ORIGINAL RESEARCH

Effects of Qishen Dihuang Granules on Intestinal Microbiota in Experimental Autoimmune Myasthenia Gravis Model Rats

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ABSTRACT

Objective • Effects of Qishen Dihuang (QSDH) granules on intestinal flora of an experimental autoimmune myasthenia gravis (EAMG) model rat were investigated (CNBI:PRJNA910532).

Methods • Thirty-six female Lewis rats were assigned to Control, EAMG, QSDH-low-dose, QSDH-medium-dose, QSDH-high-dose, and Prednisone groups using the random number table method (6 rats/group). A rat EAMG model was established by injecting Rα97-116 peptide antigen. Each day for 30 days, gavages were administered to rats in the Chinese medicine group (QSDH granules in different concentrations), Prednisone group (prednisone), and Control and Model groups (0.5% CMC). After 30-day gavages, rat fecal samples were collected and the microbial community composition and diversity differences between intestinal microbiota of EAMG and QSDH granule-treated groups were analyzed using 16S amplicon sequencing to explore the effect underlying QSDH granules alleviation of EAMG.

Results • The clinical symptoms of rats in each treatment group improved significantly after the intervention treatment with QSDH granules. Comparison of the relative abundance of microorganisms in the gut flora of different groups with that of the EAMG group rats revealed: significantly lower phylum-level Bacteroidetes abundance and significantly greater Actinobacteria abundance in the QSDH-high-dose group and a significantly greater Firmicutes/Bacteroidetes ratio in the QSDH-medium-dose group; significantly increased family-level QSDH-high-dose group abundances of Lachnospiraceae and Trichospiraceae (Firmicutes), significantly increased QSDH-medium-dose group Lactobacillaceae abundance, and significantly increased QSDH-low-dose group Bacteroidaceae abundance; genus-level, QSDH-high-dose group *Prevotella* and *Coprococcus* abundances were significantly increased and *Turicibacter* and *Lactobacillus* abundances were significantly decreased, while QSDH-medium-dose group *Akkermansia* and *Lactobacillus* abundances were significantly increased. Greater overall community richness, diversity, and genetic diversity were observed in QSDH granules-treated groups, but differences were insignificant ($P > .05$). The most significant inter-group genus-level community marker differences involved *Prevotella, Ruminococcus, Coprococcus*, and *Turicibacter*.

Conclusion • QSDH granules may regulate EAMG rat intestinal flora by decreasing relative abundances of *Turicibacter* and *Clostridium* and increasing relative abundances of *Bifidobacterium,* Lachnospiraceae, and *Prevotella*. (*Altern Ther Health Med.* 2023;29(5):342-352).

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INTRODUCTION

Myasthenia gravis (MG) is a rare neurological disorder associated with neuromuscular junction (NMJ) transmission impairment, due to the production of pathogenic acetylcholine receptor-binding autoantibodies (AChR-Abs). AChR-Abs

disrupt AChR function by blocking AChR aggregation, thereby interfering with signal transmission from nerve cells to muscle cells within the neuromuscular junctions. In turn, blocked neuromuscular signal transmission causes typical MG symptoms. It is characterized by lighter symptoms in the morning and heavier symptoms in the evening and is aggravated by activity and can be alleviated by rest.[1] MG global prevalence is (150-250)/million, the estimated annual incidence is (4-10)/million.[1] MG is rare in China, with results of a large study demonstrating MG incidence and prevalence rates there of 1.55/100 000 and 3.66/100 000, respectively.[2] MG peak incidence rates are associated with young adult women and older men,[3] who thereafter are forced to live with this chronic, refractory autoimmune disease and its serious effects on their health and quality of life. Currently, western medical treatment of MG largely relies on the administration of cholinesterase inhibitors, glucocorticoids, and immunosuppressants.[1] However, newer targeted biological therapies, such as intravenous immunoglobulin, plasma replacement, and thymectomy, are being administered in clinical practice with gradually increasing frequency in spite of their high costs and side effects[4] and high relapse and exacerbation rates.[5-7] Meanwhile, in recent years several Chinese medicinal preparations have been shown to be more effective than Western medicines in alleviating MG symptoms.[8,9] Nonetheless, the development of effective treatments for MG has been hampered by a lack of understanding of specific factors underlying MG susceptibility, although results of recent clinical studies suggest that intestinal flora plays a key role in MG pathogenesis.[10]

Studies have shown that a large number of symbiotic microorganisms that reside in the human intestine provide health benefits. The major component of the gut flora are bacteria that maintain health by regulating immune and metabolic system functions.[11-13] Mechanistically, intestinal flora secretes both anti-inflammatory and inflammatory factors and activates host cells that maintain immune system equilibrium and self-tolerance, while protecting the host from foreign bacterial invasion. Nevertheless, when the gut microbiota is altered due to the effects of various factors, immune system imbalances may occur that can trigger autoimmune disease development.[14] The results of previous studies have revealed intestinal flora imbalances in MG patients[15] and in experimental autoimmune myasthenia gravis (EAMG) model rats.[16] Other studies have showed that Foxp3+ CD4+ Treg cells are the most prominent regulatory cells in the body. The frequency of Foxp3+ CD4+ Treg cells is notably higher in colonic lamina propria than in any other organ.[17] Furthermore, the Foxp3+ CD4+Treg cells in intestinal lamina propria have unique characteristics, which are markedly affected by the composition of gut microbiota.[18] These data provide new insights indicating that Foxp3+ CD4+Treg cells in the intestine maintain homeostasis of the gut microbiota. Indeed, the gut microbiota could affect the number and T-cell receptor (TCR) repertoire of Foxp3+ CD4+ Treg cells. The TCRs on Foxp3+ CD4+ Treg cells can recognize subsets of commensal bacteria, inducing naive CD4+ T cells to differentiate into antigen-specific Foxp3+ CD4+Treg cells, leading to an increased amount of Foxp3+ CD4+ Treg cells.[19] Remarkably, Foxp3+ CD4+ Treg cells is significantly deficient in MG patients and has become the major focus of interpreting the pathogenesis of MG. Therefore, we hypothesized that perturbations in the composition of gut microbial communities may be associated with intestinal bacteria-induced Foxp3+ CD4+Treg cell deficiency. Modifying the intestinal microbiome could be crucial for the design of therapeutic interventions toward MG.

