Chang-Kang-Fang Ameliorates Experimental Diarrhea Predominant Irritable Bowel Syndrome by Regulating Gut Microbiota and Gut -Brain Axis

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Research

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Abstract

Background: Chang-Kang-Fang formula (CKF), a multi-herb traditional Chinese medicinal formula, has been clinically used for treatment of irritable bowel syndrome with diarrhea (IBS-D). Though we have reported the compounds of CKF and the therapeutic effect on IBS-D rats, the exact mechanism underlying is still not clear. The aim of this study is to clearly define the effect of CKF on IBS-D by regulating gut microbiota and gut-brain axis.

Method: We investigated the effects of CKF on IBS-D rat model, established by psychosocial stress (restraint) combined with the peripheral stimulation (senna leaf gavage) stress. The changed of body weight and the number of fecal pellets was investigated during the experiment. The effect on intestinal sensitivity was assessed based on the abdominal withdrawal reflex (AWR) scores and the intestinal permeability, the expression of ZO-1, measured by immunohistochemistry. The effect of CKF on gut-brain axis was evaluated by the expression of 5-HT through immunohistochemistry. The composition of gut microbiota was detected through 16sRNA.

Results: Administration of CKF significantly have shown the therapeutic effect on IBS-D rats, involving decreased the score of AWR and increased the number of pellets, though there were no different on body weight change. In addition, CKF could upregulated the expression of ZO-1 in colon and downregulated the expression of 5-HT in colon and brain Moreover, CKF could rebalance the gut microbiota of IBS-D, increasing the abundance of Lactobacillu, Allobaculum, Roseburia and Lachnospiraceae_NK4A136.
Conclusion: CKF potentially alleviates IBS-D through regulating gut microbiota and gut-brain axis.

Background

Irritable bowel syndrome (IBS) is a kind of functional gastrointestinal disorder which characterized by abdominal pain and changed of defecation, leading to reduce quality of life. It can be divided into four categories, according to the predominant feature of the bowel symptoms, as follows: IBS with constipation (IBS-C), IBS with diarrhea (IBS-D), mixed-type IBS (IBS-M), and unclassified IBS (IBS-U). Prevalence of IBS vary between 1.1% and 45%, with a pooled global prevalence of 11.2%. Among the four subtypes, IBS-C and IBS-D each account for one-third of the affected population[1]. Though the mechanism of IBS is still unknown, intestinal motility dysfunction, visceral hypersensitivity of the colon and gut microbiota may play a vital role in this disorder and the gut-brain axis (GBA) may play the critical role in IBS. In the past decades, with the development of microbiology and metabonomics, studies showed that distension of gut resulted in activation of key pathways within the brain and that such pathways are exaggerated in IBS with dysregulated microbiota[2]. The microbiota, a new player, has displayed as a key regulator of the gut-brain axis.

5-HT, also named serotonin, may the key mediator in gut-brain axis and the perturbed 5-HT may be a pathogenesis of IBS[3]. It has been reported that visceral sensitivity was increased by 5-HT, and then triggers the diarrhea in IBS. In the gut, tryptophan, at the action of tryptophan hydroxylase 1 enzyme

(TpH1)[4], was transformed into 5-hydroxytryptophan (5-HTP), which is further metabolized into 5-HT[5]. The production of 5-HT was accounted for 90% of the body's 5-HT. In brain, the neurotransmitter 5-HT was produced through tryptophan hydroxylase 2 enzyme (TpH2). Resent studies have shown that gut microbiota, acting as a virtual endocrine organ, is a major actor in intestinal 5-HT production. It has been reported that the germ-free mice exhibit impaired 5-HT production[5]. As for this, regulation of gut microbiota and GBA may be the new frontier of IBS-D[3].

Traditional Chinese medicine (TCM), as a complementary and alternative medicine strategies, has been widely used in Asian countries to treat chronic diarrhea for long time. CKF, which is derived from an ancient traditional Chinese formula, is composed of 7 herbs including Baishao (Paeoniae Alba Radix, the root of Paeonia lactiflora Pall.), Fang Feng (TotalFagopyri Dibotryis Rhizoma, the rhizome of Fagopyrum dibotrys (D.Don) Hara), Shu Dihuang (Saposhnikoviae Radix, the root of Saposhnikovia divaricate (Turcz.) Schischk.), Tu Sizi (Cuscutae Semen, the seed of Cuscuta chinensis Lam.), Jin Qiaomai (Rehmanniae Radix, the root of Rehmannia glutinosa Libosch.), Huang Lian (Coptidis Rhizoma, the rhizome of Coptis chinensis Franch.) and Chan Tui (Periostracum Cicadae, the exuviae of Cryptotympana pustulata Fabricius.). We have been proved that CKF could introduce the remission of IBS-D in clinical trial. And in experimental studies, we have found that CKF protected the IBS-D rats, which may relate to attenuate visceral hypersensitivity[6]. Moreover the active constituents in CKF, including berberine, paeoniflorin, acteoside, flavonoids and chromones, have been determined using high-performance liquid chromatography (HPLC)[6].

