Beijing Co., LTD. China) as per the manufacturer's protocol, and the purity and concentration of DNA were tested by agarose gel electrophoresis. The DNA obtained was used to amplify V4 region of 16S rRNA genes, with the forward primer 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and the reverse primer 806R (5′-GGACTACHVGGGTWTCTAAT-3′). The production of PCR was detected using 2% agarose gel electrophoresis. 16S rRNA gene sequence libraries were generated using TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) and quantified by Qubit and Q-PCR, then HiSeq2500 PE250 was used for sequencing.

After the sequences of barcode and primers were trimmed from sequence reads, raw tags were spliced using FLASH (V.1.2.7) [18], then effective tags were performed using quantitative insights into microbial ecology by QIIME [19,20], including truncating of raw tags, filtering of tag length, and wiping off chimera [21] compared with Gold database using UCHIME Algorithm [22]. Operational Taxonomic Units (OTUs) were delineated at the cutoff of 97% using Uparse (v.7.0.1001) [23]. Taxon-dependent analyses of OTUs were performed using Mothur method in comparison with SSUrRNA Database of SILVA [24,25]. Tax4Fun [26] was used to predict functional profiles from metagenomic 16S rRNA data based on KEGG Orthology database. Alpha- and Beta-diversity analyses were performed in QIIME (v1.7.0) based on normalized OTU relative abundance, and LEfSe algorithm was performed by Galaxy [27] with the cutoff of LDA score equaling 3.0.

2.6. Short chain fatty acids measurement in feces

Two hundred micrograms of freeze-dried fecal sample was acidified by 0.5 mL of hydrochloric acid (pH = 1) and extracted by vortex with 500 μL of ethyl acetate for 30 min. After being centrifuged at 12000 $r \cdot min^{-1}$ for 10 min, supernatants were obtained to be analyzed.

The determination of fatty acid composition was carried out using Zhao's method [28] with minor modification. A GC/quadrupole MS system (Agilent 7890B_5977A) was used to determine the contents of SCFAs, with a column of DB-FFAP (30 m × 0.32 mm i.d., 0.25 μm film thickness, Agilent, USA). The oven temperature program was as follows: 0–1 min 50 °C; 1–11 min 50–170 °C; 11–14 min 170–230 °C; 14–17 min 230 °C; 17–25 min 230–50 °C. The carrier gas was helium at a constant flow of 1 $mL \cdot min^{-1}$. Injector and detector were kept at 250 and 230 °C, respectively. Splitting ratio was 50:1. Electron impact mass spectra were taken at −70 eV.

2.7. Data and statistical analysis

Potential microbes were identified from thousands of microbes, which LDA Score were bigger than 3.0, and the predicted pathways from Tax4fun were selected, which LDA Score were bigger than 2.0, by LEfSe. All data in this study are presented as the means ± standard error. Differences among six groups were examined by one-way ANOVA followed by LSD post hoc test, which was run only if F achieved $P < 0.05$ and there was no significant variance inhomogeneity. A P value < 0.05 was considered statistically significant. All statistical analyses were performed using SPSS software, version 24.0. Graphs were drawn using GraphPad Prism (version 7.0 for Windows).

3. Results

3.1. MA improves gastric emptying rate of FD rats

As illustrated by gastric histological analysis, there was no ulcer,

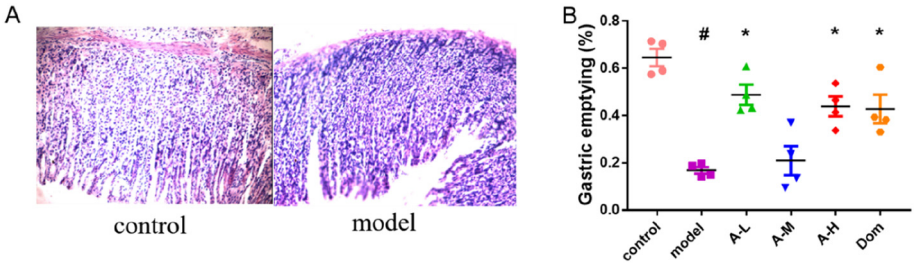

Fig. 2. MA improves gastric emptying rate of FD rats induced by transient neonatal gastric irritation (0.2 mL of 0.1% iodoacetamide (IA) in 2% sucrose daily for 6 days by oral gavages) and mature alternate-day fasting (ADF) for 2 weeks. (A) Representative H&E-treated sections in stomach samples of control rat (left) and model rat (right). (B) The gastric emptying rates of tested rats in different groups. The data are presented as the means ± SEM of $n = 4$. Control group: 0.2 mL of 2% sucrose daily for 6 days by oral gavages from 10 days old. Model group: 0.2 mL of 0.1% IA in 2% sucrose daily for 6 days by oral gavages from 10 days old, mature ADF for 2 weeks from week 6 to week 8, and given saline by oral gavages (8 $mL \cdot kg^{-1}$, per day) for 3 weeks. MA treated groups: 0.2 mL of 0.1% IA in 2% sucrose daily for 6 days by oral gavages from 10 days old, mature ADF for 2 weeks from week 6 to week 8, and given MA by oral gavages (5, 10 and 20 $mg \cdot kg^{-1}$, per day) for 3 weeks, named A-L, A-M, and A-H group, respectively. Dom group: 0.2 mL of 0.1% IA in 2% sucrose daily for 6 days by oral gavages from 10 days old, mature ADF for 2 weeks from week 6 to week 8, and given domperidone by oral gavages (3 $mg \cdot kg^{-1}$, per day) for 3 weeks. $^{\#}P < 0.05$ versus the control group, $^{*}P < 0.05$ versus the model group.

inflammatory infiltration, and glandular epithelial lesion in the model group (Fig. 2A), which was consistent with the description of no evidence of structural disease in FD, based on the diagnostic criteria of Rome IV, for functional gastrointestinal disorders.