In recent years, herbal medicines have been found to alleviate MG symptoms.[20] Traditional Chinese Medicine considers that the pathogenesis of this disease is a "deficiency of the spleen and kidney and deficiency of the brain marrow". Based on this premise, MG treatment should be administered according to the basic principle of "strengthening the spleen, benefiting the qi and nourishing the brain marrow". Qishen Dihuang granules (QSDH granules) are an innovative herbal compound based on this theory containing Astragalus, *Codonopsis pilosula*, Atractylodes, Rehmanniae Radix, *Rhizoma cibotii*, *Angelica sinensis*, etc. (see Table 1). This formulation consists of 17 traditional Chinese herbs that exert warming effects on the spleen and kidney. In fact, results of a previously reported clinical study indicated that the overall patient-reported effectiveness rate of QSDH granules in alleviating MG symptoms approached 90% after 6-month treatment, while the total QSDH effective rate, as based on traditional Chinese medicine (TCM) symptom scores, approached 96.70%. Taken together, these results indicate that QSDH granules predictably relieved muscle fatigue and improved MG symptoms. Meanwhile, results of

Table 1. Components of the QSDH Granules

	Chinese name	Latin name	Family	Part used
1	Huang qi	Astragalus membranaceus	Leguminosae	Root and stem
2	Dang shen	Codonopsis pilosula Nannf.	Campanulaceae	Root
3	Bai zhu	Atractylodes macrocephala Koidz.	Compositae	Rhizome
4	Sheng di	Rehmannia glutinosa Libosch.	Scrophulariaceae	Root
5	Gou ji	Cibotium barometz J. Sm.	Diclsoniaceae	Rhizome
6	Gou qi zi	Lycium barbarum L.	Solanaceae	Fruit
7	Ba ji tian	Morinda officinalis How	Rubiaceae	Root
8	Huang jing	Polygonatum kingianum Coll. et Hemsl.	Liliaceae	Rhizome
9	Shan yao	Dioscorea opposite Thunb.	Dioscoreaceae	Rhizome
10	Zhi gan cao	Glycyrrhiza uralensis Fisch.	Leguminosae	Rhizome
11	Dang gui	Angelica sinensis Diels	Umbelliferae	Root
12	Di long		Megascolecidae	Whole animal
13	Gou teng	Uncaria rhyunchophylla Jacks.	Rubiaceae	Rhizome
14	Chen pi	Citrus reticulata Blanco	Ruraceae	Pericarp
15	Chai hu	Bupleurum chinense DC.	Umbelliferae	Root
16	Sheng ma	Cimicifuga heracleifolia Kom.	Ranunculaceae	Rhizome
17	Ge gen	Pueratia lobata Ohwi	Leguminosae	Root

our previous study[21] revealed that T lymphocyte subpopulation differentiation in EAMG model group rats appeared to differ from that of healthy control group rats, with CD4+ T cell populations and subpopulations in EAMG model rats exhibiting significantly greater tendencies to differentiate into Th1 and Th17 cells instead of Th2 and Treg cells. Intriguingly, QSDH treatment of EAMG rats restored a state of T lymphocyte immune balance among Th1/Th2/Th17/Treg cell populations to inhibit EAMG development. Moreover, our team investigated not only the T-cell-mediated immune mechanism of QSDH granules in EAMG model rats but also the B-cell-mediated immune mechanism.[22] At the same time, a comprehensive quality control system was established[23]; liquid mass spectrometry, chemical fingerprinting, HPLC-ELSD multi-component content determination, and other quality control methods were used to quickly and intuitively determine the material basis of the pharmacological effects of QSDH granules (Jianpi Yiqi Busui prescription), which provided a scientific basis for the study of the key quality attributes of QSDH granules and ensured the stability and reliability of its clinical efficacy. In this study, our results suggested that QSDH granules regulate intestinal flora composition, prompting our team to investigate the potential mechanism underlying the QSDH granules-mediated alleviation of MG symptoms using an EAMG rat model. Using this model, the intestinal flora composition of the EAMG group was compared to the gut flora compositions of QSDH-low-dose, QSDH-medium-dose, and QSDH-high-dose groups. The results of this study should provide a theoretical and practical foundation on which QSDH granules-based MG treatments can be developed along with the methods for their administration under clinical settings.

MATERIALS AND METHODS

Experimental Animals

A total of 36 SPF female Lewis rats aged 6-8 weeks with weights of 160-180 g/rat were provided by Beijing Weitong Lihua Laboratory Animal Technology Co., LTD. (License No.: SCXK (Beijing) 2016-0006; Certificate of Compliance No.: NO.110011210109257156). Animals had free access to pellet feed and sterile water and were maintained under specific pathogen-free (SPF) conditions (constant room temperature of 24 ± 2°C, relative humidity of 40%-70%, alternating 12-h light/ 12-h dark cycle); rats were exposed to these conditions for 1 week prior to use in experiments. The animal experiment protocol was approved by the Animal Experiment Ethics Committee of Changchun University of Traditional Chinese Medicine (approval number 2021226) and was strictly followed to comply with animal protection, animal welfare, ethical principles, and relevant regulations of the National Experimental Animal Welfare Ethics Committee.

Experimental Reagent

(1) Rat-derived AChRα subunit 97-116 peptide (production lot number 04010001391) was purchased from Suzhou QiangYao Biotechnology Co. (2) Complete Freund's adjuvant (CFA, production lot number F5881-10ML) and incomplete Freund's adjuvant (IFA, production lot number F5506-10) were purchased from Sigma-Aldrich, USA. (3) *Mycobacterium tuberculosis* H37RA (dry powder, production lot number 231141-6) was purchased from Difco Bacto, USA. (4) Phosphate-buffered saline (PBS, production lot number D8537) was purchased from Thermo, USA. (5) Isoflurane (production lot number R510-22-8) was purchased from Shenzhen Rayward Life Science & Technology Co.

Experimental Drugs

The Prednisone (positive medicine) group was administered prednisone tablets (5 mg-100 tablets, production lot number 2103352) that were purchased from Shandong Xinhua Pharmaceutical Co. Control and EAMG (model) groups were administered carboxymethylcellulose Na (CMC, 250 g/bottle, product lot number: 20210604) that was purchased from Tianjin Bailens Biotechnology Co. QSDH-high-dose, QSDH-medium-dose, and QSDH-low-dose groups were administered QSDH granules formulated according to a prescription consisting of 17 TCM herbs: Astragalus, *Codonopsis pilosula,* Atractylodes, Rehmanniae Radix, *Rhizoma cibotii*, *Angelica sinensis*, etc. The QSDH granules formulation was a light-yellow powder with a sweet and bitter taste that was formulated as a 33.9% ointment. QSDH was produced and vacuum packaged (1 kg per bag, production lot number: 210303T) by Jilin Yatai Yongan Tang Pharmaceutical Co., a national GMP-certified company.

