Modified Guishen Pill Ameliorates Polycystic Ovary Syndrome in Rats Through Anti-inflammation and Modulating Gut Microbiota

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Abstract

Background

Polycystic ovary syndrome (PCOS) is a complex endocrine and metabolic disorder often accompanied by chronic low-grade inflammation. Western medicine often causes negative side effects, such as premature ovarian failure. Therefore, this study evaluates the efficacy of modified Guishen pill (MGP) in ameliorating letrozole-induced PCOS in rats.

Methods

Six-week-old specific-pathogen free (SPF) female Sprague-Dawley rats were randomly divided into a blank group (Blank), model group (Model), positive group (Diane-35, 0.2 mg/kg BW), high dose MGP group (H.MGP, 23.96 g/kg BW), medium dose MGP group (M.MGP, 11.98 g/kg BW), and low dose MGP group (L.MGP, 5.99 g/kg BW). After 4 weeks of treatment, plasma, ovaries, and fecal samples were collected from the rats for analysis.

Results

MGP treatment improved ovarian function by improving estrous cycle, ovarian morphology, and endocrine functions in letrozole-induced PCOS rats. MGP alleviated inflammation by reducing macrophage infiltration and inhibiting the activation of NF- κ B and MAPK pathways in the ovaries of letrozole-induced PCOS rats. 16S rDNA sequencing of the intestinal microbiome indicated that MGP regulated gut microbiota structure and restored gut microbiota dysbiosis. MGP significantly increased the relative abundance of bifidobacteria at the order, family, and genus levels. Moreover, correlation analysis showed that gut microbiota were correlated with the host phenotype of letrozole-induced PCOS rats.

Conclusion

MGP treatment could ameliorate letrozole-induced PCOS by improving ovarian function, reducing inflammation, and regulating the gut microbiota.

1 Background

Polycystic ovary syndrome (PCOS), a common endocrine disorder in women of childbearing age, is mainly characterized by androgen excess, ovulatory dysfunction and polycystic ovarian morphology[1]. PCOS is often accompanied by hyperglycemia, insulin resistance, and obesity, which can lead to metabolic disorder, infertility and psychiatric and cardiovascular diseases[1, 2]. Epidemiological studies have shown that the incidence of PCOS is as high as 15% – 20%, and is the main cause of about 75% of

anovulatory infertility[3, 4]. Although PCOS has been extensively studied, its pathogenesis remains unclear[5].

Several studies suggest that in the pathogenesis of PCOS, chronic low-grade inflammation is a crucial factor [6–8]. Elevated levels of interleukin-6 (IL-6), C-reactive protein (CRP) and tumor necrosis factor- α (TNF- α) influence the development and progression of PCOS[9]. High levels of IL-1 negatively affects the balance between sex hormones and inhibit follicular development and fertility[10]. A correlation study between IL-10 and the risk of PCOS found that IL-10 haplotype A-C-G could indicate a reduced risk of PCOS[11–13]. Moreover, serum levels of NF- κ B, TSP-1 and TGF- β 1 were significantly increased in patients with PCOS in northern China, suggesting a close relationship between PCOS and chronic inflammation [7]. While the association between inflammation and PCOS is well supported in the literature, no further studies have elucidated the direct causal relationship. During recent years, an increasing number of studies have focused on the relationship between hyperandrogenemia and insulin resistance in PCOS and chronic low-grade inflammation. Studies have shown that chronic inflammatory response increases NF- κ B activation and oxidative stress production, and increased reactive oxygen species (ROS) production alters the physiologic ovarian dynamics and adversely affects reproductive function. Unbalanced ROS production stimulates inflammatory signaling pathways and induces the release of a variety of pro-inflammatory cytokines. These cytokines enhance androgen production with upregulation of steroidogenic enzymes and stimulation of theca cells proliferation, and interact with activated NF- κ B to lead to β cell dysfunction and abnormal insulin secretion in PCOS, thus, resulting in insulin resistance, which ultimately disrupts the normal ovulation and fertilization process [14]. Meanwhile, chronic low-grade inflammation can induce immune function and ovulation disorder, and may act together with insulin resistance, hyperandrogenemia, and sympathetic dysfunction in a vicious cycle in the pathophysiology of PCOS [6]. Therefore, anti-inflammatory treatment is one of the best strategies to mitigate the pathogenesis of PCOS.

However, the occurrence of several diseases, including PCOS could be linked to gut health, which is related to the composition, structure, and function of gut microbiota. Gut microbiota are closely related to immune, metabolic, cardiovascular diseases, and PCOS [15–17]. Imbalance in gut microbiota can induce inflammation and disrupt energy balance, which is closely related to the composition of gut microbiota in PCOS patients. Particularly, probiotics can improve the reproductive function of PCOS rats by regulating gut microbiota which reduces inflammation [18–23]. These studies suggest that the intestinal microbiome is related to the development of PCOS.

Presently, the treatment of PCOS mainly involves lowering androgen level (Diane-35), improving insulin resistance (metformin), and inducing ovulation (clomiphene). However, western medicine is associated with some side effects, such as ovarian hyperstimulation syndrome, premature ovarian failure, and thromboembolism[24–26]. Therefore, it is necessary to seek a safe and effective therapeutic strategy.

Traditional Chinese medicine can alleviate the symptoms of PCOS with high efficacy, with minimal toxicity and side effects [18, 27, 28]. Guishen pill (GSP) is a well-known prescription first recorded in

Jingyue Quanshu (1624) and consists of eight herbs: *Rehmannia glutinosa* (Gaetn.) Libosch. ex Fisch. et Mey, *Dioscorea oppositifolia* L, *Cornus officinalis* Sieb. et Zucc, *Wolfiporia cocos*, *Angelica sinensis*, *Fructus Lycii*, *Eucommia ulmoides* Oliver, and *Cuscuta chinensis*. Studies have shown that GSP can improve ovarian function in diminished ovarian reserve (DOR) and PCOS mice[29–34]. However, GSP cannot fully meet the clinical needs because most PCOS patients show different symptoms. Therefore, the modified Guishen pill (MGP) has been widely used in clinical trials to improve ovarian function[35, 36]. Considering most PCOS patients experience Yang deficiency, phlegm dampness, liver-qi stagnation, and turbid stasis obstructing collaterals, we have developed MGP by adding *Epimedium brevicornu* Maxim, *Salvia miltiorrhiza* Bunge, *Rhizoma Cyperi*, *Trichosanthes kirilowii* Maxim, and *Tree Peony Bark*(*Cortex Moutan*). These Chinese herbs can improve ovarian function, regulate gut microbiota, and have anti-inflammatory properties [37–46]. We hypothesize that this MGP can improve PCOS, via its anti-inflammatory effects and regulation of gut microbiota.