FD involves complex pathophysiological mechanisms including visceral hypersensitivity, delayed gastric emptying, etc., and delayed gastric emptying is a feature present in 24% -31.8% of FD patients [29,30]. The gastric emptying rate was used herein as an index to assess the improvement of FD. As shown in Fig. 2B, compared with the control group, the gastric emptying rate of the model group significantly delayed, while after treated with MA, it improved, especially, significantly improved in A-L and A-H groups, which means that MA can improve the gastric emptying rate of FD rats.

3.2. MA modulates the secretion of BGPs of FD rats

In recent years, accumulating evidence has revealed that the dysfunction of brain-gut axis is closely linked to FD, and the imbalance of BGPs is regards as one of the pathogeneses of FD. For example, as neurotransmitter responsible for modulating gastrointestinal motor behavior, nNOS, VIP, and 5-HT were significantly high in FD patients and rats [31–33]. Lower levels of GAS and MTL were observed in FD patients and rats, and the increasing of MTL and GAS can induce alterations of gastric motility, resulting in a significant symptom improvement of FD patients and rats [34,35]. The levels of GAS, MTL, NOS, VIP, SS, and CGRP in serum and 5-HT in colon were thus tested and the results were shown in Fig. 3. For model rat, the levels of GAS and MTL were significantly decreased, while the levels of VIP and 5-HT were significantly increased, and NOS was increased but not significant, compared with those in the control group. After treated with MA, GAS and MTL were significantly increased, while VIP and 5-HT were significantly decreased and NOS decreased but not significant, compared with the models. In addition, dose-response effects were found in the cases of NOS and 5-HT, and the highest levels of GAS and MTL and the lowest level of VIP were found in A-H group while the effects induced by A-L and A-M were similarly. Besides the gastroduodenal motor dysfunction, sensory dysfunction also played an important role in FD. In model rat, the level of CGRP, a sensory neuropeptide, was significantly increased, compared with control one, while it was decreased in a dose-dependent way after treated with MA and the significant decrease was found in A-H group, compared with the model rats, indicating that MA treatment could decrease the visceral hypersensitivity. Whether SS plays a passive or active role in the pathogenesis of FD is still a matter of debate. Some studies found that FD patients have been shown to display higher levels of SS compared with healthy individuals [36], in other studies no significant difference of SS was found [37]. In our study, no significant difference of SS was found either between the model and control groups or between the model and MA-treated groups, although a tendency considering the decrease of SS in model group, while the increase of SS in MA treated groups, especially in A-H group, was observed.

3.3. MA attenuates inflammatory responses of FD rats

The levels of inflammatory factors in spleen were increased in a significant (IL-4) or non-significant (IFN-γ) way in model group versus the control group, because of the inflammation and hypoxia stimulated by IA [38–40], while, they were decreased (IFN-γ) and significantly decreased (IL-4) after the treatment of MA, and a dose-dependence decrease was observed for IL-4 (Fig. 4).

In addition, the level of LD in blood was significantly increased in model group versus the control group, while, it was significantly decreased after the treatment of MA and a dose-response relationship was observed (Fig. 4). The opposite phenomenon was observed for AMS, i.e., it was significantly decreased in model group versus the control group and significantly increased in A-M and A-H groups with a dose-response relationship. And the deficiency in AMS was also believed to be one of the contributing factors for FD [41].