Experimental Animal Group Assignments

Using the random number table method, 36 female Lewis rats were assigned to six groups (6 rats/group): Control group, EAMG group (model group), Prednisone group (positive medicine group), QSDH-low-dose group, QSDH-medium-dose group, and QSDH-high-dose group. Dosing of rats was conducted according to the table of equivalent ratios that is used to convert doses of treatments from human to rat doses, based on the rat-specific surface area/human-specific surface area ratio of 6.3 as provided in the book *Pharmacological Laboratory Methodology*.[24] Rats of Control and EAMG groups were each gavaged daily with 15 ml/kg of 0.5% CMC for 30 d. Rats in QSDH-low-dose, QSDH-medium-dose, and QSDH-high-dose groups were each gavaged daily with 4.8 g/kg/d, 9.6 g/kg/d, or 19.2 g/kg/d, respectively, for 30 d. Rats in the Prednisone group were each gavaged daily with 9.6 g/kg/d for 30 d.

Establishment of the Rat EAMG Model

Referring to well-established model-making methods,[21] rat-derived AChRα subunit 97-116 peptide was ultrasonically powdered then added to dried *M. tuberculosis* H37RA powder, and the mixture was resuspended in PBS. Next, the peptide-*M. tuberculosis* immunogen suspension was thoroughly mixed with an equal volume of CFA to form a white, viscous

emulsion, which is the immunogenic emulsion. The second- and third-time immunogen preparations replaced CFA with IFA. For the control group immunogen preparation, no other addition was needed, only CFA was mixed with PBS.

The immunogen preparations (40 μL each, total 200 μL) were injected subcutaneously with a 1 ml sterile syringe at five sites on the foot pads, both sides of the back, and the base of the tail of rats. The first immunization was recorded as day 0, and a booster was given on day 30 and day 45. On day 60 (15 days after the second booster immunization), the successful establishment of the EAMG rat model was confirmed as based on four indicators: body weight, Lennon Score, forelimb grip strength, and peripheral serum AChR-Ab level. Thereafter, model and control rats were assessed using these indicators during the 30-day treatment period, as specified below.

Weight. The body weights of rats were measured every 2 days. In addition, rats were monitored for changes in respiration, feeding, activity, resting posture, hair color, and growth of lower teeth.

Lennon Score. Rats were assessed at 2-day intervals for MG symptoms by an evaluator using the Lennon clinical symptom grading method.[25] Briefly, each rat was manually suspended upside down by grasping the distal end of the tail, which caused the rat to use its forelimbs to grasp the rat cage bars. Thereafter, the rat was dragged backward and upwards until it released its grip on the cage bars then the entire procedure was repeated several times for a total duration of 30 s. Scores were interpreted as follows: 0 points, no weakness in performance; 1 point, mildly reduced activity, grasping and hissing weakness, easily fatigued; 2 points, significantly reduced activity and weight loss, head and tail drooping, forelimbs toes bent, trembling during activity; 3 points, severe weakness performance, no grasping and hissing action, respiratory distress, near death; 4 points, death. If the symptom assessment score fell between two levels, scores of 0.5, 1.5, 2.5, and 3.5 were assigned, as appropriate.

Forelimb Grip Strength Assessment. To avoid subjective bias while assessing Lennon scores, clinical signs and forelimb grip strength were also assessed beginning on day 60 (after the first injection). Briefly, the evaluator lifted the rat's tail with one hand so that the rat's front paws grasped the grip plate then the evaluator gently pulled the rat towards the rear parallel grip plate to trigger the grip force tester to automatically read the maximum force value (g). The average of five consecutive measurements was used to assess rat forelimb grip force value.

Peripheral Serum AChR-Ab Levels. 1 ml of blood was drawn from the tail vein of the rats. After centrifugation, the upper serum layer was collected and the level of AChR-Ab in the serum sample was measured by enzyme-linked immunosorbent assay (ELISA).

Successful EAMG model generation was confirmed based on EAMG group values as compared to corresponding Control group values to reveal significant inter-group differences ($P < .05$) in the following indicators: decreased weight; Lennon Score difference > 1; significantly reduced forelimb grip strength test value; significantly increased peripheral blood AChR-Ab level.

Fecal Collection and DNA Extraction

Treatments were administered by gavage for 30 consecutive days beginning on day 45 after day 0 of model induction. Then stool samples were collected at the end of the experiment (days 75). Fecal samples collected from animals in the same cage were immediately placed in sterile freezing tubes, rapidly frozen in liquid nitrogen, and stored in a -80°C freezer. DNA extractions were performed using a fecal DNA extraction kit (Norgen) then sequencing, sequence processing, and sequence analysis were performed to determine gut flora microbial diversity for each group of rats.

Microbial Diversity Sequencing Method

Extraction of Genomic DNA. Total genomic DNA from samples was extracted using the cetyltrimethylammonium bromide/sodium dodecyl sulfate (CTAB/SDS) method. DNA concentration and purity were monitored via electrophoresis on 1% agarose gels and then the DNA concentration of each sample was adjusted to 1 ng/μl by the addition of sterile water.

Amplicon Generation. Primers included primer sets for amplification of 16S regions V3-V4 (341F/806R) and ITS1 (ITS1F/ITS2R). 16S rRNA genes were amplified using primers with added barcode sequences. Each PCR reaction was carried out in a 30 μL reaction volume containing 15 μL of Phusion® High-Fidelity PCR Master Mix (New England Biolabs), 0.2 μM of forward and reverse primers, and ~10 ng of template DNA.[26] Thermal cycling consisted of initial denaturation at 98°C for 1 min followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 50°C for 30 s, and elongation at 72°C for 60 s and a final cycle at 72°C for 5 min.

PCR Product Quantification and Quality Assessments. After PCR completion, a volume of 1× loading buffer (containing SYB green) was added to an equal volume of a portion of each DNA sample then the mixture was electrophoresed on 2% agarose gels for detection of PCR products.[27] Samples containing PCR products, as identified based on observed bright main bands between 400-450 bp in size on gels, were chosen for subsequent experiments.

Preparation of PCR Product Mixtures and Purification. After adjusting PCR product concentrations to the same concentration, equal volumes of PCR products were combined, then the mixtures of PCR products were purified using an AxyPrepDNA Gel Extraction Kit (AXYGEN).