Based on this, we hypothesize that CKF has a therapeutic effect on IBS-D by regulating gut microbiota and BGA. And in this study, we estimated the therapeutic effect of CKF by AWR, fecal pellets and the expression of ZO-1 in colon. Moreover, we observed the structure changes of the gut microbiota in IBS-D model rats after CKF treatment using 16S rRNA method. we also investigated the expression of 5-HT in both colon and brain with or without CKF treatment.

1 Materials And Methods

1.1 Composition and preparation of CKF

All the seven medical plants were purchased from Jiangsu Province Integrated of Chinese and Western medicine (Nanjing, China). The seven medicinal herbs of CKF raw powder, as shown in Table 1, were soaked together in 10 volumes (w/v) of distilled water and heated to boiling and maintained at 100℃ for 1.5 hours. The decoction was filtered through a multi-layer gauze and the residue was diluted 8 volumes (w/v) using distilled water for a second extraction. The filtrates obtained from two cycles of extraction were mixed for use.

1.2 Animal

All animal experiments were approved by the Experimental Animal Ethics Committee of Nanjing University of Chinese Medicine (Nanjing, Jiangsu, China). Male Sprague-Dawley rats (4 weeks old,

weighing 140±10g) were purchased from Vital River Laboratories Animal Technology Co, Ltd (Beijing, China). On arrival, all rats were fed in an SPF environment at animal center of Jiangsu institute of traditional Chinese medicine and randomized and transferred to plastic cage in an air-conditional room (temperature23±3℃ , humidity 55±5%) under a 12-h light/dark cycle for 1 week before experiments and allowed water and standard chow ad libitum. All mice were quarantined for one week before starting the experiment.

1.3 Induction of IBS-D and treatment

In this studies, IBS-D model was described as previously reported with little modification[7], psychosocial stress (restraint) combined with the peripheral stimulation (senna leaf gavage) stress. In brief, the model group, CKF group, TM group, PFK group, CKF+TM group and CKF+PFK group rats were gavaged with senna decoction (3g/kg) plus restraint stress (for a duration of 2 starting from 1h after the gavage) for 2 weeks. After the model was established, the Corio Rectal Distention (CRD) method was used to screen the abdominal withdrawal reflex (AWR) score and the Bristol fecal score. The control and model groups were treated orally with distilled water. In CKF group, the rats were given 2mL CKF (18.8g/kg rat weight) via oral gavage per day. In Model group, the rats were given 2mL distilled water per day. At the end of experiment, all rats were killed by cervical dislocation after evaluated abdominal withdrawal reflex.

1.4 Abdominal withdrawal reflex

After 14 days of the treatment, the abdominal withdrawal reflex (AWR) score was evaluated. The 8 Fr (diameter 2.7 mm) double-chambered pediatric end-balloon catheter was coated with paraffin oil and inserted slowly into the rat anus so that the proximal end of the balloon was 1 cm away from the anus. The catheter was connected to the syringe. The rat was fixed in a transparent plastic box so that it could not move back and forth, and after the rat was completely adapted to the environment, the syringe slowly injected into the balloon 2 mL of ambient temperature water to observe the retreat of the abdominal wall and to induce AWR. The pressure of the balloon was increased stepwise, 20, 40, 60 and 80mmHg with duration of 30s and was repeated three times with 5-min intervals. The AWR score presenting the behavioral responses was observed and evaluated. Briefly, rats were scored as follows: 0=the rats are emotionally stable and free to move; 1=the rats are emotionally unstable and occasionally twist their heads; 2=the abdominal muscles contracted slightly, but the abdomen did not lift off the ground; 3 = the abdominal back muscles contracted strongly, and the abdomen was lifted off the ground; and 4= the abdominal back muscles contracted strongly, the back was arched, and the abdomen, pelvis and perineum were lifted off the ground.

1.5 Immunohistochemistry

After rats were killed, colon sections were rinsed with ice-cold PBS, blot up excess PBS solution and then immediately fixed in 10% buffered formalin, dehydrated and paraffin embedded. Sections (5 μm thick) were stained with

Standard immunohistochemical procedures were performed. Tissue sections were incubated with the primary antibody ZO-1 (sc-33725, Santa cruz) and 5-HT (ab6336, abcam) for 2 h at room temperature, followed by 30 min incubation with the pre-diluted horseradish peroxidase-conjugated secondary antibody (1:400). For negative controls, 1% non-immune serum in PBS replaced the primary antibodies. The immunohistochemistry staining of ZO-1 and 5-HT was scored by measuring the integrated optical density of at least three visions of each slice using Image pro-plus 6.0 software. Data are expressed as mean± s.e.m. of six mice.