3.4. MA makes alterations to gut microbiota of FD rats

In recent years, accumulating evidence has revealed that gut

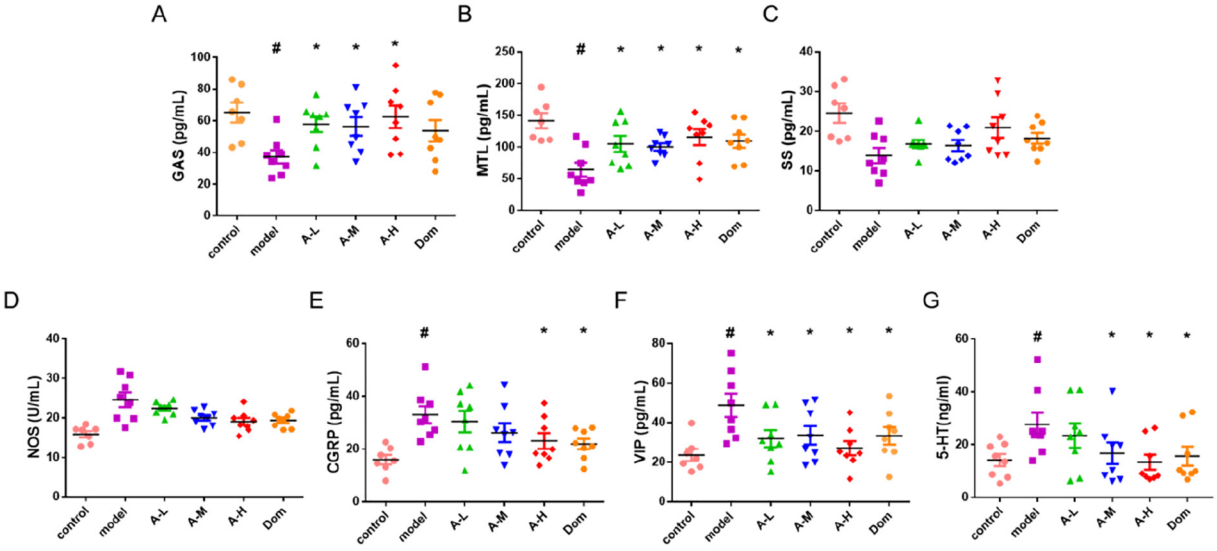

Fig. 3. MA modulates the secretion of brain-gut peptides of FD rats. The levels of gastrin (GAS) (A), motilin (MTL) (B), somatostatin (SS) (C), nitric oxide synthase (NOS) (D), calcitonin gene related protein (CGRP) (E), and vasoactive intestinal peptide (VIP) (F) in serum and 5-hydroxytryptamine (5-HT) (G) in colon are shown. The data are presented as the means ± SEM. One-way ANOVA followed by LSD post hoc test for multiple comparison of GAS, MTL, CGRP, VIP and 5-HT. As for SS and NOS, there is significant variance inhomogeneity. Control group (n = 7): 0.2 mL of 2% sucrose daily for 6 days by oral gavages from 10 days old. Model group (n = 8): 0.2 mL of 0.1% IA in 2% sucrose daily for 6 days by oral gavages from 10 days old, mature ADF for 2 weeks from week 6 to week 8, and given saline by oral gavages (8 mL·kg^{-1}, per day) for 3 weeks. MA treated groups: 0.2 mL of 0.1% IA in 2% sucrose daily for 6 days by oral gavages from 10 days old, mature ADF for 2 weeks from week 6 to week 8, and given MA by oral gavages (5, 10 and 20 mg·kg^{-1}, per day) for 3 weeks, named A-L (n = 8), A-M (n = 8), and A-H group (n = 8), respectively. Dom group (n = 8): 0.2 mL of 0.1% IA in 2% sucrose daily for 6 days by oral gavages from 10 days old, mature ADF for 2 weeks from week 6 to week 8, and given domperidone by oral gavages (3 mg·kg^{-1}, per day) for 3 weeks. $^{\#}P < 0.05$ versus the control group, $^{*}P < 0.05$ versus the model group.

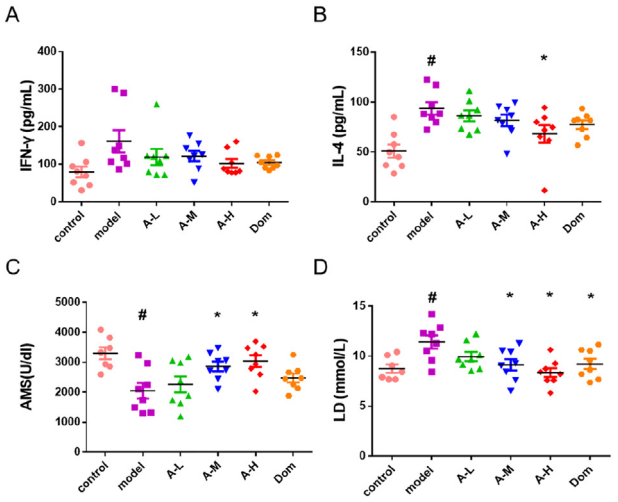

Fig. 4. MA attenuates inflammatory responses of FD rats. The levels of interferon-γ (IFN-γ) (A) and interleukin 4 (IL-4) (B) in spleen; amylase (AMS) (C) and lactic acid (LD) (D) in serum were shown. The data are presented as the means ± SEM. One-way ANOVA followed by LSD post hoc test for multiple comparisons of IL-4, LD and AMS. Control group (n = 7): 0.2 mL of 2% sucrose daily for 6 days by oral gavages from 10 days old. Model group (n = 8): 0.2 mL of 0.1% IA in 2% sucrose daily for 6 days by oral gavages from 10 days old, mature ADF for 2 weeks from week 6 to week 8, and given saline by oral gavages (8 mL·kg^{-1}, per day) for 3 weeks. MA treated groups: 0.2 mL of 0.1% IA in 2% sucrose daily for 6 days by oral gavages from 10 days old, mature ADF for 2 weeks from week 6 to week 8, and given MA by oral gavages (5, 10 and 20 mg·kg^{-1}, per day) for 3 weeks, named A-L (n = 8), A-M (n = 8), and A-H group (n = 8), respectively. Dom group (n = 8): 0.2 mL of 0.1% IA in 2% sucrose daily for 6 days by oral gavages from 10 days old, mature ADF for 2 weeks from week 6 to week 8, and given domperidone by oral gavages (3 mg·kg^{-1}, per day) for 3 weeks. $^{\#}P < 0.05$ versus the control group, $^{*}P < 0.05$ versus the model group.