Library Preparation and Sequencing. Sequencing libraries were generated using a NEBNext® Ultra™ DNA Library Prep Kit for Illumina (NEB, USA) following the manufacturer's instructions using primers with added indexing barcodes. Library quality was assessed using a Qubit@ 2.0 Fluorometer (Thermo Scientific, Waltham, MA, USA) and an Agilent Bioanalyzer 2100 system. The library was sequenced using the Illumina Miseq/HiSeq2500 platform, resulting in the generation of 250-bp to 300-bp paired-end reads.

Gut Microbiota Analysis

Amplicon sequence variant (ASV) clustering and classification analysis methods were used to analyze processed intestinal flora data using SPSS 24.0 statistical software. Alpha diversity index values were determined using QIIME2 (2019.4). Principal coordinates analysis (PCoA) was performed using R analysis based on the Jaccard distance algorithm, with the significance of results assessed based on an elliptical confidence level of 0.95.[28] Differences between groups were analyzed using similarity analysis (Adonis) assessments of results obtained using the Jaccard distance algorithm. LEfSe was used to conduct quantitative analysis of biomarkers within different groups when the data obtained for each group represented a number of species that were much greater than the number of samples.[29] Use of this method provided biological class explanations for use in determining statistical significance, biological consistency, and effect-size estimations of predicted biomarkers. Orthogonal projections to latent structures-discriminant analysis (OPLS-DA) was performed using R analysis with significance of results determined based on an elliptical confidence level of 0.95.

Statistical Analysis

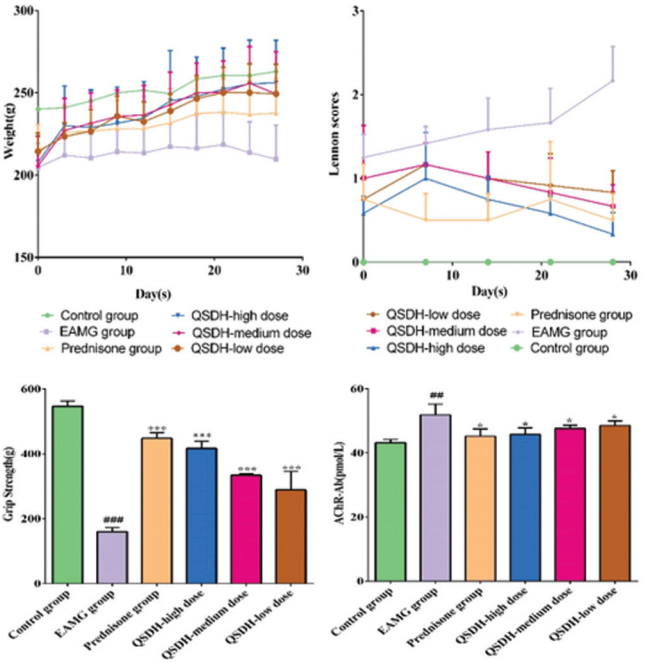
Weights of rats in different groups were expressed as the mean ± standard deviation. One-way analysis of variance (one-way ANOVA) with SPSS 25 software was used to compare differences among multiple groups. Analysis of gut microbiome data was conducted as mentioned above, with $P < .05$ and $q < .05$ considered statistically significant.

RESULTS

Improvement of Model Rat EAMG Symptoms After QSDH Granules Treatment as Assessed Based on Weight, Lennon Score, Forelimb Grip Strength, and AchR-Ab Level

After experimental and control group rats were gavaged with corresponding treatments, the weights of rats were measured and recorded every 2 days for 30 days and then the general condition of rats in each group was noted. In terms of weight changes during treatment, the mean weights of Control group rats were greater than the mean weights of EAMG group rats throughout the 30-day treatment cycle, with inter-group differences reaching statistical significance ($P<.05$). Meanwhile, as compared with the mean weights of EAMG group rats, the mean weights of rats in the three QSDH treatment groups and the Prednisone group gradually increased during the 30-day treatment cycle, with inter-group mean weight differences reaching statistical significance ($P < 0.05$), as shown in Figure 1A. However, no significant inter-group differences in weight gain were observed among the drug treatment groups ($P>.05$). Notably, over time the EAMG group rats exhibited a reduced range of motion and slower movement, loss of coat color and luster, and decreased vocalization strength. By contrast, rats in the three QSDH-treated groups and the Prednisone group exhibited improvement in range of motion and mobility and increased coat color, luster, and vocalization strength as compared to corresponding EAMG group characteristics.

Figure 1. Improvement of EAMG symptoms in each group after 30-day treatment with QSDH granules. (A) Changes in weights of rats in each group after drug administration; (B) Effect of QSDH granules and prednisone on Lennon clinical scores of EAMG group rats; (C) Effect of QSDH granules and prednisone on forelimb grip strengths of EAMG group rats; (D) Effect of QSDH granules or prednisone treatments on serum AChR-Ab levels of EAMG group rats.



Clinical Lennon score tests were performed beginning on the day of the first gavage and every 7 days thereafter. The results showed that EAMG group Lennon scores were higher than Control group scores, with inter-group score differences reaching statistical significance ($P < .05$). Of note, clinical symptoms of EAMG group rats did not improve during the 30-day treatment period, indicating good model stability. Moreover, as compared with Lennon scores of EAMG group rats, Lennon scores of rats in the three QSDH treatment groups and the Prednisone group decreased during the 30-day treatment cycle, with inter-group Lennon score differences reaching statistical significance ($P<.05$), as shown in Figure 1B.

Furthermore, forelimb grip strength of EAMG group rats was always lower than that of the Control group during the 30-day treatment period, with inter-group differences reaching statistical significance ($P < .05$). Meanwhile, as compared with the forelimb grip strength of EAMG group rats, the forelimb grip strengths of rats in the three QSDH treatment groups and the Prednisone group showed significant improvement during the 30-day treatment period, with inter-group forelimb grip strength differences reaching statistical significance ($P<.05$), as shown in Figure 1C.