1.6 Microbial DNA extraction and PCR amplification

DNA extraction and amplification

Total genomic DNA was extracted was extracted from every stool sample using DNA Extraction Kit following the manufacturer's instructions. Quality and quantity of DNA was verified with NanoDrop and agarose gel. Extracted DNA was diluted to a concentration of 1 ng/μl and stored at -20°C until further processing. The diluted DNA was used as template for PCR amplification of bacterial 16S rRNA genes with the barcoded primers and Takara Ex Taq (Takara). For bacterial diversity analysis, V3-V4 variable regions of 16S rRNA genes was amplified with universal primers 343F (5'- TACGGRAGGCAGCAG -3') and 798R(5'- AGGGTATCTAATCCT-3').

Library Construction

Amplicon quality was visualized using gel electrophoresis, purified with AMPure XP beads (Agencourt), and amplified for another round of PCR. After purified with the AMPure XP beads again, the final amplicon was quantified using Qubit dsDNA assay kit. Equal amounts of purified amplicon were pooled for subsequent sequencing.

Bioinformatic analysis

Raw sequencing data were in FASTQ format. Paired-end reads were then preprocessed using Trimmomatic software to detect and cut off ambiguous bases (N). It also cut off low quality sequences with average quality score below 20 using sliding window trimming approach. After trimming, paired-end reads were assembled using FLASH software. Parameters of assembly were: 10bp of minimal overlapping, 200bp of maximum overlapping and 20% of maximum mismatch rate. Sequences were performed further denoising as follows: reads with ambiguous, homologous sequences or below 200bp were abandoned. Reads with 75% of bases above Q20 were retained. Then, reads with chimera were detected and removed. These two steps were achieved using QIIME software (version 1.8.0).

1.7 Statistical analysis

Data are expressed as Mean ± SEM. P values calculated using one-way or two-way analysis of variance (ANOVA) for multiple comparisons or two-tailed Student's t-test for paired comparisons.

Results

CKF ameliorated symptoms in IBS-D rats

Body weight change, fecal pellets and AWR score were observed to assess the induction of IBS-D in rats. After 14 days the weight of IBS-D rats and the number of fecal pellets were lower than that of normal rats, and AWR score was increased in IBS-D rats when compared to those in rats without IBS-D, which are strongly supported that the successful establishment of IBS-D rat model. Then the IBS-D rats were treated without or with CKF for 14 days (Fig.1A). After that, the body weight changed of IBS-D rats in different groups was also significantly lower than that in the control group and there were no different in IBS-D rats treated with or without CKF (Fig.1B). There was no different in AWR scores among the groups for 20mmHg distention pressure. And the AWR score were remarkably increased when the distention pressure was 40, 60 and 80mmHg in Model group. However, the AWR score of CKF group was significantly reduced at the 40, 60 and 80 mmHg distention pressure (Fig.1C). Moreover, the number of fecal pellets in IBS-D rats significantly increased compared with that of normal rats, while rats treated with CKF could produce fewer fecal pellets than the Model group (Fig.1D). All of this indicated that CKF could significantly ameliorated symptoms in IBS-D rats. Since ZO-1 are the most important transmembrane proteins that impact the permeability of tight junctions in colonic tissues, we detected their expression in colonic tissues by Immunohistochemical staining. As shown in our result, the content of ZO-1 was downregulated in the Model group compared with that in the Ctrl group, which suggesting that intestinal mucosal barrier function was impaired in IBS-D rats. The level of ZO-1 was upregulated after CKF-treated when compared to that of Model group. The above results strongly suggested that CKF could significantly enhance intestinal mucosal barrier function in IBS-D rats (Fig.1E, F).

CKF downregulated the expression of 5-HT in IBS-D rats

It has been reported that IBS-D is caused by stress-induced brain-intestinal axis (BGA) changes, leading to excessive intestinal permeability, which aggravates submucosal abnormal immune responses. So next we investigated the expression of 5-HT in colon and brain by immunohistochemistry. The level of 5-HT in colon was higher in the IBS-D group than in the control group. CKF treatment decreased the level of 5-HT in colon (Fig.2A, B). Moreover, we also found the increased level of 5-HT in brain of IBS-D rats and decreased expression after CKF treatment (Fig.2C, D). The above results strongly suggested that CKF could significantly regulate the stress-induced brain-intestinal axis in IBS-D rats.

CKF reversed gut dysbiosis of the IBS-D rats

Because of the vital part of gut microbiota in IBS, we then analysised the structural of the gut microbiota in different group. Firstly, we noted that α-diversity, the representative parameter of richness and diversity of flora as calculated by the chao index, was no significant different between Ctrl group and model group, and CKF could increase the diversity of gut microbiota (Fig.3A). Moreover, we calculated the Shannon index and found that it was decreased in IBS-D group compared with normal rats. Treatment with CKF significantly upregulated the index, which means restoration of α-diversity of gut microbiota (Fig.3A).