microbiota-host interactions are closely linked to human health, and the imbalance in the structural and functional properties of gut microbiota may be a driving force for systemic inflammation and has been shown to be related to some diseases [42,43]. In our study, as shown in Fig. 5A, *Firmicutes* and *Bacteroidetes* are the most prevalent phyla, > 90%, in all the tested rats, as other literature reported [43]. ACE was used herein to indicate the richness of gut microbiota, and the foundation considering the decreased ACE in FD rats and the increased ACE in MA-treated groups suggested that MA can regulate gut dybiosis induced by IA and ADF (Fig. 5B). To be specific, 11 differential microbes were identified from thousands of microbes, with LDA Score bigger than 3.0, by LEfSe. The relative abundances of *Pseudomonas*, *Subdoligranulum* and *Akkermansia* showed higher only in MA groups, especially in A-M group, compared with others (Fig. 6A). As shown in Fig. 6B, the relative abundances of *Christensenellaceae_R-7_group* and *Ruminococcaceae_UCG-005* were increased in model group versus the control group, and they were also increased in MA administration groups versus the model group. As shown in Fig. 6C, the relative abundances of *Proteobacteria*, *Gammaproteobacteria*, *Alteromonadales* and *Ruminococcus_2* were reduced in model group versus the control group, while they were found increased in MA and Dom groups. As shown in Fig. 6D, the relative abundances of *Prevotellaceae_NK3B31_group* and *Lachnospiraceae_NK4A136_group* were increased in model group versus control one, but reduced by both MA and Dom.

Further, the correlations between 9 differential microbes (shown in Fig. 6A, C, D) with BGPs and inflammation factors were analyzed by Spearman Correlation in SPSS (Fig. 7). A significant positive correlation was found between *Alteromonadales* and GAS, while significant inverse correlations were found between *Akkermansia* and SS, *Prevotellaceae_NK3B31_group* and 5-HT. Taken together, MA made some alterations to gut microbiota of FD rats.

The functional profiles (KEGG) of the microbial communities were predicted by Tax4Fun R package [26], and group differences in microbial functions were mainly related to amino acid metabolism and carbohydrate metabolism. To be specific, seven pathways were found to be related with amino acid metabolism, degradation and biosynthesis, in which the pathways of *glycine, serine, and threonine metabolism*, *tryptophan metabolism*, *phenylalanine metabolism*, *valine, leucine, and isoleucine degradation*, and *lysine degradation* were significantly decreased in model group compared with control group, and they were significantly increased by MA versus model group. While, the pathways of *phenylalanine, tyrosine, and tryptophan biosynthesis* and *valine, leucine, and isoleucine biosynthesis* were significantly increased in model group compared with control group, then they were decreased by MA administration. Among 6 pathways related with carbohydrate metabolism, the pathways of *starch and sucrose metabolism* and *pentose and glucuronate interconversions* were significantly increased in model group compared with control group, and they were significantly decreased by MA, especially by A-M, compared with model group. While, the pathways of *butanoate metabolism, propanoate metabolism, citrate cycle*, and *glyoxylate and dicarboxylate metabolism* were significantly decreased in model group compared with control group, then they were increased through MA, especially A-M administration.

3.5. MA affected on SCFAs of FD rats

As we all know, SCFAs, as the major metabolites of intestinal floras, play an important role in the maintenance of health and the development of disease [44]. The concentrations of SCFAs (including acetic acid (AA), propanoic acid (PA), butyric acid (BA), valeric acid (VA), hexanoic acid (HXA), isobutyric acid (IBA), isovaleric acid (IVA), and heptanoic acid (HPA)) were detected in feces of rats by GC/MS.

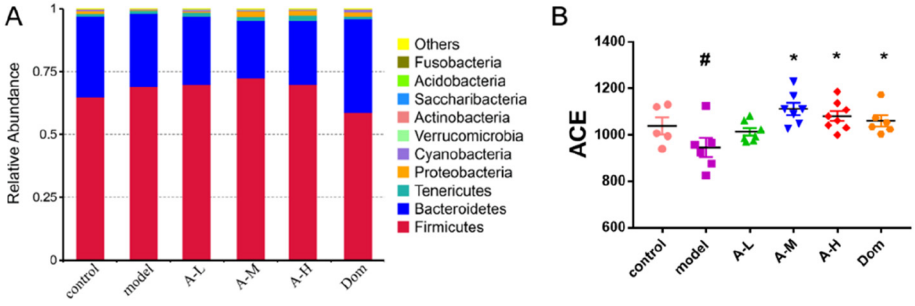

Fig. 5. MA makes alterations to gut microbiota of FD rats. (A) Average phylum distribution of gut microbiota in tested groups. (B) The ACE levels of gut microbiota. The data are presented as the means ± SEM. One-way ANOVA followed by LSD post hoc test for multiple comparisons. Control group (n = 5): 0.2 mL of 2% sucrose daily for 6 days by oral gavages from 10 days old. Model group (n = 6): 0.2 mL of 0.1% IA in 2% sucrose daily for 6 days by oral gavages from 10 days old, mature ADF for 2 weeks from week 6 to week 8, and given saline by oral gavages (8 mL·kg^{-1}, per day) for 3 weeks. MA treated groups: 0.2 mL of 0.1% IA in 2% sucrose daily for 6 days by oral gavages from 10 days old, mature ADF for 2 weeks from week 6 to week 8, and given MA by oral gavages (5, 10 and 20 mg·kg^{-1}, per day) for 3 weeks, named A-L (n = 7), A-M (n = 7), and A-H group (n = 8), respectively. Dom group (n = 8): 0.2 mL of 0.1% IA in 2% sucrose daily for 6 days by oral gavages from 10 days old, mature ADF for 2 weeks from week 6 to week 8, and given domperidone by oral gavages (3 mg·kg^{-1}, per day) for 3 weeks. $^{\#}P < 0.05$ versus the control group, $^{*}P < 0.05$ versus the model group.