At the end of the treatment period, rats were anesthetized using a gas anesthesia apparatus, blood was collected via the abdominal aorta, serum was isolated by centrifugation, then peripheral blood serum AChR-Ab level was measured by

Figure 2. Alpha-Diversity Analysis

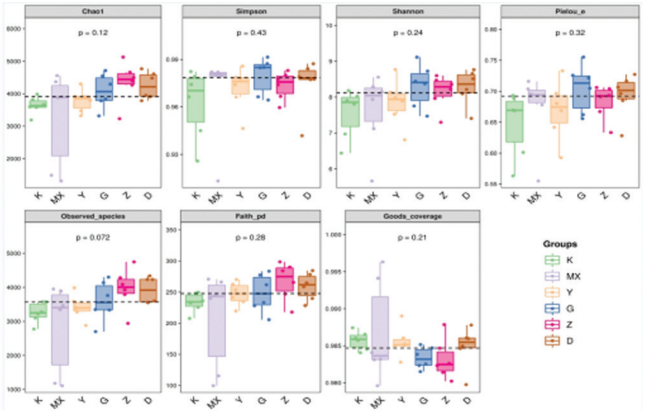


Figure 3. Differences in microbial communities between the Control group, EAMG group, and each drug-treated group. (A) PCoA analysis results showing differences among the six groups; (B) Analysis of differences between the EAMG group and other groups. K, Control group; MX, EAMG group; Y, Prednisone group; G, QSDH-high-dose group; Z, QSDH-medium-dose group; D, QSDH-low-dose group.

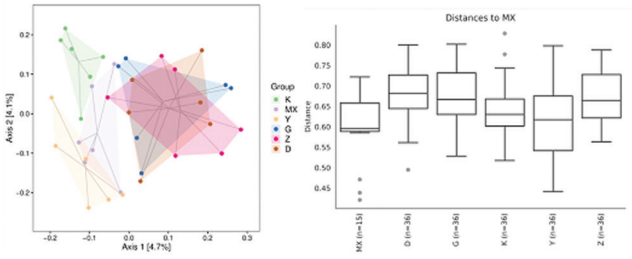
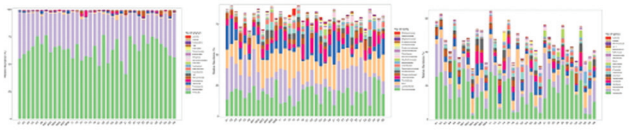


Figure 4. Gut microbiota abundance compositions. (A) Abundance composition at the phylum level; (B) Abundance composition at the family level; (C) Abundance composition at the genus level. K, Control group; MX, Model group; Y, Prednisone group; G, QSDH-high-dose group; Z, QSDH-medium-dose group; D, QSDH-low-dose group.



ELISA. Notably, the serum AChR-Ab level of the EAMG group was significantly greater than that of the Control group, with the inter-group difference reaching statistical significance ($P < .05$). Moreover, as compared with the serum AChR-Ab level of the EAMG group, serum AChR-Ab levels of the three QSDH treatment groups and the Prednisone group were significantly lower, with inter-group differences reaching statistical significance ($P < 0.05$), as shown in Figure 1D.

Effect of QSDH Granules on Intestinal Microbiota of EAMG Model Rats

Gut Microbiota Alpha Diversity and Beta Diversity Analyses. To enable a comprehensive assessment of microbial community diversity, alpha diversity was analyzed for the six groups based on the Chao1 index and Observed species richness indices, Shannon and Simpson diversity indices, Faith's PD genetic diversity index, Pielou's evenness index, and Good's coverage index. Our results revealed increased community richness, diversity, and degree of genetic diversity in rats of each of the three QSDH treatment groups as compared to corresponding indicators of the EAMG group, although these differences were not statistically significant ($P > .05$), as shown in Figure 2.

Results of beta diversity analysis and PCoA analysis of bacterial flora of stool samples revealed completely separate clustering of results obtained for Control and EAMG groups (Figure 3A), which indicated that intestinal flora community composition differed significantly between EAMG model rats and Control (healthy) rats. Meanwhile, results obtained for samples of the Prednisone group and the three QSDH granules-treated groups were also completely separate, indicating that QSDH granules and prednisone regulated the intestinal flora of EAMG rats differently (by acting in opposite directions). Moreover, Adonis difference analysis ($P < .01$) and permutational analysis of variance (PERMONOVA) analysis of inter-group differences indicated that community compositions of the EAMG group versus those of groups treated with QSDH granules or prednisone differed significantly ($P < .05$), as shown in Figure 3B.

Gut Microbiota Taxonomic Composition Analysis

At the phylum level, Firmicutes (64.42%) was present in greatest average abundance in EAMG group intestinal flora, followed by Bacteroidetes (30.29%), with similar results obtained for the Prednisone group (Firmicutes, 59.57%; Bacteroidetes, 35.76%; Actinobacteria 1.71%). Phyla present in greatest average abundance in the intestinal flora of the three QSDH granule-treated groups were Firmicutes and Bacteroidetes. The average abundance of Bacteroidetes in the QSDH-high-dose group was found to be significantly lower than that in the gut flora of the EAMG group ($P < .05$) and the Prednisone group (see Figure 4A). Whereas, the abundance of Actinobacteria was found to be significantly higher in the gut flora of the EAMG group than in the gut flora of the QSDH-high-dose group. Meanwhile, comparisons of Firmicutes/Bacteroidetes (F/B) ratios of the EAMG group to ratios obtained for other groups revealed the F/B ratio was significantly higher ($P < .05$) in the QSDH-medium-dose group and lower in the Prednisone group.

At the family level, Ruminococcaceae (Firmicutes, 20.10%) was present in greatest average abundance in EAMG group intestinal flora, followed by Lachnospiraceae (15.25%) and Prevotellaceae (6.48%). The family with the highest average abundance in the Prednisone group gut flora was Lactobacillaceae (18.73%), followed by Ruminococcaceae (Firmicutes, 13.32%) and Prevotellaceae (7.72%). The family with the highest average abundance in the QSDH-high-dose group gut flora was Ruminococcaceae (Firmicutes, 19.98%), followed by Prevotellaceae (12.30%) and Lactobacillaceae (8.90%). Meanwhile, the average abundance of

Lactobacillaceae in the intestinal flora of the QSDH-medium-dose group was significantly increased ($P < .05$), while the average abundance of Bacteroidetes increased to varying degrees in all three QSDH granule-treated groups as compared to their corresponding average abundances in EAMG group gut flora, with most significant increases found in the QSDH-low-dose group. Moreover, the average abundance of Lachnospiraceae (Firmicutes) was most markedly elevated in the gut flora of the QSDH-high-dose group, while Bifidobacteriaceae was present in significantly greater abundance in the Prednisone group gut flora (see Figure 4B).