In this study, the active ingredients in MGP were identified using high-performance liquid chromatography-mass spectrometry (HPLC-MS), and the effects of MGP on ovarian function, inflammatory responses, and gut microbiota composition in letrozole-induced PCOS rats were examined. Additionally, we analyzed the correlation between host phenotype and gut microbiota.

2 Methods

2.1 Drugs and Reagents

Rehmannia glutinosa (Gaetn.) Libosch. ex Fisch. et Mey. (Henan, China), *Dioscorea oppositifolia* L. (Henan, China), *Cornus officinalis* Sieb. et Zucc. (Henan, China), *Wolfiporia coco*. (Yunnan, China), *Angelica sinensis*. (Gansu, China), *Fructus Lycii*. (Ningxia, China), *Eucommia ulmoides* Oliver. (Sichuan, China), *Cuscuta chinensis*. (Liaoning, China), *Epimedium brevicornu* Maxim. (Sichuan, China), *Salvia miltiorrhiza* Bunge. (Sichuan, China), *Rhizoma cyperi*. (Sichuan, China), *Trichosanthes kirilowii* Maxim. (Sichuan, China), and tree peony bark (*Cortex Moutan*). (Sichuan, China) were purchased from Chongqing Shangyao Huiyuan Pharmaceutical Co., Ltd. (Chongqing, China). The plants were identified by Prof. Xuekuan Huang of the Chongqing Medical University and saved in Chongqing Key Laboratory of Traditional Chinese Medicine for Prevention and Cure of Metabolic Diseases. Letrozole was purchased from HengRui Pharmaceutical Factory (2.5 mg/piece, Jiangsu, China). Diane-35 was purchased from Schering GmbH & Co. Produktinos KG. (Guangzhou, China). ELISA kits for T, E2, LH, FSH, and PROG, IL-6, IL-1 β , TNF- α , CRP, and IL-10 assays were purchased from Bioscience Co., Ltd. (QZ-20078, QZ-20001, QZ-22009, QZ-20015, QZ-15042, QZ-20037, QZ-20049, QZ-20160, QZ-20051, QZ-20026, Chongqing, China). TRIzol, PrimeScript RT reagent Kit with gDNA Eraser (RR047A), and TB Green Premix Ex Taq II (RR820A) were purchased from Takara Bio (Kusatsu, Japan). All primers for qPCR were synthesized by Tsingke Biology Technology (Beijing, China). Gram stain kits were purchased from Beijing *Solarbio* Science and Technology Co., Ltd. (# G1060, Beijing, China)

Antibodies against NF- κ B P65 (# 59674), phospho-NF- κ B P65 (Ser536,# 3036), I κ B α (# 4812), phospho-I κ B α (Ser32, # 2859), ERK1/2 (# 4695), Phospho-ERK1/2 (Thr202/Tyr204, # 8544), JNK (# 9252), phospho-JNK (Thr183/Tyr185, # 9251), P38 (# 14451), Phospho-P38 (Thr180/Tyr182, # 4511), and β -actin (# 4970) were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against CD68 (# ab31630) were purchased from Abcam (Cambridge, United Kingdom).

2.2 Preparation of Modified Guishen Pill

MGP was formulated using 13 different herbs, including ShuDiHuang (*R. glutinosa*), ShanYao (*D. oppositifolia*), ShanZhuYu (*C. officinalis*), FuLing (*W. cocos*), DangGui (*A. sinensis*), GouQiZi (*F. Lycii*), DuZhong (*E. ulmoides*), TuSiZi (*C. chinensis*), YinYangHuo (*E. brevicornu*), DanShen (*S. miltiorrhiza*), XiangFu (*R. Cyperi*), GuaLou (*T. kirilowii*), and MuDanPi (Cortex Moutan). The composition ratios and herbal information are shown in Table 1. The daily dose of MGP decoction per adult was 230 g. In accordance with the conversion ratio of surface area between rats and humans (6.25), the daily dose for rats was calculated to be 23.96 g/kg, which was the high dose used in this study. The medium and low doses of MGP were 11.98 and 5.99 g/kg, respectively[47]. According to the ratio, the herbs were mixed and added to the approximately 700 ml distilled water and boiled to make different concentrations of MGP oral liquids. Part of the MGP oral liquid was stored at -80°C for UHPLC-MS analysis.

Table 1
Composition of Modified Guishen Pill.

Scientific name	Pinyin name	Medicinal parts	Batch number	Adult daily dose of herb dose (g)	Composition (%)
<i>Rehmannia glutinosa</i> (Gaetn.) Libosch. ex Fisch. et Mey.	ShuDiHuang	Rhizome	200801	20	8.7
<i>Dioscorea oppositifolia</i> L.	ShanYao	Tuber	200402	15	6.5
<i>Cornus officinalis</i> Sieb. et Zucc.	ShanZhuYu	Mature fruit	191101	15	6.5
<i>Wolfiporia cocos</i>	FuLing	Sclerotium	200601	15	6.5
<i>Angelica sinensis</i>	DangGui	Root	200501	15	6.5
<i>Fructus Lycii</i>	GouQiZi	Mature fruit	200710463	15	6.5
<i>Eucommia ulmoides</i> Oliver	DuZhong	Bark	200501	15	6.5
<i>Cuscuta chinensis</i>	TuSiZi	Mature seed	191101	15	6.5
<i>Epimedium brevicornu</i> Maxim.	YinYangHuo	Rhizome,leaf	200901	30	13.1
<i>Salvia miltiorrhiza</i> Bunge	DanShen	Root	200201	30	13.1
<i>Rhizoma Cyperi</i>	XiangFu	Rhizome	191210613	15	6.5
<i>Trichosanthes kirilowii</i> Maxim.	GuaLou	Pericarp	200201	15	6.5
<i>Tree Peony Bark,Cortex Moutan</i>	MuDanPi	Velamen	200401	15	6.5