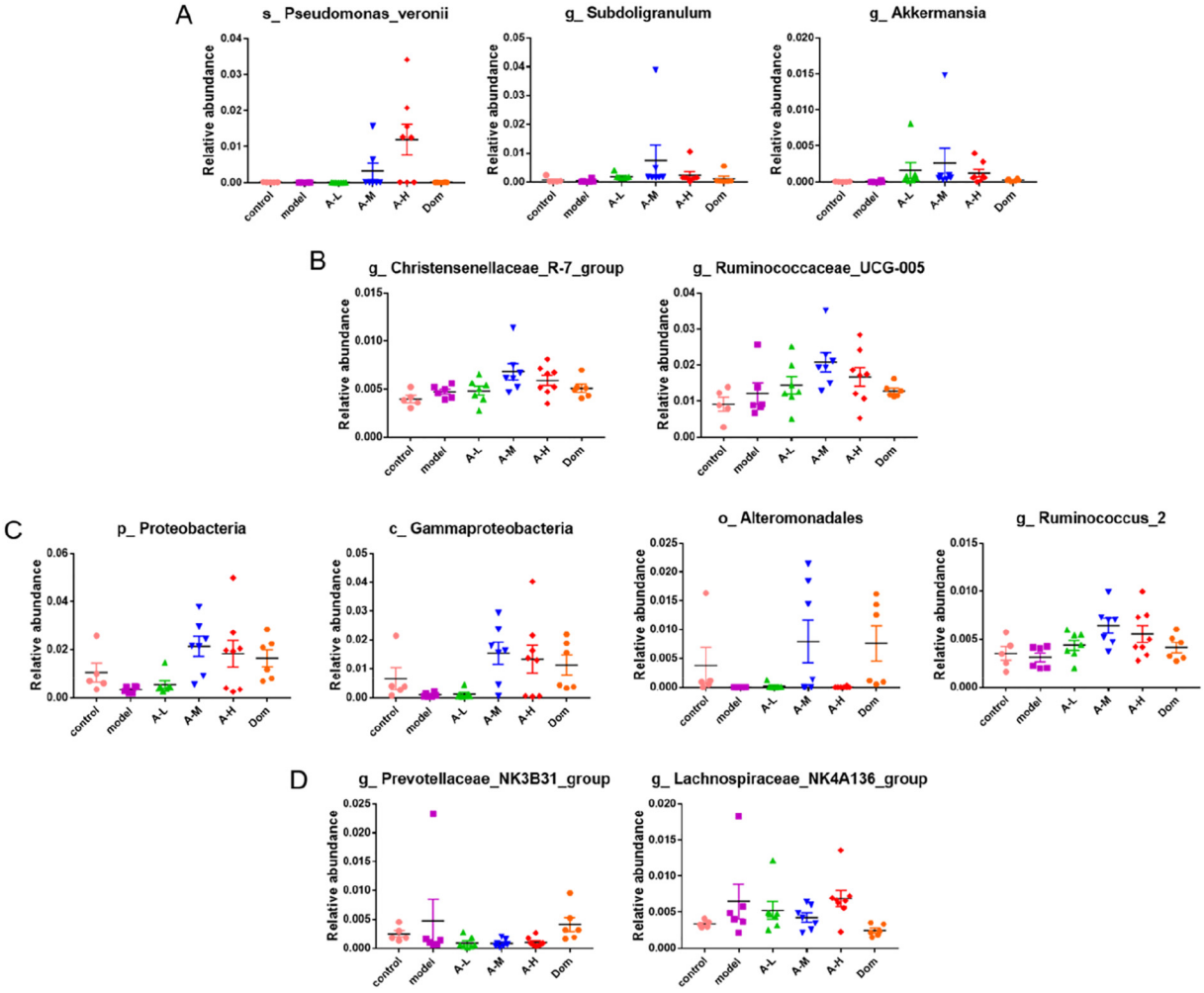

Fig. 6. Differential microbes existed among control, model, MA-treated, and Dom-treated rats. (A) Three microbes increased only by MA in FD rats. (B) Two microbes increased in model, MA, and Dom group. (C) Four microbes reduced in model group but increased by MA and Dom. (D) Two microbes increased in model group but reduced by MA and Dom. "p_" stands for phylum, "c_" stands for class, "o_" stands for order, "g_" stands for genus; "s_" stands for species. The data were presented as the means ± SEM and analyzed by LEfSe. Control group (n = 5): 0.2 mL of 2% sucrose daily for 6 days by oral gavages from 10 days old. Model group (n = 6): 0.2 mL of 0.1% IA in 2% sucrose daily for 6 days by oral gavages from 10 days old, mature ADF for 2 weeks from week 6 to week 8, and given saline by oral gavages (8 mL·kg^{-1}, per day) for 3 weeks. MA treated groups: 0.2 mL of 0.1% IA in 2% sucrose daily for 6 days by oral gavages from 10 days old, mature ADF for 2 weeks from week 6 to week 8, and given MA by oral gavages (5, 10 and 20 mg·kg^{-1}, per day) for 3 weeks, named A-L (n = 7), A-M (n = 7), and A-H group (n = 8), respectively. Dom group (n = 8): 0.2 mL of 0.1% IA in 2% sucrose daily for 6 days by oral gavages from 10 days old, mature ADF for 2 weeks from week 6 to week 8, and given domperidone by oral gavages (3 mg·kg^{-1}, per day) for 3 weeks. $^{\#}P < 0.05$ versus the control group, $^{*}P < 0.05$ versus the model group.