At the genus level, *Lactobacillus* was present in greatest average abundance in intestinal flora of all groups, while the average abundance of *Bifidobacterium* was lower. The greatest average abundance of *Lactobacillus* was observed in gut flora of the EAMG group, followed by *Prevotella* (6.38%) and *Ruminococcus* (4.57%). In the Prednisone group gut flora, genera *Prevotella* (7.53%) and *Oscillospira* (3.90%) were present in greatest average abundance, while in the gut flora of the QSDH-high-dose group, genera present in greatest average abundance included *Prevotella* (12.08%) and *Ruminococcus* (4.86%). Meanwhile, the average abundances of *Bifidobacterium* and *Lactobacillus* in the Prednisone group gut flora were significantly greater than their average abundances in the intestinal flora of the EAMG group. Whereas, the average abundances of *Prevotella* and *Coprococcus* in the QSDH-high-dose group were significantly greater, and average abundances of *Turicibacter* and *Lactobacillus* were significantly lower than their respective abundances in the EAMG group gut flora. Finally, average abundances of *Akkermansia* and *Lactobacillus* were significantly greater in the QSDH-medium-dose group gut flora compared to that in the EAMG group gut flora (see Figure 4C).

OPLS-DA

To determine whether differences in microbial community compositions among groups were related to different taxonomic distributions, we carried out PCoA and OPLS-DA at the genus level to obtain OPLS-DA plots and a loading plot. PCoA results revealed that the interpretation degrees of PC1 and PC2 dimensions were 40.3% and 15.9%, respectively. OPLS–DA results indicated that projection distances between the Control group and groups treated with QSDH granules or prednisone in the PC1 dimension were large, suggesting that there were obvious differences in taxonomic abundance between the two groups (see Figure 5A). The loading plot showed that the variable importance projection (VIP) values for genera *Prevotella* (78.52%), *Ruminococcus* (18.25%), *Coprococcus* (14.19%), and *Turicibacter* (13.30%) were higher in the PC1 dimension than PC2 dimension. Thus, these four genera contributed greatly to the inter-group microbial community composition differences (see Figure 5B), as confirmed by the results of taxonomic composition analysis (see Figure 5C).

Figure 5. Microbial community composition differences and taxonomic markers among individual groups. (A) OPLS-DA genus-level plot; (B) Genus-level loading plot; (C) Taxonomic composition analysis of different groups at the genus level.

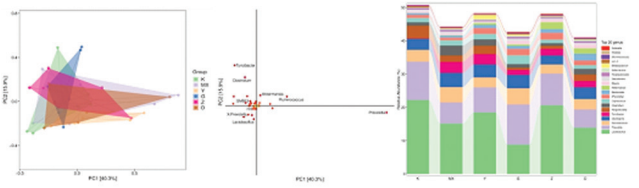
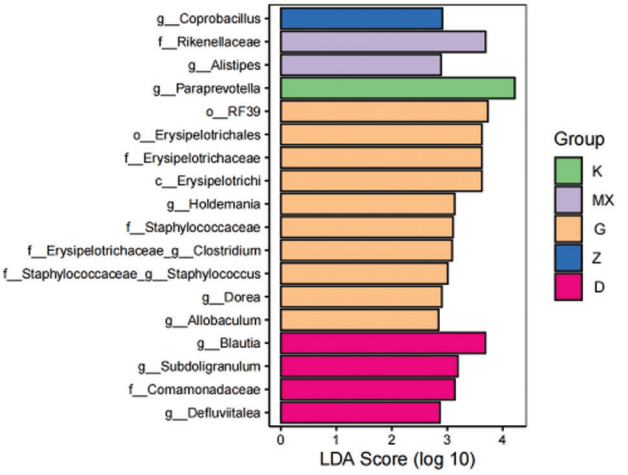


Figure 6. Histogram showing distribution of the Linear Discriminant Analysis (LDA) scores. LDA scores greater than 2 were regarded as biomarkers associated with statistically significant inter-group differences in representation. Lengths of histogram bars represent effect sizes of different species.



LEfSe Analysis

The use of the LEfSe tool to compare multiple groups revealed that *g_Paraprevotella* was the dominant bacteria genus in the Control group, f_Rikenellaceae and *g_Alistipes* were dominant bacterial genera in the EAMG group, f_Erysipelotrichaceae, c_Erysipelotrichi, *g_Holdemania*, etc. were dominant bacterial genera in the QSDH-high-dose group, *g_Coprobacillus* was the dominant bacterial genus in the QSDH-medium-dose group, and *g_Blautia*, *g_Subdoligranulum*, f_Comamonadaceae, and *g_Defluviitalea* were dominant bacterial genera in the QSDH-low-dose group (see Figure 6).

DISCUSSION

MG, an intractable and recurrent autoimmune neurological disorder, is caused by an unclear pathogenic process that has been a main focus of investigations conducted in recent years by numerous scholars, including our research group. Our group has been dedicated to investigating the pathogenesis of MG and the mechanism of clinical and basic experimental effects of TCM intervention in MG. We proposed that the key to the pathogenesis of MG is a "deficiency of spleen and kidney and deficiency of brain

marrow", therefore, the main point of treatment is "strengthening the spleen, benefiting the qi and nourishing the brain marrow". The QSDH granules are an innovative herbal compound based on this theory which consists of 17 herbs, including Astragalus, *Codonopsis pilosula*, Atractylodes, Rehmanniae Radix, *Rhizoma cibotii*, *Angelica sinensis*, etc. It is thought to alleviate MG symptoms by correcting MG-related deficiencies of both the spleen and kidney. An analysis of the therapies currently used for the treatment of MG reveals that the herbal medicinal treatments for MG are dominated by deficiency-tonifying herbs, such as Astragalus, *Codonopsis pilosula*, Radix Liquiritiae, and Rhizoma Atractylodes macrocephala, with Astragalus and *Codonopsis pilosula* being most commonly used. These results align with MG etiological and pathogenic characteristics and effects of treatments as previously evaluated in clinical and basic research studies conducted by our research group,[30] which explored the relationship between MG and gut microbiota by studying the effects of potential MG treatments on gut microbiota composition. Meanwhile, Prednisone was chosen as a positive agent because glucocorticoids, such as prednisone acetate, are the first-line immunosuppressive therapy for patients with MG who remain symptomatic or wish to better control their symptoms while on AChE inhibitors. Moreover, early initiation of steroids therapy during the disease course may allow for early and long-term remission, with 70–80% of patients on steroids achieving marked improvement or complete resolution of symptoms as opposed to 10–20% who achieve spontaneous remission.[31]