Principal coordinate analysis (PCoA) revealed a distinct shift of gut microbiota composition in IBS-D rats treated with CKF compared with IBS-D rats (Fig. 3C). The system clustering tree showed that difference existed in the three groups, and the level of the CKF group was close to that of the ctrl group (Fig. 3D).

CKF regulated the certain microbiota

In order to understand the impact of CKF on gut microbiota, we then analysed different taxonomic level of gut microbiota detailly. As show in our results, at phylum level (Fig. 4A), we have detected nine kinds of gut microbiota ant the most abundant phyla were Firmicutes and Bacteroidetes. In IBS-D rats the relative abundance of Firmicutes was decreased and the abundance of Bacteroidetes was increased. However, after treated with CKF, this phenomenon was reversed (Fig.4 B, C). At the family level (Fig.4 D), the relative abundances of Lachnospiraceae, Ruminococcaceae and Lactobacillaceae were lower in model group, compared with normal rats. After treated with CKF, the relative abundance of these three kinds of microbiota were increased (Fig.4 E-G). Moreover, the relative abundance of Prevotellaceae was higher in IBS-D rats, and CKF reversed this phenomenon (Fig.4 H). At the genus level, the relative abundance of *Allobaculum*, *Lactobacillus*, *Lachnospiraceae_NK4A136* and *Lactobacillus*, which are the short chain fatty acids (SCFAs) productors, were lower in IBS-D rats compared with normal rats, where CKF treatment could increase these microbiota level in IBS-D rats (Fig.4 I-L). Taken together, these results demonstrate that CKF could increase the numbers of bacteria, which product SCFAs.

Discussion

In this study, we established an IBS-D model rats through psychotic stress (binding) combined with peripheral stimulation (folium sennae gavage) stress for 2 weeks, and some of them were treated with CKF while other were given the distilled water. As shown in our result, though there is no different in body weight changed between CKF group and model group, and the number of fecal pellets, in CKF group, was increased, which indicating that CKF could relieve diarrhea in these rats. The intestinal barrier, containing the physical barrier, secretory barrier and immunological barrier, maintains the homeostasis by separating the internal milieu from the external environment[8]. The single layer of epithelial cells along the intestinal tract and tight junctions (TJ) formed by the ability of neighboring epithelial cells are critical to formation and maintenance of the intestinal barrier, controlling the movement of water and other small molecules from the luminal into the mucosa. TJ, consisting of a heterogeneous group of transmembrane proteins such as zona occludens-1, occludins and claudins, plays vital role in intestinal health, and decreased the expression and activity of TJ proteins have been shown to be a pathogenic factor for IBS-D.

Recent studies suggested that structural and functional disruptions in BGA cause changes in perceptual and reflexive responses of the nervous system and may lead to IBS-D. 5-HT is widely present in the central nervous and gastrointestinal systems in which it plays an important role in the GBA[9]. On one hand, alterations in gut motility are linked to 5-HT dysmetabolism, in which the contents of 5-HT are higher in IBS-D. And the role of 5-HT has already been exploited as a therapeutic target with the use of 5-HT_3, a subtype of 5-HT, receptor agonists[10]. On the other hand, perturbed central serotonin action may

participate in IBS-D pathogenesis. Our studies demonstrated that CKF may decrease the level of 5-HT in colon and brain which means that CKF could regulate the balance of BGA through modulating the release of 5-HT.

Lots of studies have found that dysregulated gut microbiota was associated with IBS. Compared with healthy controls, IBS patients have shown the lower abundance in *Lactobacillus*, *Bifidobacterium*, and *Faecalibacterium prausnitzii*[2, 11]. Furthermore, disturbed microbiota structure is linked to co-morbidities of IBS, such as anxiety, depression and migraine[12, 13]. On clinical trials, Lactobacillus probiotics[14, 15] and multispecies probiotics[16], which contains the SCFAs-producing bacteria, have shown improvement symptoms in IBS patients. Targeting the gut microbiota may be a new treatment of IBS. In our resents studies, we have found CKF could significantly rebalance the gut microbiota in IBS-D rats. Especially, the abundance of *Lactobacillu, Allobaculum, Roseburia* and *Lachnospiraceae_NK4A136* was increased after CKF treatment.

Conclusion

The protective effects of CKF in treating IBS-D were potentially through upregulating the expression of TJ such as ZO-1, balancing gut microbiota and regulating the GBA mediated by decreasing the expression of 5-HT.

Abbreviations

IBS: Irritable bowel syndrome; AWR: abdominal withdrawal reflex; HPLC: high-performance liquid chromatography; 5-HT: 5-hydroxytryptamine; TpH1: tryptophan hydroxylase 1 enzyme; 5-HTP: 5-hydroxytryptophan; GBA: gut-brain axis; TCM: Traditional Chinese medicine; CKF: Chang-Kang-Fang; TJ: tight junction; ZO-1: zona occludens 1; CRD: Corio Rectal Distention; PCoA: principal coordinate analysis.