2.3 UHPLC-MS analysis

The active ingredients in MGP were determined by LC-MS/MS analysis using Agilent UHPLC system (Agilent 1290 UPLC) with Waters UPLC BEH C18 column (1.7, 2.1 × 100 mm). The multi-step linear elution gradient program was as follows: 0–3.5 min, 95–85% A; 3.5–6 min, 85–70% A; 6–6.5 min, 70–70% A; 6.5–12 min, 70–30% A; 12–12.5 min, 30–30% A; 12.5–18 min, 30–0% A; 18–25 min, 0–0 % A; 25–26 min, 0–95% A; 26–30 min, 95–95% A. According to the IDA acquisition mode, A Q Exactive Focus mass spectrometer coupled with Xcalibur software was used to acquire the MS and MS/MS data. Aux gas flow rate: 15 Arb; capillary temperature: 400°C; full MS resolution: 70000; MS/MS resolution: 17500; collision energy: 15/30/45 in NCE mode; spray voltage: 4.0 kV (positive) or -3.6 kV (negative). The raw mass spectra were imported using the XCMS software. Retention time correction, peak identification, peak

extraction, peak integration, and peak alignment were performed. The secondary mass spectrometry database provided by Shanghai BIOTREE Biotech Co., Ltd. and the corresponding cleavage law matching method were used for material identification of peaks including MS data.

2.4 Experimental animals

Sprague Dawley (SD) rats were maintained in a temperature-controlled facility ($21 \pm 1^\circ\text{C}$ constant temperature) under a 12 h/12 h light/dark cycle and allowed free access to food and water. The rats were allowed to adapt to the feed for two weeks prior to the start of the experiment.

Six-week-old specific-pathogen free (SPF)-level female rats were supplied by the Laboratory Animal Center. The rats were randomly divided into two groups: a blank group ($n = 10$, 0.5% carboxymethyl cellulose sodium (CMC-Na) by gavage) and PCOS group ($n = 50$, 1 mg/kg letrozole in 0.5% CMC-Na by gavage). After letrozole treatment for 21 d, the SD rats were evaluated by estrous cycle and hormonal profiles of the orbital venous blood to establish the PCOS model. PCOS was successfully established when the estrous cycle and hormonal profiles were altered, otherwise, the rats were excluded. The included rats were randomly divided into six groups: 1) a blank group (blank, $n = 10$); 2) model group (model, $n = 9$); 3) oral positive drug group (Diane-35, $n = 8$); 4) oral high-dose MGP group (H.MGP, $n = 7$); 5) oral medium-dose MGP group (M.MGP, $n = 9$); and 6) oral low-dose MGP group (L.MGP, $n = 10$). The treatments were administered orally once daily for 30d by gavage. Feed intake and body weight were measured every 2 d and 3 d, respectively. At the end of the treatment, the rats were anesthetized with isoflurane, and blood was collected from the abdominal aorta. After the rats were sacrificed, ovaries were immediately collected, weighed, left-side ovaries were immediately fixed in 4% paraformaldehyde, and right ovaries were immediately frozen at -80°C for further use.

2.5 Estrous cycle determination

Vaginal smears were obtained at the same time every day for 10 consecutive days at the end of modeling and after the third week treatment with MGP. The estrous stage was confirmed through microscopic (BX53, Olympus Corporation, Japan) analysis of the predominant cell type in vaginal smears by using gram stain kits.

2.6 Plasma Sex hormones and inflammatory cytokines assessments

Briefly, blood samples were centrifuged at 3,500 rpm for 20 min, and plasma was then obtained for analysis. According to the manufacturer's instructions, the plasma levels of testosterone (T), estradiol (E2), progesterone (PROG), follicle stimulating hormone (FSH), luteinizing hormone (LH), interleukin-6 (IL-6), interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α), interleukin-10 (IL-10), and C-reactive protein (CRP) were detected using ELISA kits.

2.7 Histopathology and Immunohistochemistry

Hematoxylin-Eosin Staining: The left-side ovaries were quickly fixed in 4% paraformaldehyde for 24 h, dehydrated, paraffin-embedded, and cut into 5- μ m sections, which were stained with hematoxylin and eosin (H&E) and successively dehydrated with ethanol and xylene, and sealed with neutral resin. All sections were observed under a microscope (BX53, Olympus Corporation, Japan).

Immunohistochemistry: These sections were blocked with 3% bovine serum albumin, then first incubated with primary antibody (TNF- α , IL-6, IL-1 β , IL-10, and CD68, 1:100) overnight, and then with HRP labeling antibody, according to the manufacturer's instructions. The cumulative optical density was determined and calculated using Image-Pro Plus software (Media Cybernetics, USA).

2.8 Reverse Transcription and Quantitative Polymerase Chain Reaction (RT-qPCR).

The mRNA expression levels of TNF- α , IL-6, IL-1 β , IL-10, CRP, CD68, CD86, CD163, CD206, CD200R, NF- κ B P65, I κ B α , ERK, JNK, and P38 in the ovaries were examined by RT-qPCR. TRIzol was used to extract total RNA from ovarian tissues and reverse transcribed into cDNA, according to the manufacturer's protocols. TB Green Premix Ex Taq II and the specific primers (Table 2) were used to detect the relative mRNA levels of target gene transcripts relative to the control β -actin. The PCR reaction cycle was as follows: 95°C for 30 s, followed by 40 cycles at 95°C for 5 s, and finally at 60°C for 30 s (CFX Connect, Bio-Rad, United States). The $2^{-\Delta\Delta C_t}$ method was used to analyze the acquired data.

Table 2
Primer sequences used for PCR.

Gene	Oligonucleotide sequence(5'-3')	
IL-1 β	Forward	GACTTCACCATGGAACCCGT
	Reverse	CAGGGAGGGAAACACACGTT
IL-6	Forward	TCCTACCCCAACTTCCAATGC
	Reverse	GGTCTTGGTCCTTAGCCACT
TNF- α	Forward	CGTCAGCCGATTTGCCATTT
	Reverse	TCCCTCAGGGGTGTCCTTAG
CRP	Forward	GCAGTAGGTGGGCCTGAAAT
	Reverse	CCCGTCAAGCCAAAGCTCTA
IL-10	Forward	TTGAACCACCCGGCATCTAC
	Reverse	CCAAGGAGTTGCTCCCGTTA
CD68	Forward	GCCTCTCTGTATTGAACCCG
	Reverse	AGGGAATGAGAGAGCCAAGT
CD86	Forward	CGAACACTATTTGGGCGCAG
	Reverse	CAAGCCCGTGTCCTTGATCT
CD163	Forward	CCCTGCATAGCAAGTGGTCA
	Reverse	GTCCCTTCCCCAAAGTAGGC
CD206	Forward	GGGTTCACCTGGAGTGATGG
	Reverse	ATTGTCTTGAGGAGCGGGTG
CD200R	Forward	TCCAGCATGAAGGGCGTTAC
	Reverse	GCAATCGCCTCACAAACTGC
NF- κ B P65	Forward	GATGGCTTCTATGAGGCTGAACTCTG
	Reverse	CTTGCTCCAGGTCTCGCTTCTTC
I κ B α	Forward	TATGTACTGCTTCAGCCCCG
	Reverse	GCCTCCAATCTTCATCACGC
MAPK1(ERK)	Forward	GTTTGACATGGAGCTGGACG
	Reverse	AGCCCTTGTCCTGACCAATTT