Compared with control group, the concentrations of total SCFAs and two major SCFAs (AA and PA) were all increased in model group, and they were decreased after treated by MA, especially in A-M and A-H groups, compared with model group. It's interesting to note that A-M group looks to readjust the levels of total SCFAs, AA and PA to normal one better than A-L group (Fig. 8).

The concentrations of SCFAs were correlated with 9 differential microbes (shown in Fig. 6A, C, D) and also with the levels of BGPs, IFN-γ, IL-4, AMS, and LD by Spearman Correlation in SPSS (Fig. 9). Briefly, significant inverse correlation was found between *Subdoligranulum* and both of VA and HXA; between *proteobacteria* and both of HXA and IVA; between *gammaproteobacteria* and both of HXA and IVA; between *alteromonadales* and HPA. While, significant positive correlation was found between *lachnospiraceae* and HPA, between *pseudomonas_veronii* and total SCFAs (Fig. 7). On the other hand, GAS was found to be significantly correlated with AA and PA in a positive way; while, correlated with HXA, IVA and HPA in a negative way. All of the SCFAs but AA and PA had positive correlation with VIP, especially in a significant way for BA-VIP, HEA-VIP and HPA-VIP. In addition, AA, PA and total SCFAs were significantly correlated with 5-HT in a negative way, and only PA was significantly correlated with CGRP in a negative way.

4. Discussion

Houpo, a main ingredient in > 200 types of Chinese formulae commonly used in clinics, is a widely used TCM for symptomatic treatment of FD. In previous study, we focused on the study of water soluble compounds and isolated amounts of PhGs, including newly found PhGs. Although amounts of literatures reported diversity pharmacological activities of PhGs isolated from other medicinal plants [45], little was considering on PhGs in Houpo, which contained an allopyranose moiety rarely in the plant kingdom. Also in our previous study, the aqueous decoction of Houpo improved the gastrointestinal motility dysfunction of experimental animals [46], and MA showed inhibition against acetylcholine induced isolated colon contraction [13]. The purpose of present study focused on two aspects, 1) if MA is beneficial to FD, 2) if the answer is positive, then how it works? In order to answer the two questions, we isolated and purified MA from Houpo and investigated its effect on FD rats induced by IA and ADF. The ability of A-L and A-H significantly fastening the delayed gastric empty, of FD

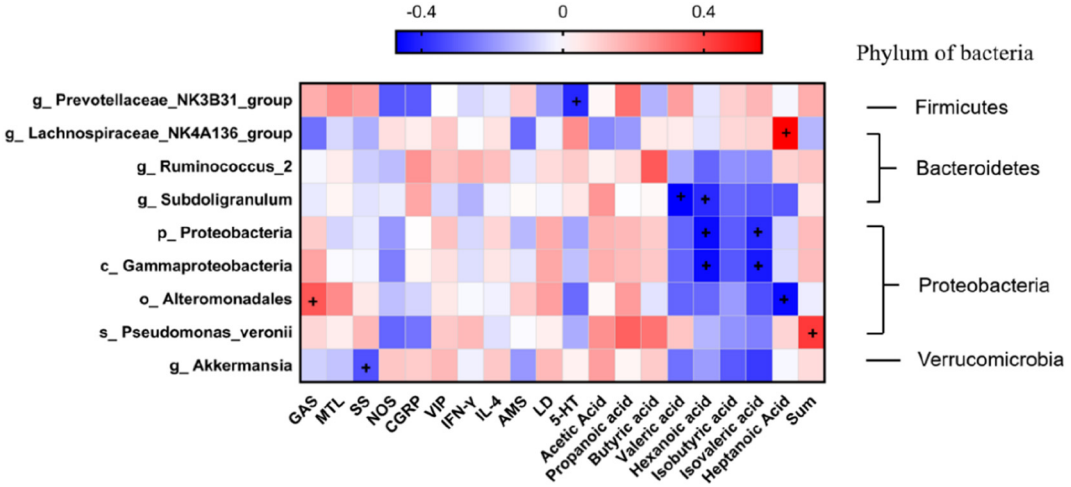

Fig. 7. Spearman correlations of 11 differential microbes with brain-gut peptides (BGPs), short chain fatty acids (SCFAs), and other biochemical factors. The R value is shown as a heat map, with blue cell indicating inverse correlation and red cell indicating positive correlation. +, $P < 0.05$ by Spearman's correlation. The names of microbes at phylum level are labeled to the right. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