The intestinal microbiota, which consists of a variety of bacteria, archaea, viruses, and monocytes that live symbiotically with the human body, has a unique composition of microorganisms that results in production of metabolites that can effectively regulate host metabolic and immune system functions.[32,33] Nevertheless, once the intestinal microbes are disturbed, the intestinal mucosa cannot effectively function as a barrier, leading to the development of a variety of diseases.[34-36] In recent years, several studies have shown that intestinal flora plays an important regulatory role in the development of autoimmune diseases.[37,38] Indeed, diversity and abundance of intestinal microbiota are increasingly viewed as key determinants of host health since they can influence the biological functions of immune effector cells. Intestinal flora modulates immune system functions by producing molecules with immunomodulatory and anti-inflammatory functions, such as short-chain fatty acids (SCFAs), indols and their derivatives, and secondary bile acids that regulate responses of immune cells (including T cells, B cells, dendritic cells, and macrophages, etc.). Intriguingly, results of one study have shown that CD4+ T cells play a key role in MG,[39] while results of another study have shown that CD4+ FoxP3+ Treg cells regulate the production of AChR-specific antibodies.[40] In the latter study, CD4+ FoxP3+ Treg cells, by affecting the number of self-reactive T cells and suppressing the activity of self-reactive B cells, were shown to control the severity of MG clinical symptoms, with the level of CD4+ FoxP3+ Treg cells in MG patients found to be decreased as compared to that of healthy people. Qiu et al.[15] compared the intestinal flora of healthy subjects with that of MG patients and found reduced diversity of intestinal microorganisms, reduced fecal levels of SCFAs, and altered microbial community structure in MG patients. Importantly, SCFAs are key metabolites that have been shown to directly regulate the differentiation of T cells into CD4+ FoxP3+ Treg cells.[41,42] Indeed, the inhibitory function of SCFAs acting through histone deacetylases (HDACs) has been shown to affect peripheral T cell activities that prevent Treg cells from performing their normal inhibitory function, leading to the development of autoimmune diseases, including MG. Additionally, results of other studies[43,44] have revealed that the production of most SCFAs within mouse colons is induced by HDACs, which also support FoxP3 regulatory functions involved in Treg differentiation and stimulate expression of this master transcription factor to promote colon homeostasis-restoring Treg cell functions. Furthermore, SCFAs have been shown to promote G protein-coupled receptor (GPR)43-mediated Th1 cell IL-10 production and IL-22 production by CD4+ T cells and innate immune system lymphocytes by activating GPR41 and inhibiting HDAC activities.[45] Meanwhile, secondary bile acids interacting with Takeda G-protein receptor (TGR)5 can inhibit the Nuclear factor kappa B (NF-κB)-dependent inflammatory response mediator expression in macrophages by reducing the nucleotide-binding domain, leucine-rich–containing family, pyrin domain–containing-3 (NLRP3) inflammasome activation, by inhibiting NF-κB signaling to down-regulate macrophage pro-inflammatory cytokine production, and by co-blocking protein-mediated chromatin modifications through nuclear receptor-farnesoid X receptor (FXR) interactions.[46] In addition, *Bacteroides fragilis* polysaccharide A is able to induce Th1 cell development, while also interacting with T cells through toll-like receptor (TLR)2 binding that promotes immune tolerance by inhibiting Th17 differentiation and enhancing regulatory T cell (Treg cell) activity.[47] Taken together, the abovementioned results indicate that the intestinal flora is crucial for the immune system development and maintenance of immune system functions, such that gut flora dysbiosis may contribute to the development of immune disorder-related diseases. Therefore, therapeutic interventions that alter gut microbiota composition and metabolite production hold promise as potential MG treatments. In this study, we investigated the effects of QSDH granules on the intestinal flora of EAMG rats in terms of intestinal flora abundance, intra-group and inter-group diversity, taxonomic differences, and levels of inter-group discriminatory markers.

Importantly, results obtained through alpha diversity analysis revealed no significant inter-group differences in gut microbiota abundance, genetic diversity, and coverage. However, results of previous studies[48-50] demonstrated associations between reduced microbial diversity and several autoimmune diseases, with the reduction in alpha diversity

correlated with the disease course. Regardless, no significant inter-group differences in alpha diversity were observed in the current study. By contrast, results of beta diversity analysis revealed significant inter-group differences in microbiome diversity between EAMG and Control groups, indicating that the MG disease state may modulate intestinal flora composition. In addition, inter-group differences between the EAMG group and each of the three groups treated with different doses of QSDH granules indicate that QSDH granules may alleviate MG symptoms by altering intestinal flora composition, prompting us to analyze relative abundances of different gut flora taxonomic groups. Our results revealed that in the EAMG group, the gut flora contained a significantly greater abundance of the genus *Turicibacter* and significantly lower abundances of the genera *Lactobacillus* and *Prevotella* as compared to their respective abundances in the gut flora of the Control group. At the phylum level, compared to the gut flora of the EAMG group, abundances of Bacteroidetes and Actinobacteria were significantly lower and higher, respectively, in the gut flora of the QSDH-high-dose group, while the Firmicutes/Actinobacteria ratio was significantly higher in the QSDH-medium-dose group. At the family level, compared to the gut flora of the EAMG group, Lactobacillaceae was present in significantly greater abundance in the QSDH-medium-dose group, while the abundance of Lachnospiraceae (Firmicutes) was increased to varying degrees in the three groups treated with different doses of QSDH granules (and most significantly in the QSDH-low-dose group), and the abundance of Lachnospiraceae (Firmicutes) was increased most significantly in the QSDH-high-dose group. At the genus level, compared to the gut flora of the EAMG group, the abundances of *Prevotella* and *Coprococcus* were significantly greater, and abundances of *Turicibacter* and *Lactobacillus* were significantly lower in the QSDH-high-dose group, while abundances of *Akkermansia* and *Lactobacillus* were significantly greater in the QSDH-medium-dose group. Taken together, these results showed that the intestinal microbiota characteristics of the EAMG group were significantly altered after treatment with QSDH granules, as reflected by phylum-level changes in abundances of Firmicutes and Bacteroides. Notably, these results are consistent with those reported by Qiu et al.[15] showing a significantly different F/B ratio in MG patients relative to that of healthy subjects, which indicated greater pro-inflammatory effects of MG patient gut flora as compared to that of healthy controls. In fact, gut flora with pro-inflammatory characteristics has been shown to disrupt the intestinal epithelium and trigger an immune response that can lead to immune imbalances resulting in autoimmune diseases, as reported by Andoh et al.[51-53] Interestingly, the results of the current study showed significant differences in F/B ratios between the EAMG group and groups treated with three different doses of QSDH granules or prednisone, suggesting that QSDH granules may suppress MG-associated processes by regulating the F/B ratio.