Gene		Oligonucleotide sequence(5'-3')
MAPK8(SAPK/JNK)	Forward	TGCTGGTGATAGATGCGTCC
	Reverse	CCAGACGTTGATGTACGGGT
MAPK14(P38)	Forward	GGATATTTGGTCCGTGGGCT
	Reverse	CGCATTATCTGCTGAAGCTGG
β-actin	Forward	AGATCAAGATCATTGCTCCTCCT
	Reverse	ACGCAGCTCAGTAACAGTCC

2.9 Western Blot.

Proteins from ovarian tissues were extracted using RIPA buffer containing phosphatase and protease inhibitors, and the concentration was measured using the BCA method. The protein expression level of CD68 (1:1000), IκBα (1:1000), phospho-IκBα (1:1000), NF-κB P65 (1:1000), phospho-NF-κB P65 (1:1000), ERK1/2 (1:1000), phospho-ERK1/2 (1:1000), SAPK/JNK (1:1000), phospho-SAPK/JNK (1:1000), P38 (1:1000), phospho-P38 (1:1000), and β-actin (1:1000) were determined according to the western blotting protocol using chemiluminescence imaging system (Odyssey Fc, LI-COR Biosciences, United States).

2.10 16S rDNA gene sequencing

The total bacterial DNA was extracted by using the Power Soil DNA Isolation Kit (MO BIO Laboratories). The V3-V4 region of the 16S rRNA gene was amplified through the common primer pair (forward primer, 5'- ACTCCTACGGGAGGCAGCA-3'; reverse primer, 5'- GGACTACHVGGGTWTCTAAT-3') combined with adapter sequences and barcode sequences. PCR amplification was implemented in a total volume of 50 µl. The thermal cycling conditions were as follows: an initial denaturation at 95°C for 5 min, followed by 15 cycles at 95°C for 1 min, 50°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 7 min. The PCR products from the first PCR step were purified by VAHTSTM DNA Clean Beads. The second step of PCR was then carried out in a 40 µl volume. The following thermal cycling conditions were used: an initial denaturation at 98°C for 30 s, followed by 10 cycles at 98°C for 10 s, 65°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 5 min. Finally, the PCR products were quantified using Quant-iT™ dsDNA HS Reagent and pooled together. The Illumina Hiseq 2500 platform (2×250 paired ends, San Diego, CA, USA) was used to sequence the purified, pooled samples for high-throughput sequencing analysis.

2.11 statistical analysis

IBM SPSS software (version 25.0; IBM, Armonk, New York, USA) was used to perform all data analyses and one-way analysis of variance (ANOVA) followed by the LSD test. All data were expressed as mean ± SEM and $p < 0.05$ represents statistical significance.

3 Results

3.1 UHPLC-MS /MS analysis of active components in MGP.

Total positive and negative ion chromatograms of MGP showed the representative herbal compounds (Fig. 1A-B). As shown in Fig. 1C, eighteen components were identified in MGP: *8-prenylnaringenin*, *eudesmin*, *salvianolic acid c*, *paeonol*, *quercetin*, *hyperoside*, *rutin*, *chlorogenic acid*, *gallic acid*, *citric acid*, *apigenin*, *kaempferol*, *vanillic acid*, *isorhamnetin*, *eupatilin*, *icariin*, *cryptotanshinone*, *naringenin*. The mass spectrogram of these substances are listed in the Additional file 1.

3.2 MGP improved endocrine abnormalities and pathological changes in PCOS model rats

Prior to the start of the treatments, 60 female Sprague Dawley (SD) rats had normal estrus cycle of 4–5 d, based on the major cell types of vaginal exfoliated cells in vaginal smears for 10 consecutive days, as confirmed by microscopic observation (Fig. 2B). After intragastric treatment with letrozole for 21 d, 43 rats in the PCOS group showed endocrine abnormalities and altered estrous cycle, most of which were in the diestrus phase. After 4 weeks of treatment, the blank group maintained normal estrous cycles, while the model group remained acyclic. However, normal estrous cycle was restored in six rats in the Diane-35 group ($n = 8$), four rats in the H.MGP group ($n = 7$), two rats in the M.MGP group ($n = 9$), and one rat in the L.MGP group ($n = 10$), and their recovery rates were 75% (6/8), 57.14% (4/7), 22.22% (2/9), and 10% (1/10), respectively (Fig. 2C-I). These results suggest that Diane-35 and MGP treatments were effective in treating estrous cycle disorder.

The feed intake and the body weight of the rats were not significantly affected ($p > 0.05$) by the treatments (Fig. 3A-B). As shown in Fig. 3C, the ovarian coefficient in the model group was obviously higher than that in blank group and was obviously decreased after treatment with Diane-35 or MGP. Moreover, there was a significant improvement in the plasma levels of T, E2, PROG, FSH, and LH, and LH/FSH ratios of the PCOS rats after Diane-35 and MGP treatments (Fig. 3D-I). H&E staining analysis (Fig. 3J-L) showed that Diane-35 or MGP administration reduced the number of cystic follicles and promoted the formation of granulosa cells and corpus lutea in PCOS-induced rats, indicating that MGP and Diane-35 treatments could improve endocrine functions and alleviate pathological changes in PCOS rats. MGP treatment alleviated PCOS in a dose-dependent manner, with rats treated with the high-dose MGP having the most significant result, which was equivalent to the results obtained in positive drug Diane-35 group. Therefore, to further explore the mechanism of MGP, follow-up experiments were carried out on the blank, model, and H.MGP groups.