Declarations

Acknowledgements

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Author's contributions

Min Lu designed the animal experiments; Xinyu Fan performed the animal experiments; Jingyi Hu wrote the article; Yuanyuan Zheng and Ruiyi Ji prepared CKF samples and helped the animal experiment; Weiqian Kong analyzed part of the data; Hui Xie conceived the study and revised the 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 experiments were approved by the Experimental Animal Ethics Committee of Nanjing University of Chinese Medicine (Nanjing, Jiangsu, China).

Consent for publication

All authors agree to publish this paper.

Competing interests

The authors declare that they have no competing interests

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Tables

Table 1.

The composition of CKF

Chinese name	Latin name	Dry weight of crude drugs in CKF (g)
Bai Shao	*Paeoniae radix albe*	15
Fang Feng	*Saposhnikoviae radix*	6
Shu Dihuang	*Rehmanniae radix praeparata*	5
Tu Sizi	*Cuscutae semen*	5
Jin Qiaomai	*Fagopyri dibotryis rhizoma*	8
Huang Lian	*Coptidis rhizoma*	3
Chan Tui	*Cicadae periostracum*	3
Total		45

Figures

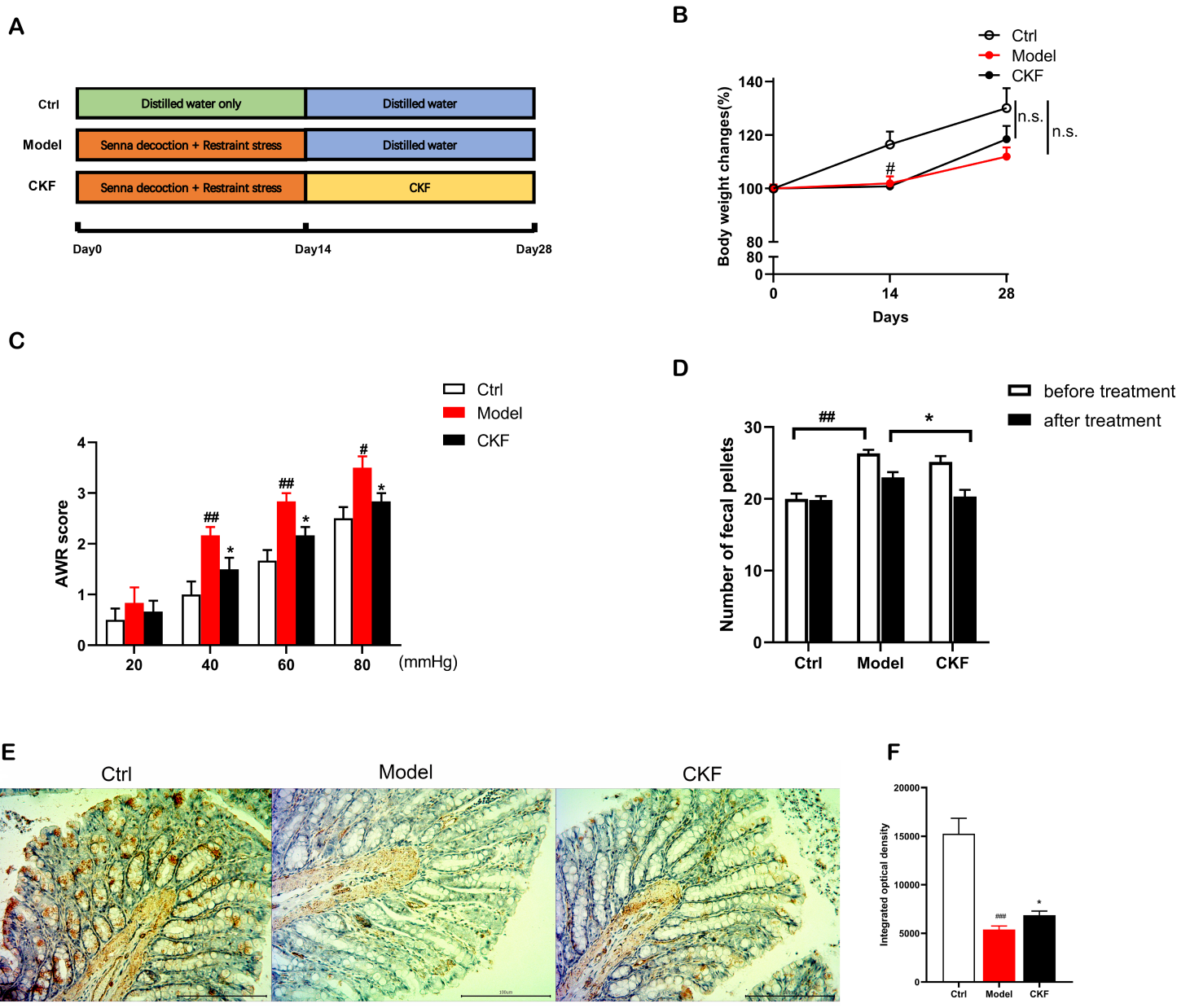

Figure 2