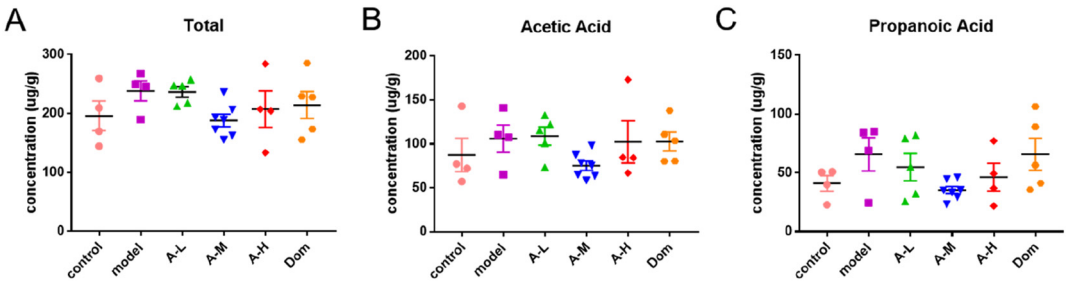

Fig. 8. MA affected on short chain fatty acids (SCFAs) of FD rats. (A) The concentration of total SCFAs. (B) The concentration of acetic acid (AA). (C) the concentration of (propanoic acid) PA. The data are presented as the means ± SEM. Control group ($n = 4$): 0.2 mL of 2% sucrose daily for 6 days by oral gavages from 10 days old. Model group (n = 4): 0.2 mL of 0.1% IA in 2% sucrose daily for 6 days by oral gavages from 10 days old, mature ADF for 2 weeks from week 6 to week 8, and given saline by oral gavages (8 mL·kg^{-1}, per day) for 3 weeks. MA treated groups: 0.2 mL of 0.1% IA in 2% sucrose daily for 6 days by oral gavages from 10 days old, mature ADF for 2 weeks from week 6 to week 8, and given MA by oral gavages (5, 10 and 20 mg·kg^{-1}, per day) for 3 weeks, named A-L (n = 5), A-M (n = 7), and A-H group (n = 4), respectively. Dom group (n = 5): 0.2 mL of 0.1% IA in 2% sucrose daily for 6 days by oral gavages from 10 days old, mature ADF for 2 weeks from week 6 to week 8, and given domperidone by oral gavages (3 mg·kg^{-1}, per day) for 3 weeks. $^{\#}P < 0.05$ versus the control group, $^{*}P < 0.05$ versus the model group.

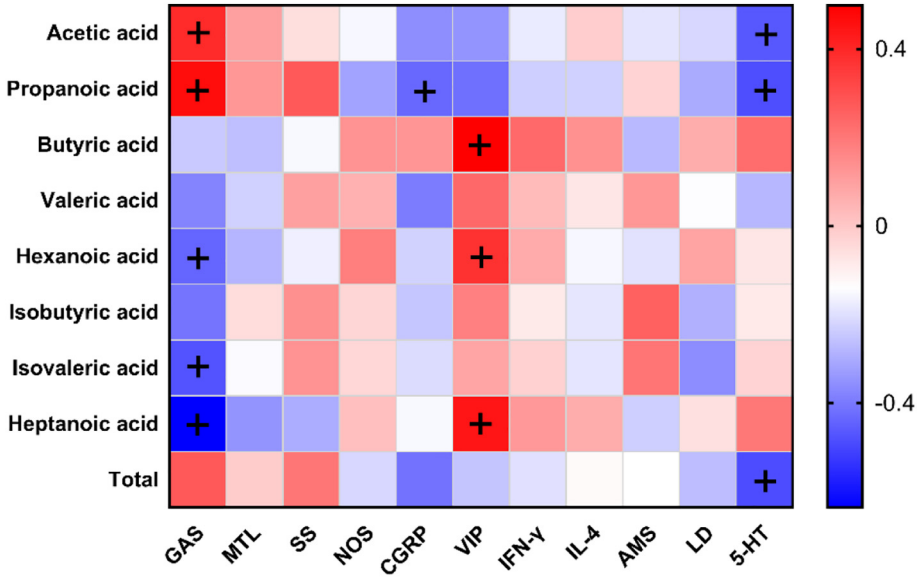

Fig. 9. Spearman correlations of short chain fatty acids (SCFAs) with brain-gut peptides (BGPs), interferon-γ (IFN-γ), interleukin 4 (IL-4), amylase (AMS) and lactic acid (LD). The R value is shown as a heat map, with blue cell indicating inverse correlation and red cell indicating positive correlation. +, $P < 0.05$ by Spearman's correlation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

rats showed the potency of MA in FD treatment, although no dose-dependence relationship was observed, and A-M showed weaker ability to fasten the delayed gastric empty than A-L and A-H, possibly because of the individual differences of the tested rats.

The pathophysiology of FD is likely complex and multifactorial, and not completely elucidated. Gastroduodenal motor and sensory dysfunction, as well as impaired mucosal integrity, low-grade immune activation, and dysregulation of the gut-brain axis have all been implicated [1]. Accumulated information indicated the changes of BGPs played an important role in gastroduodenal motor dysfunction. Among the involved BGPs, GAS and MTL were primarily responsible for enhancing gastric mucosal growth and gastric motility, etc. [34,35]. VIP, one important BGP released from the ENS to many gastrointestinal tract regions, has been shown to directly stimulate the production of NO by increasing NOS activity, and regulate the gastrointestinal motility by relaxing the smooth muscle [47]. Up-regulation of 5-HT transporter level in the midbrain and thalamus may underlie the pathogenesis of FD such as abdominal and psychological symptoms via a brain-gut interaction. In our study, MA increased the levels of GAS and MTL and decreased the levels of VIP, NOS, and 5-HT, indicating that MA can improve the symptom of FD rats by regulating the secretion of BGPs to adjust gastric motor dysfunction. Visceral hypersensitivity is also considered as an important phenomenon that can clarify many unexplained gastrointestinal symptoms. In FD significantly negative correlations between discomfort and pain thresholds and antral mucosal concentrations of CGRP was observed, suggesting that sensory neuropeptides are involved in FD pathophysiology. In our study, MA decreased the level of CGRP, which may result in the decrease of visceral sensitivity of FD rats. In conclusion, the changes of BGPs levels induced by IA and ADF can be improved by MA.