3.3 MGP Treatment reduce inflammation in PCOS rats.

As PCOS is closely related to chronic low-grade inflammation, the levels of anti-inflammatory and pro-inflammatory cytokines in the plasma and ovaries were evaluated using ELISA kits. Fig. 4A-J shows that

MGP significantly reduced the levels of IL-1 β , TNF- α , IL-6 and CRP, and significantly increased IL-10 levels in the plasma and ovaries of PCOS-induced rats. Additionally, the immunohistochemical results revealed that MGP markedly reversed the increase in immune infiltration areas of IL-1 β , TNF- α , IL-6, and the decrease in IL-10 immune infiltration areas in ovaries of PCOS rats (Fig. 4K-O).

Macrophages are a critical factor in the inflammatory response, and the balance between M1 and M2 macrophages determines their physiological or pathological outcomes [48]. Therefore, the effect of MGP on macrophage infiltration in the ovaries of PCOS rats was evaluated. Compared with the blank group, CD68 expression at the protein level was significantly increased in PCOS rats; however, MGP treatment reduced CD68 expression (Fig. 5A-B), as confirmed by the immunohistochemical distribution of macrophages in the ovaries. Similarly, the number of CD68 (+) macrophages in the granulosa cell layer of the ovaries and stromal tissue of PCOS rats were increased; however, MGP treatment reduced the number (Fig. 5C-D). The mRNA expression profiles of M1 and M2 macrophages indicated that there was a significant increase in ovarian M1 macrophages (CD68+, CD86+) expression in PCOS rats compared with that of the blank group. In contrast, the expression of M2 macrophages (CD206+) was not significantly affected by the treatments, indicating that only M1 macrophages were responsible for the increase in macrophages in PCOS rats. MGP treatment significantly reduced the expression of M1-type surface markers and increased the expression of M2-type surface markers at the mRNA level. These results showed that MGP modulated macrophage polarization (Fig. 5E). Additionally, MGP treatment significantly decreased the relative mRNA expression of TNF- α , IL-6, IL-1 β and CRP, and increased IL-10 expression (Fig. 5F). Overall, these results suggest that MGP has an anti-inflammatory effect on letrozole-induced PCOS in rats.

3.4 Effect of MGP on NF- κ B signaling pathway

The NF- κ B signaling pathway plays a key role in the inflammatory response, hence we determined the effect of MGP treatment on NF- κ B p65 activation. The results showed a significant increase in NF- κ B p65 expression at the mRNA levels, p-NF- κ B p65 expression at the protein levels and an increased p-NF- κ B p65/NF- κ B p65 ratio in PCOS rats; however, this was reversed by MGP treatment. Additionally, the effect of MGP treatment on I κ B α , a major endogenous inhibitory protein of NF- κ B, expression in PCOS rats was examined. MGP treatment significantly increased I κ B α expression and decreased the expression of phosphorylated-I κ B α at the protein levels. Overall, the mRNA expression level of I κ B α was not significantly affected by the treatments, indicating that MGP inhibited NF- κ B P65 activation by inhibiting I κ B α protein degradation, which resulted from the inhibition of I κ B α phosphorylation, rather than by the upregulation of the transcription of I κ B α (Fig. 5G-J). Therefore, MGP might reduce inflammation in letrozole-induced PCOS by blocking the activation of the NF- κ B signaling pathway.

3.5 Effect of MGP on MAPK signaling pathway

The MAPK pathway is a key pathway regulating inflammatory responses. The protein expression levels of ERK, P-ERK, JNK, P-JNK, P38, and P-P38 in the ovaries of the rats were determined. There was a significant increase in P-ERK/ERK, P-JNK/JNK, and P-P38/P38 ratios in PCOS rats; however, this was

decreased by MGP treatment (Fig. 5K-N). Interestingly, MGP treatment significantly restored mRNA expression of ERK, JNK, and P38 in PCOS rats (Fig. 5O). The results suggest that MGP inhibited the activation of the MAPK signaling pathway.

3.6 MGP treatment alleviated gut microbiota dysbiosis in letrozole-induced PCOS rats

16S rDNA sequencing (<https://www.ncbi.nlm.nih.gov/sra/PRJNA747735>) of fecal samples was performed to determine the effect of MGP on the composition and relative abundance of intestinal microflora in PCOS rats. The rarefaction curves and rank abundance curves analyzed the sample number, species richness, and evenness, indicating that the quantity and quality of the fecal samples were sufficient for sequencing and follow-up analysis. When the sequencing number increased to 30,000, the rarefaction curve tended to flatten, suggesting that the amount of sequencing data was reasonable (Fig. 6A-B). Four α -diversity indices (Simpson, abundance-based coverage estimator (ACE), Chao1, and Shannon index) were used to evaluate gut microbiota abundance and diversity. Gut microbiota abundance and α -diversity indices of the rats were not significantly affected by the treatments (Fig. 6C-F), indicating that letrozole treatment or MGP intervention did not significantly affect the α -diversity of gut microbiota. Principal coordinate (PCoA) and non-metric multi-dimensional scaling (NMDS) analyses were performed based on the Bray–Curtis distance to evaluate the β -diversity of gut microbiota. Although the H.MGP group could not be completely separated from the model group, it was somewhat different from the model group, implying that MGP intervention affected the gut microbial profile of the letrozole-induced PCOS rats. PCoA and NMDS analyses revealed that the overall composition of gut microbiota among each group of rats was different, and the Bray-Curtis Anosim analysis further indicated that the differences in the three groups were significant ($R = 0.42$, $p = 0.001$) (Fig. 6G-I). Overall, these results showed that treatment with letrozole or MGP significantly affected the β -diversity of the gut microbiota.

The gut microbiota composition of rats in the three groups was compared by analyzing the taxonomic similarity of bacteria at the phylum and family levels. At the phylum level, the three groups were mainly composed of Firmicutes and Bacteroidetes. Letrozole treatment increased the abundance of Verrucomicrobiota, Campilobacterota, Chloroflexi, and Enttheonellaeota, and decreased the abundance of Actinobacteria, Euryarchaeota, Desulfobacterota, and Gemmatimonadota; however, MGP treatment reversed this phenomenon (Fig. 7A-B). At the family level, Lactobacillaceae and Muribaculaceae were the dominant families in the three groups. Letrozole significantly increased the abundance of Lactobacillaceae and Akkermansiaceae, and decreased the abundance of Bifidobacteriaceae, Methanobacteriaceae, Tannerellaceae, Unidentifiedclostridia-ruminococcaceae, Sutterellaceae, Erysipelotrichales-Erysipelotrichaceae, Desulfovibrionaceae, and Clostridiaceae; however, these changes were reversed by MGP treatment (Fig. 7C-D). Additionally, the significant differences between the model group and the H.MGP group were identified by using the linear discriminant analysis (LDA) effect size (LEfSe) method, which showed that MGP could improve the gut microbiota structure of PCOS rats (Fig. 7E-F). Particularly, there was a significant increase in bifidobacteria abundance in the guts of the

H.MGP-treated rats at the family, genus, and order levels (Fig. 7G). These data showed that MGP could remodel the structure of the gut microbiota in letrozole-induced PCOS rats.