CKF ameliorates symptoms in IBS-D rats (A) Experimental design. (B) Body weight changed in experimental duration. Date are plotted as percentage of basal body weight. (C) AWR score was measured on d28. (D) Number of fecal pellets was measured on d28. (E) Representative images of immunohistochemistry staining for ZO-1 (brown) of colon tissue from different groups. Bar = 100 μm. (F) Quantitative analysis of ZO-1 in different group. Results are mean ± S.D. of six to eight mice in each group. P values calculated using one-way or two-way analysis of variance (ANOVA) for multiple comparisons or two-tailed Student's t-test for paired comparisons. # P<0.05，## P<0.01, ##P<0.001 versus normal; * P<0.05，** P<0.01, ***P<0.001 versus model group.

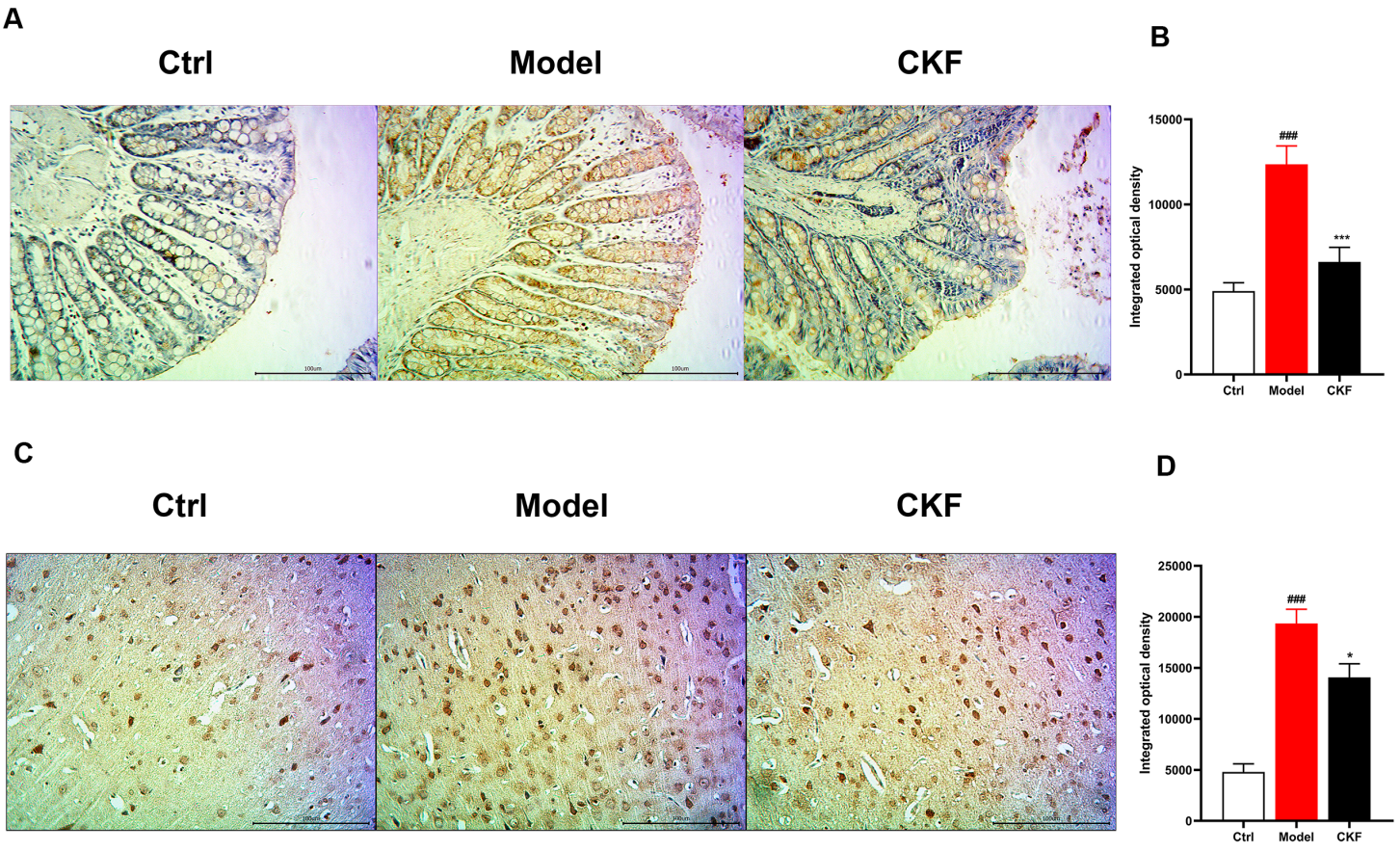

Figure 4

CKF downregulated the expression of 5-HT in IBS-D rats (A) Representative images of immunohistochemistry staining for 5-HT (brown) of colon tissue from different groups. Bar = 100 μm. (B) Quantitative analysis of 5-HT in different group. (C) Representative images of immunohistochemistry staining for 5-HT (brown) of brain tissue from different groups. Bar = 100 μm. (D) Quantitative analysis of 5-HT in different group Results are mean ± S.D. of six mice in each group. P values calculated using one-way or two-way analysis of variance (ANOVA) for multiple comparisons or two-tailed Student's t-test for paired comparisons. # P<0.05 ## P<0.01, ##P<0.001 versus normal; * P<0.05 ** P<0.01, ***P<0.001 versus model group.