Gut microbiome could affect host metabolism through providing energy, nutrients, and immunological protection, resulting from degrading non-enzymatically digestible foods, and synthesis of metabolites, such as SCFAs, amino acids, etc. In recent years, a large number of studies have shown that herbal medicines including single compounds, single herbals and herbal formulas, are capable of reversing the abnormal gut microbiota composition in diseased human cohorts and model animals [48]. Here, MA-treated alteration of gut microbiota and its correlation with amelioration of FD rats were studied for the first time.

Under IA and ADF treatment, disturbances of the gut microbes lead to the decrease of community richness and the increase in microbial richness was found in MA-treated FD rats. It needs to mention that A-M resulted in not only the richest gut microbiome but also the relative abundances of some microbiomes, such as *Subdoligranulum* and *Akkermansia*, etc., among the three doses of MA. Compared to A-M, A-H seems to be a saturation level, a possible explanation was that like some PhGs reported in [45], MA may have anti-bacterial effect, so high level of MA will reduce the richness and abundance of some microbiomes. Further, the association or causation relationship between the changed gut microbiota and the improvement of FD rat were studied. First, the establishing gut microbiota partially contributed to the secretion of BGPs, for example, the significant positive correlation founded between *Alteromonadales* and GAS indicated the high level of *Alteromonadales* in MA treated group contributed or partially contributed to GAS production, which was benefit to the improvement of FD rat. Second, the changed gut microbiota was found to have positive relation with AA, PA and BA, and negative relation with other four SCFAs. Particularly, *Proteobacteria* showed significant negative relation with HXA and IVA, which enriched our knowledge on gut microbiota and its metabolites, besides the common consideration that acetate and propionate are mainly produced by *Bacteroidetes* whereas *Firmicutes* are the primary contributors of butyrate [49]. Third, most SCFAs showed relationship (significant or non-significant) with BGPs either in a positive or negative way. Worth notice is that although SCFAs are the main metabolites of gut microbiota, SCFAs can also directly impact gut microbiota composition. Being similar to the report that high concentrations of SCFAs could exhibit toxic effects on some gut microbiota species [50], the increased contents of SCFAs in FD models may contribute to the decreased richness of gut microbiota in our study. In addition, although significant inverse correlations were found between *Prevotellaceae_NK3B31_group* and 5-HT, *Akkermansia* and SS, no reasonable explanation could be given possibly due to the complex of gut microbiota and also the fact that multiple parameters involved in the secretion of SS and 5-HT.

Considering dose response, A-H is suggested to be more active than either A-L or A-M on adjusting the changes of BGPs, inflammatory factors and other factors of FD rats induced by IA and ADF, according to the following clues: (1) the lowest levels of NOS, CGRP and 5-HT were observed in A-H group with proportional dose-dependence relationship, (2) the highest levels of GAS, MTL, and SS, and the lowest level of VIP were found in A-H group, although no dose-dependence relationships were observed, (3) the lowest levels of IL-4, MAS (with dose-dependence relationship) and INF-γ (with no dose-dependence relationship) and the highest level of LD (with dose-dependence relationship) were found in A-H group. All of which improved the treatment of FD rats. While, A-M looks to make more alteration to gut microbiota than either A-L or A-H, and on SCFAs also, a similar phenomenon was reported in Yan's study, in which low dose of total saponins from *Polygonatum kingianum* could decrease total SCFAs, AA, PA, and BA, whereas high dose could increase total SCFAs and PA [51]. The multi-relationships among herbal medicine, gut microbiota, and SCFAs are rather complicated as mentioned in [48], in which herbal medicine is thought to be an important resource provider for generation of SCFAs, it also can intervene the generation of SCFAs by modulation of the composition of gut microbiota and the bioactivity of enzymes which catalyze the production of SCFAs. Moreover, some herbal medicine can produce SCFAs but not gut microbiota, and SCFAs can also impact gut microbiota composition [48]. Therefore, further steps are needed to reach a valid conclusion on the dose response effect of MA, and also on the toxic of MA, no report being found till now.

In conclusion, we isolated and purified MA from Houpo and investigated its effect on FD rats induced by IA and ADF, and demonstrated that MA treatment ameliorated FD rats, mainly through (1) direct or indirect modulation of the secretion of related BGPs, beneficial to the gastric motor and visceral hypersensitivity, (2) partial modulation of intestinal microbiota composition, resulting in the secretion of BGPs and SCFAs. This is the first time to study the effect of MA on FD and its potential mechanism.

Author contributions

Z.X. isolated MA, performed the experiments, and prepared the manuscript. C.W. analyzed the data and prepared the manuscript. J.W. designed the study and prepared the experiments. M.X. and T.W. performed the overall experiments. M.C. prepared the manuscript. B.Y. designed the study and prepared the manuscript.

Declaration of competing interest

The authors declare that there are no conflicts of interests.

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