3.7 Correlation between gut microbiota and host phenotype

To determine the potential association between gut microbial genera and host phenotype, Spearman's correlation analysis was performed to exam the association between 43 genera and 14 host phenotypes (Fig. 8). There were two host phenotypes (body weight, IL-1 β) that were not significantly correlated with any genus. Nine host phenotypes (CRP, IL-6, TNF- α , LH, LH/FSH, PROG, T, Diestrous and Ovarian coefficient) were significantly negatively correlated with at least ten genera (*Bifidobacterium*, *Collinsella*, *Eggerthella*, *Gordonibacter*, *Parabacteroides*, *Tannerella*, *Catenibacterium*, *[Clostridium]innocuum_group*, *Allobaculum*, and *Holdemania*, *[Eubacterium]_hallii_group*, *Blautia*), and positively correlated with approximately six genera (*Dielma*, *unidentified_Muribaculaceae*, *Vagococcus*, *[Eubacterium]_xylanophilum_group*, *UCG-009*, and *UBA1819*). Interestingly, the other three host phenotypes (E2, FSH, and IL-10) were positively correlated with the above ten genera and negatively correlated with the above six genera. The results showed that gut microbiota were significantly correlated with PCOS-related symptoms, including endocrine abnormalities and inflammation.

4 Discussion

PCOS is one of the prime causes of anovulatory infertility, which induces metabolic syndrome, cardiovascular and cerebrovascular diseases, and other serious complications, affecting a large part of the global population[49]. Long-term use of ovulation-inducing drugs such as clomiphene can easily cause irreversible premature ovarian failure, hence a safe and effective treatment strategy is warranted [26]. In the present study, MGP was formulated using 13 herbs, and 18 main active ingredients were identified by UHPLC-MS analysis (Fig. 1). Several active ingredients with positive effects in treating PCOS have been reported in the literature, including *cryptotanshinone*, *quercetin*, *naringenin*, and *apigenin*[50–53]. Moreover, 15 active ingredients, including *isorhamnetin*, *salvianolic acid C*, and *paeonol*, have been proved to have anti-inflammatory effects[54–56]. Therefore, these compounds may play important roles in the treatment of PCOS with MGP. Herein, we showed that MGP could improve estrous cycle disorder, ovarian morphology damage, and endocrine abnormalities in letrozole-induced PCOS rats (Fig. 2, 3), suggesting that MGP could improve ovarian dysfunction in PCOS rats.

Chronic low-grade inflammation is closely associated with PCOS[10], of which macrophage infiltration is the primary mechanism[48]. Anti-inflammatory and pro-inflammatory cytokines are released by ovarian macrophages at different sites of the ovary, and these cytokines are involved in the regulation of tissue apoptosis and remodeling associated with luteinization, follicular development, and ovulation [57]. Pro-inflammatory cytokines, such as IL-1 β and TNF- α , are produced and released by activated M1 macrophages and play a key role in host defense, whereas M2 macrophages are activated to produce anti-inflammatory cytokines, such as IL-10, to help control inflammation and repair tissue[58]. The imbalance between M1 and M2 macrophages induces ovarian granulosa cell death, thereby causing

ovarian dysfunction[59]. The transfer of bacterial endotoxins from the intestinal cavity to the circulation may interfere with the production of progesterone and lead to luteal deficiency, leading to a decline in ovarian function, indicating that chronic inflammation is an important factor in the development of PCOS [60]. The expression levels of serum IL-1 β and IL-6 in PCOS mice implanted with testosterone (T) pellets were obviously increased, which improve the understanding of the relationship between hyperandrogens, inflammation, and ovarian dysfunction[61]. Similarly, TNF- α and IL-6 levels in PCOS patients were higher, while IL-10 levels were significantly lower than that of normal people[62–65]. In the present study, MGP blocked CD68(+) macrophage infiltration in the granulosa cell and stroma tissue of the ovaries of PCOS rats, and reduced CD68 expression at the mRNA and protein levels. Moreover, MGP significantly reduced the expression of M1-type surface markers and increased the expression of M2-type surface marker at the mRNA level, suggesting that MGP could modulate macrophage polarization and promote the transformation of M1 macrophages to M2 macrophages. Although changes in the expression of M2-type macrophages CD163 (+) and CD200R (+) were observed in the letrozole-treated rats compared with the normal rats, which may be related to the differential expression in different tissues [66], there was a significant increase in the mRNA expression of CD163 (+) and CD200R (+) in H.MGP-treated rats, which was consistent with our conclusion (Fig. 5A-E). Moreover, MGP treatment reduced the plasma and ovarian levels of TNF- α , IL-6, IL-1 β and CRP, and increased the plasma and ovarian levels of IL-10, which was consistent with the mRNA levels of these cytokines in the ovaries and the results of immunohistochemical analysis (Fig. 4, Fig. 5F). Overall, these findings suggest that MGP can reduce inflammation by inhibiting the production of pro-inflammatory cytokines by M1 macrophages and activating the production of anti-inflammatory cytokines by M2 macrophages.

Studies have shown that selective inhibition of the NF- κ B pathway has ovarian protective effect, which can regulate IL-1 β , TNF- α , and other genes[63, 67]. In resting cells, most of the NF- κ B dimers are inactive by non-covalent bonding with one of the three cytoplasmic inhibitors (I κ B α , I κ B β , and I κ B γ). Various signals activate NF- κ B by degrading I κ B α , such as phosphorylation and proteolysis of I κ B α , which can accelerate the degradation of I κ B α . Activated NF- κ B enters the nucleus and binds to DNA-binding dimers by transcription factor P50/p65 to induce transcription of target genes[68, 69]. In this study, MGP significantly inhibited the phosphorylation of NF- κ B P65 and I κ B α and enhanced the expression of I κ B α protein. Additionally, qPCR analysis showed a significant decrease in NF- κ B P65 expression in H.MGP-treated rats; however, I κ B α expression was not affected by the treatments. These results show that MGP inhibited NF- κ B P65 activation by inhibiting the degradation of I κ B α protein through inhibiting I κ B α phosphorylation, rather than by upregulating the transcription of I κ B α (Fig. 5G-J). Furthermore, it is well-known that the activation of the NF- κ B pathway is mainly regulated by the MAPK pathway. For instance, MAPK pathway activation is observed in PCOS rats, whereas inhibiting the MAPK pathway can reduce inflammation and protect ovarian function[70, 71]. MGP treatment reduced P-ERK, P-JNK, and P-P38 expression compared with that of the model group, suggesting that MGP could block the MAPK pathway (Fig. 5K-O).