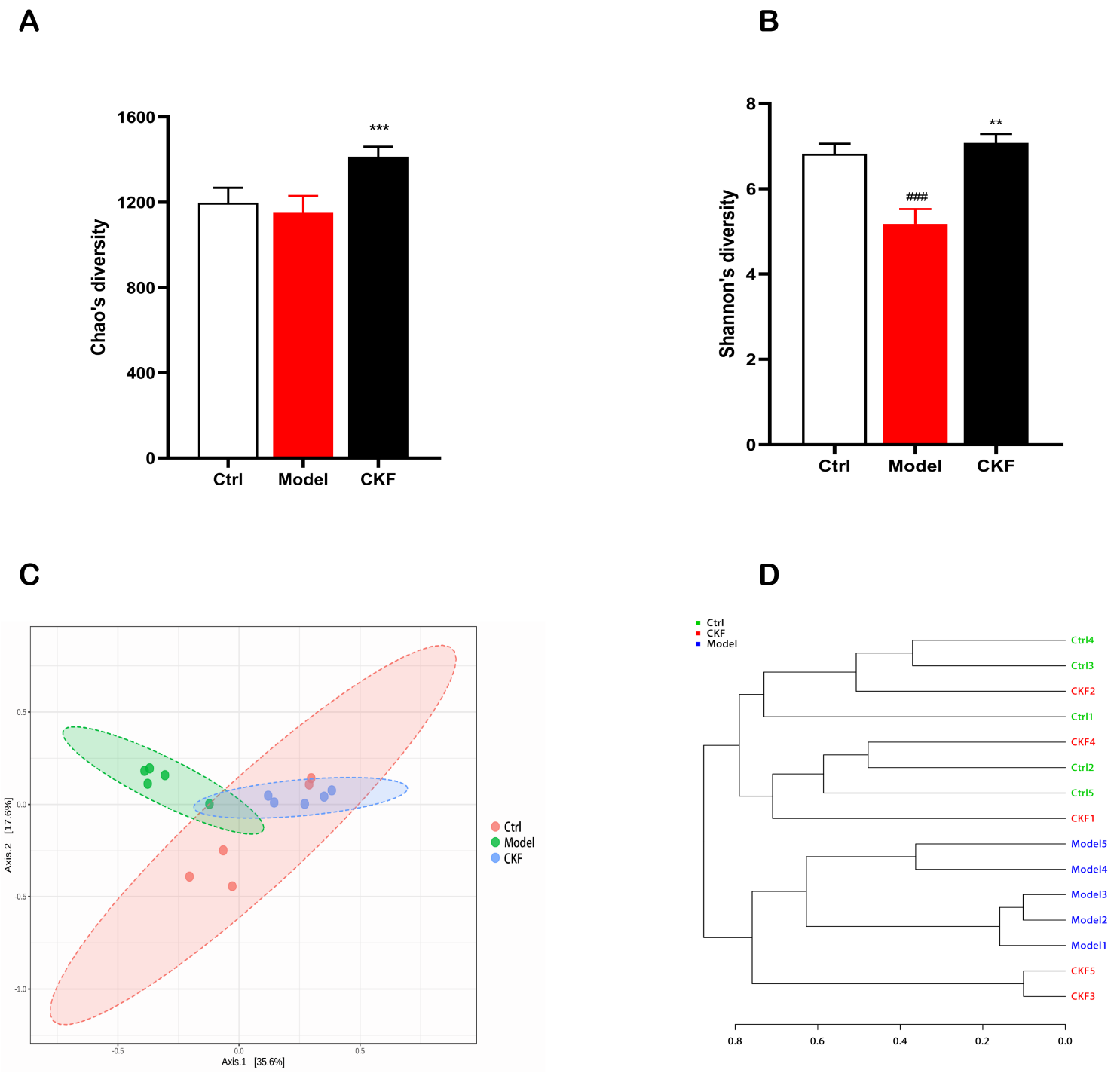

Figure 6

CKF reversed gut dysbiosis of the IBS-D rats (A) Gut microbial chao diversity analysis. (B) Gut microbial shannon diversity analysis. (C) PCoA plot of fecal microbiota of each group. (D) Multiple sample similarity tree.