Other results of this study revealed significant inter-group gut flora community differences related to relative gut flora abundances of specific bacterial genera, including *Prevotella*, *Ruminococcus*, *Coprococcus*, and *Turicibacter*. Bacteria belonging to the genus *Prevotella* that have often been associated with a healthy plant-based diet appear to act as "probiotics" in the body by providing health benefits while also interfering with activities of opportunistic gut pathogens that cause inflammatory disorders.[54,55] Nonetheless, results of one study suggested that the colonization of the gut by *Prevotella* led to altered microbiota metabolism and reduction of colon IL-18 production that exacerbated intestinal inflammation to support development of a systemic autoimmune disease.[56] Moreover, *Prevotella* has been found to reside in relatively low abundance in gut microbiota of patients with the autoimmune disease multiple sclerosis (MS). The treatment of MS model mice with *Prevotella* alone or in combination with the MS drug COPAXONE® was shown to reduce the *Prevotella*-associated pro-inflammatory response to alleviate MS symptoms. [57] Similarly, an increased abundance of *Prevotella* has been found in the gut microbiota of patients affected with the autoimmune disease rheumatoid arthritis (RA), but not in their first-degree relatives.[35] Meanwhile, the pro-inflammatory Th17 and Th1 cellular immune responses in RA patients have been shown to be induced by *Prevotella*-specific antibodies. This suggests that increased *Prevotella* abundance in the gut microbiota may be used to identify individuals at increased risk of contracting RA. Notably, the observation that the gut microbiota of MG patients contains a greater abundance of *Prevotella* organisms than the gut microbiota of healthy individuals suggests that Th17 and Th1 cells may also play regulatory roles in MG pathogenesis.[58]

Ruminococcus, one of the first discovered stomach bacteria, plays a crucial role in host metabolism, while also stabilizing the intestinal barrier. In fact, *Ruminococcus* has been shown to produce metabolites consisting of glucomannan polysaccharides, which are known to activate the immune system by inducing the production of cytokines that include tumor necrosis factor-alpha (TNF-α), an inflammatory biomarker of Crohn's disease[59], may also be significantly associated with MG disease.[60] Furthermore, *Ruminococcus* has also been associated with several immune-related diseases,[61] and was observed in a cross-sectional study to be five times more abundant in stool samples of patients with systemic lupus erythematosus.[62] Interestingly, *Ruminococcus* effects were not limited to the immune response, since *Ruminococcus* was also observed to decarboxylate tryptophan to produce the neurotransmitter tryptamine, an evidence that this gut organism interacts with both intestinal and central nervous systems. In the current study, *Ruminococcus* abundance was reduced in groups of rats treated with three different doses of QSDH granules or prednisone as compared to its abundance in gut microbiota of the EAMG group, suggesting that QSDH granules may effectively alleviate MG symptoms by modulating the abundance of *Ruminococcus* in gut microbiota.

Coprococcus, an important genus of gut bacteria within the Lachnospiraceae family (phylum Firmicutes), mainly isolated from feces, actively ferments carbohydrates and is one of the important producers of butyric acid. Additionally, *Coprococcus* abundance is viewed as a microbial biomarker of human gastrointestinal tract health and as a treatment to help suppress immune responses and allergic reactions,[63] thus warranting the investigation of *Coprococcus* as a potential candidate for use in MG treatment.

Interestingly, increased abundances of common probiotic genera *Bifidobacteria* and *Lactobacilli* were observed in gut microbiota of EAMG group rats after gavage treatment with QSDH granules. Significantly greater abundance of *Bifidobacteria* was observed in the QSDH-high-dose group and significantly greater abundance of *Lactobacilli* observed in the QSDH-medium-dose group as compared to their respective abundances in the EAMG group gut flora. Meanwhile, significantly greater abundances of both *Bifidobacteria* and *Lactobacilli* were observed in the gut microbiota of all three groups treated with different doses of QSDH granules compared to their abundances in the Prednisone group. Taken together, the abovementioned results suggest that probiotic supplementation may provide a new therapeutic avenue for treating autoimmune diseases such as MG. However, the efficacies and mechanisms of action of supplemental treatments used for this purpose are unclear, warranting further study. Toward this end, we plan to conduct further evaluations of the use of probiotics in MG treatments.

Limitations

A large body of evidence suggests that intestinal flora plays an important role in the pathogenesis of immune-related diseases. However, more research is needed to better elucidate the mechanisms by which gut flora is altered during development of these diseases to influence immune system functions. Toward this goal, our team has explored MG pathogenesis and potential treatment strategies for many years. The results obtained in our current study suggest that QSDH granules may modulate intestinal flora composition to alleviate MG symptoms. Nevertheless, the present study also has the disadvantage of lacking clinical trials. Precise mechanisms underlying this observed effect remain unclear. Metabolic pathway studies and metagenomic analyses that are currently planned by our laboratory will provide valuable insights upon completion in the future. In the meantime, the results obtained in this study provide new insights that will be tested using larger samples in our continuing investigation of interactions between SCFAs, intestinal microbiota, and the immune system toward the development of probiotic treatments for MG.

CONCLUSIONS

Our findings confirm that QSDH granules exert beneficial effects that alleviate EAMG disease in model rats by influencing relative gut flora abundances of organisms belonging to the Lachnospiraceae family and genera *Prevotella*, *Bifidobacterium*, *Turicibacter*, and *Clostridium*. Our results also suggest that therapies related to microbiota regulation may be developed as key targeted treatments for autoimmune and other immune system disorders. Therefore, once we gain a better understanding of the molecular mechanisms of pathogenesis associated with dysbiosis, we may be able to develop personalized gut flora-based interventions to reduce the incidence and slow the progression of immune-mediated diseases and related disorders.

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DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

ETHICS STATEMENT

The animal experiment protocol was approved by the Animal Experiment Ethics Committee of Changchun University of Traditional Chinese Medicine (approval number 2021226) and was strictly followed to comply with animal protection, animal welfare, ethical principles, and relevant regulations of the National Experimental Animal Welfare Ethics Committee.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Tong Wu, Baitong Wang and Peng Xu. These authors contributed equally to this work and share the first authorship. Conceptualization, TW, TL; writing—original draft preparation, TW; writing—review and editing, JL, TC, DZ, LW; visualization, YZ, ZC, ML; project administration, ZL, PX, JW; funding acquisition, JW. All authors gave the final approval and agreed to be accountable for all aspects of the work.

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