Several studies have shown that there is an increase in bacteroides, Escherichia/Shigella, and Streptococcus abundance, and a decrease in Akkermansia and Ruminococcus abundance in PCOS

patients[16, 17, 20]. Additionally, the restoration of gut microbiota homeostasis through probiotic supplementation or fecal microbiota transplantation in healthy people could help treat PCOS[20, 72]. Herein, the rats' gut microbiota composition was significantly affected by the treatments, most of the differences were reduced after MGP intervention. At the phylum level, MGP increased the relative abundance of Actinobacteria, which played an important anti-inflammatory role[73]. Moreover, its secondary metabolites comprised of primarily antibiotics and immunosuppressants, which have important physiological and therapeutic effects [74]. In addition, Benzene metabolites of Chloroflexi, including ktedonoketone and 2'-oxosattabacin, induce tissue inflammation and lead to inflammatory diseases[75, 76], while MGP decreased the relative abundance of Chloroflexi in letrozole-induced PCOS rats. Meanwhile, at the family level, Butyrate generated by Ruminococcaceae can inhibit the inflammatory response by inhibiting multiple mechanisms such as NF- κ B and JAK pathway; MGP significantly increases the relative abundance of Ruminococcaceae[77]. Similarly, MGP increased the relative abundance of Desulfovibrionaceae and Sutterellaceae, beneficial strains used to treat of inflammatory diseases [78]. Therefore, these data suggest that MGP might remodel the gut microbiota structure of letrozole-induced PCOS rats and may play an anti-inflammatory role by regulating the gut microbiota (Fig. 7A-F).

Bifidobacteria belong to Actinobacteria, a common probiotic. Actinobacteria are dominant in breastfed infants and account for 90% of the total bacterial count, which plays a vital role in the physiological development of newborns[79]. Probiotics, represented by bifidobacteria, can antagonize microorganisms by changing intestinal microbiota, competitively adhering to mucosal and epithelial cells, strengthening the intestinal epithelial barrier, and regulating the immune system, with positive effects on the host[80]. Several studies have confirmed that bifidobacteria can use extracellular structures or secretory substances to reduce inflammation and enhance the epithelial barrier to restore intestinal health. Moreover, bifidobacteria exhibit anti-inflammatory effects by regulating key signaling pathways, such as NF- κ B and MAPK pathways[80–82]. Interestingly, the results of this study showed that MGP treatment increased bifidobacteria abundance at the order, family, and genus levels (Fig. 7G). Therefore, we speculated that MGP may play an anti-inflammatory role by regulating gut microbiota structure, specifically by increasing bifidobacteria abundance. Unexpectedly, an elevated Firmicutes/Bacteroidetes ratio is an important feature of "obese intestinal flora" [83], while this was not significantly affected by the treatments in this study. Moreover, PCOS rats had significantly higher Lactobacillaceae abundance than the rats in the blank group. These results were contrary to the reports in the literature that the Firmicutes/Bacteroidetes ratio of PCOS rats is usually higher than that of normal rats and increasing the abundance of Lactobacillaceae could improve body weight and metabolic disorder in PCOS rats[20]. However, there are also reports that increasing Lactobacillus abundance could promote weight gain[84]. Similarly, the results of the present study showed that there was a significant increase in akkermansia abundance in PCOS rats, which was contrary to the report that akkermansia abundance was significantly reduced in PCOS patients[17]. The discrepancies in the results may be attributed to differences in the study environment[85]; moreover, contrary to the results of most previous studies, the body weight of the rats was not significantly different in this study, which may have contributed to differences in results.

The correlation analysis between gut microbiota and host phenotype indicated that gut microbiota may have key roles in the treatment of PCOS with MGP, and may assist in the prevention, treatment, and prognosis of PCOS, as well as provide a solid theoretical foundation for the treatment of PCOS with traditional Chinese medicine.

5 Conclusion

This study demonstrated that MGP can effectively improve ovarian function in letrozole-induced PCOS rats, and that it has an anti-inflammatory effect in PCOS rats, which may be associated with the reduction in macrophage infiltration and blocking of the NF-κB and MAPK pathways. Meanwhile, MGP can regulate gut microbiota homeostasis and remodel the gut microbiota structure in letrozole-induced PCOS rats. These results imply that MGP is a potentially effective drug for the treatment of PCOS.

Abbreviations

- PCOS
- Polycystic ovary syndrome
- MGP
- modified Guishen pill
- H.MGP
- high dose MGP
- medium dose MGP
- M.MGP
- low dose MGP
- L.MGP
- GSP
- Guishen pill
- T
- testosterone
- E2
- estradiol
- PROG
- progesterone
- FSH
- follicle stimulating hormone
- LH
- luteinizing hormone
- IL-6
- interleukin-6
- CRP

C-reactive protein
TNF- α
tumor necrosis factor- α
IL-10
interleukin-10
IL-1 β
interleukin-1 β
HPLC-MS
high-performance liquid chromatography-mass spectrometry
PCoA
Principal coordinate
NMDS
non-metric multi-dimensional scaling.

Declarations

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Author contributions

J-YT, J-WW, X-KH conceived and designed the study. J-YT conducted animal experiments, interpreted the results, analyzed the data, and drafted a manuscript. LZ, Q-CZ, YZ, Y-ZL assisted in the experiments and provide data. Y-QX, YX assisted in data analysis. J-WW, X-KH supervised the research and revised the manuscript. The authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All animal protocols in the study were performed in accordance with international ethical guidelines and the National Institutes of Health Guide concerning the Care and Use of Laboratory Animals. All experiments involving animals were approved by the Institutional Animal Care and Use Committee of Chongqing Medical University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Chemical components of MGP by UHPLC–MS/MS analysis. Total ion chromatography in (A) positive and (B) negative ion modes for MGP samples and their chemical molecule structure (C).