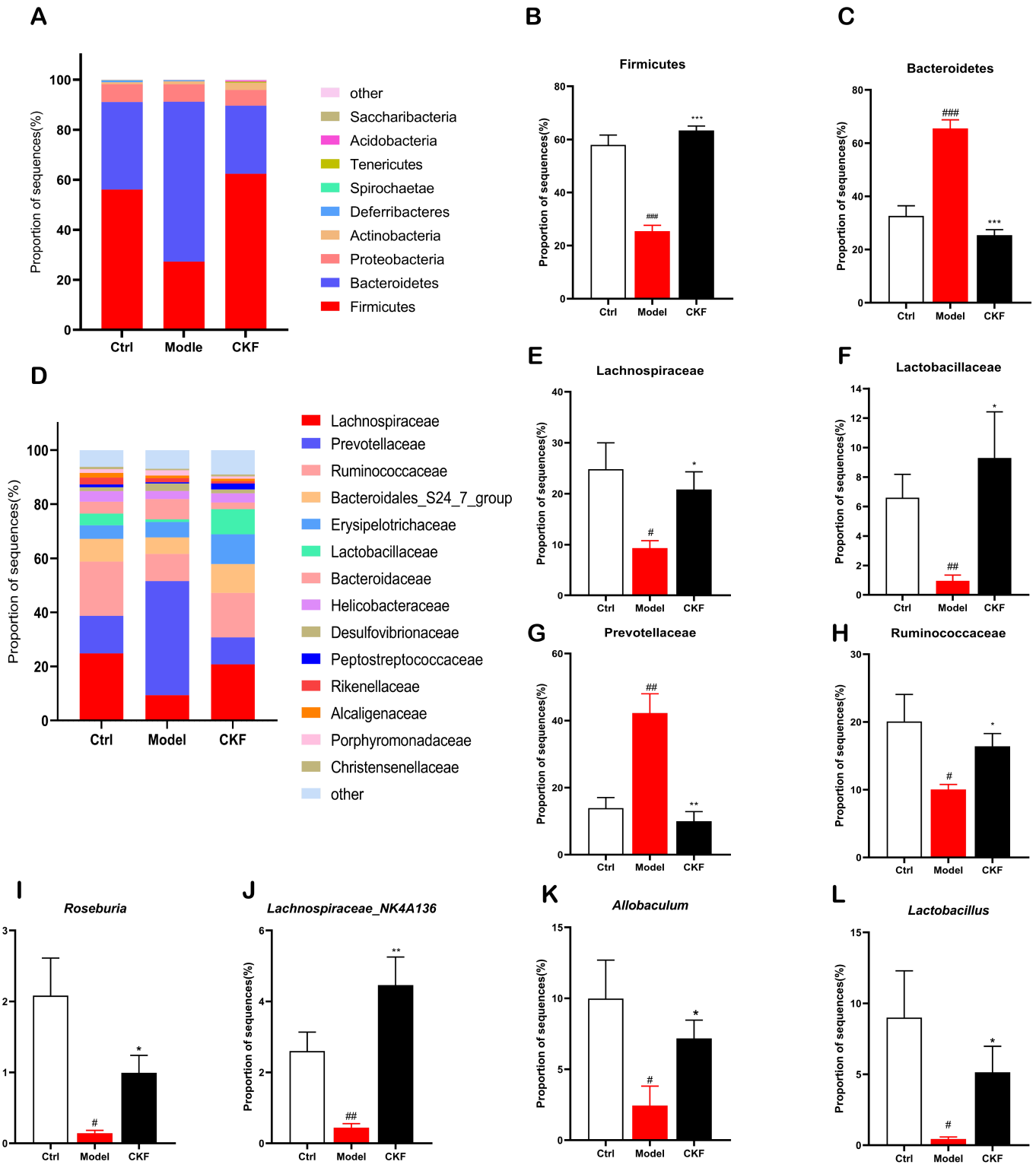

Figure 8

CKF regulated the certain microbiota (A) Stacked bar plot of the phylogenetic composition of common bacterial taxa at the phylum level. (B-C) Relative abundance of Firmicutes(B) and Bacteroides(C) (n = 5). (D) Stacked bar plot of the phylogenetic composition of common bacterial taxa at the family level. (E-H) Relative abundance of Lachnospiraceae(E), Lactobacillaceae(F), Ruminoccoccaceae(G) and Prevotellaceae(H) (n = 5). (I-L) Relative abundance of Roseburia(I), Lachnospiraceae_NK4A136(J),

Allobaculum(G) and Lactobacillu(L) at genus level (n = 5). Results are mean ± S.D. of six mice in each group. P values calculated using one-way or two-way analysis of variance (ANOVA) for multiple comparisons or two-tailed Student’s t-test for paired comparisons. # P<0.05 ## P<0.01, ##P<0.001 versus normal; * P<0.05 ** P<0.01, ***P<0.001 versus model group.