Figure 2

MGP improves acyclic estrous cycles in letrozole-induced PCOS rats. (A) Diagram of animal and therapeutic experiments. (B) representative photomicrographs of the vaginal smears by gram stain method, (B1) proestrus, (B2) estrous, (B3) metestrus, and (B4) diestrus stages. (C-H) Female rats were treated with letrozole for 21 days, followed by Diane-35 or MGP gavage for 4 weeks, and the estrous cycles were determined after three weeks of drug treatment. Representative images of estrous cycle in each group are shown. (I) The proportion of rats in the six groups in different estrous stages was analyzed by the Chi-square test and z-test to assess the differences (n=7-10). *p < 0.05 vs. Blank, # p < 0.05 vs. Model.

Figure 3

MGP improves endocrine functions and treats morphological damage in letrozole-induced PCOS rats. (A) chow intake. (B) body weight. (C) ovarian coefficient. Ovarian coefficient was calculated by the formula: ovarian coefficient = ovarian weight/ body weight (mg/g); (D–I) represented T, E2, PROG, FSH, LH, and LH/FSH ratio, respectively, (n = 7-10). (J) Representative ovary sections were stained with hematoxylin and eosin. (J1) Blank group; (J2) Model group; (J3) Diane-35 group; (J4) H.MGP group; (J5) M. MGP group; (J6) L. MGP group. *indicates the appearances of cystic follicle, □ indicates the appearances of granular cells layer, ▢ indicates the appearances of Corpus luteum (magnification, ×100), scale bars, 200 μm. The number of (K) cystic follicles and (L) corpora lutea was determined from these stained sections (n = 6). Data are presented as mean ± SEM. *p < 0.05 versus blank group, **p < 0.01 versus blank group, #p < 0.05 versus model rats, ##p < 0.01 versus model rats.

Figure 4

Effect of MGP on the expression of pro-inflammatory and anti-inflammatory factors in PCOS rats. (A-J) The ovarian and plasma levels of IL-1β, IL-6, TNF-α, CRP and IL-10 were measured by ELISA assays. (A, F) The ovarian and plasma levels of IL-1β. (B, G) The ovarian and plasma levels of IL-6. (C, H) The ovarian and plasma levels of TNF-α. (D, I) The ovarian and plasma levels of CRP. (E, J) The ovarian and plasma levels of IL-10. (K) Representative images of immune infiltration areas of IL-1β, IL-6, TNF-α and IL-10, respectively. Scale bars indicate 50 μm (400×). (L-O) Quantitative analysis of IL-1β, IL-6, TNF-α and IL-10 expression. Data are presented as the mean ± SEM (n = 7-10). *p < 0.05, **p < 0.01 vs. the blank group. #p < 0.05, ##p < 0.01 vs. the PCOS group.

Figure 5

Effect of MGP on macrophage infiltration and NF- κ B and MAPK pathway in the ovaries of letrozole-induced PCOS rats. (A) Protein levels of CD68 in ovaries were detected by Western blotting. (B) Protein concentration analysis. (C) The distribution of CD68 (+) macrophage in ovaries was detected by immunohistochemical staining, scale bars indicate 50 μ m (400 $\times$). (C1) Blank group, (C2) Model group, (C3) H.MGP group. (D) quantitative analysis for CD68 expression. (E) Relative mRNA expression levels of CD68, CD86, CD163, CD206, CD200R in the three groups. (F) Relative mRNA expression levels of IL-1 β , IL-6, TNF- α , CRP, and IL-10 in the three groups. (G) Expression of NF- κ B P65, P-NF- κ B P65, I κ B α , and P-I κ B α in ovaries at the protein level were detected by Western blotting. (H, I) Protein concentration analysis. (J) Relative mRNA expression levels of NF- κ B P65, I κ B α in the three groups. (K) Protein levels of ERK, P-ERK, JNK, P-JNK, P38, and P-P38 in the ovaries were detected by Western blotting. (L-N) Protein concentration analysis. (O) Relative mRNA expression levels of ERK, JNK, P38 in the three groups. Data are presented as the mean $\pm$ SEM (n = 6). *p < 0.05, **p < 0.01 vs. the Blank group. #p < 0.05, ##p < 0.01 vs. the Model group.

Figure 6

Effect of MGP on α -diversity and β -diversity of gut microbiota. Gut microbiota diversity was analyzed by 16S rDNA sequencing. (A) Rarefaction curves and (B) Rank Abundance curves are shown. (C) Simpson index (D) ACE index, (E) Chao1 index and (F) Shannon index were used to evaluate the α diversity of intestinal bacterial assemblages in rats in the three groups. (G) PCoA and (H) NMDS plots based on Bray-Curtis are presented. (I) Bray-Curtis Anosim analysis was used to measure differences between the groups. Data are presented as the mean $\pm$ SEM ($n = 7-10$).

Figure 7

Variation in the taxonomic composition of gut microbial at the phylum and family levels. Maximum bacteria abundance of the top 30 bacteria at the (A) phylum and (C) family levels. There were significant variations in bacterial abundance at the (B) phylum and (D) family levels. (Red) taxa represent the blank group; (Green) taxa represent the model group; (Blue) taxa represent the H.MGP group; The statistical differences in biomarker levels between the model group and the H.MGP group were detected by the linear discriminant analysis (LDA) effect size (LEfSe) method. Taxa enriched in model (green), H.MGP (red) groups are shown using LDA scores. (E) Only the taxa meeting an LDA significant threshold of four are shown, and the histogram length represents the impact of different species. (F) Cladogram visualizes the output of LEfSe algorithm. Distinctly different taxonomic nodes are colored, and the branch areas are shaded in accordance with the effect size of taxa. (G) The relative abundance of bifidobacteria at the family, genus, and order levels in the three groups. Data are presented as the mean $\pm$ SEM ($n = 7-10$); * $p < 0.05$, ** $p < 0.01$ vs. the Blank group. # $p < 0.05$, ## $p < 0.01$ vs. the Model group.

Figure 8

Associations between host phenotype and gut microbial genera. Spearman's correlation analysis between 43 genera and 14 host phenotypes in the model (n = 9) and H.MGP (n = 7) groups is shown. Red represents positive correlations between host phenotype and bacterial genera, and purple represents negative correlations between host phenotype and bacterial genera. *indicates that the correlation was significant.

Supplementary Files

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