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Research article

Spirocyclopiperazinium salt compound DXL-A-24 improves visceral sensation and gut microbiota in a rat model of irritable bowel syndrome

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ABSTRACT

Irritable bowel syndrome (IBS) is characterized by visceral pain, impaired intestinal barrier and a disorder of the microbiota. DXL-A-24 has analgesic and anti-inflammatory effects by inhibiting neuropeptides and inflammatory factors. In this study, we used chronic unpredictable mild stress (CUMS) induced IBS model, to assess the action of DXL-A-24 on visceral hypersensitivity, barrier function and microbiota. Visceral sensation was assessed by colorectal distension in a model of IBS. The expressions of substance P (SP) and calcitonin gene-related peptide (CGRP) were detected by immunohistochemistry and western blot, the contents of diamine oxidase (DAO) and D-lactic acid were detected by ELISA, and 16S rRNA to detect the diversity of gut microbiota. CUMS reduced visceral pain threshold and increased colonic permeability of rats. DXL-A-24 for 28 days inhibited these changes. DXL-A-24 also decreased the expression of SP, CGRP in colon and D-LA, DAO in serum. Besides, DXL-A-24 increased the richness and diversity of intestinal microbiota. In conclusions, DXL-A-24 reduced visceral sensitivity, improved intestinal barrier and regulated gut microbiota in rats with IBS.

1. Introduction

Irritable Bowel Syndrome (IBS) is a global intestinal disease with an overall prevalence of 11.2%. The disease is characterized by changes in bowel habits and abdominal pain, which seriously affects the normal work and life of human beings [1]. In recent years, it is believed that irritable bowel syndrome is induced by a combination of factors, including changes in visceral sensitivity, disturbance of gastrointestinal function and brain-gut axis, abnormal regulation and alteration of intestinal microorganisms [2,3]. According to the symptoms and underlying pathogenesis of irritable bowel syndrome, different drugs have been developed for treatment, such as antispasmodic, antidiarrheal, laxative etc, but these drugs have more or less adverse reactions, so the development of new drugs is of great significance.

The spirocyclopiperazinium salt compound DXL-A-24 was synthesized from its precursor substance LXM-15. LXM-15 has significant anti-inflammatory and analgesic effects in animal models of inflammation, thermal and chemical pain models, and rheumatoid arthritis. Its mechanism of action was related to the activation of peripheral α7 nicotinic acetylcholine receptor and M4 muscarinic

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acetylcholine receptor, further inhibiting of the expression of TNF-α and CGRP [4,5,6]. Compared with LXM-15, DXL-A-24, which is synthesized in a shorter and cheaper process, has significant anti-injury effects in animal neuropathic and chemically irritating pain models, and its mechanism is similar to that of LXM-15, with no obvious toxicity [7,8]. There is no study about DXL-A-24 and gastrointestinal diseases. Based on the high visceral sensitivity characteristic of IBS, we established an IBS model to evaluate the potential mechanism of DXL-A-24.

2. Materials and methods

2.1. Drug and materials

DXL-A-24 was synthesized by Shandong Newtime Pharmaceutical Co., Ltd. Linaclotide Capsules (Almac Pharma Services Limited, Jiangsu, China), Rat Diamine oxidase (DAO) ELISA Kit, Rat Tumor necrosis factor-α (TNF-α) ELISA Kit and Rat D-lactate (D-LA) ELISA Kit (All ELISA Kit were purchased from Shanghai Enzyme-linked Biotechnology Co. Ltd, Shanghai, China), Substance P polyclonal antibody (Thermo, USA), Anti-TNF alpha antibody and Anti-CGRP-I + CGRP-II monoclonal antibody (Abcam, Cambridge, MA, UK), HRP-conjugated goat anti-rabbit IgG (Sangon Biotech, Shanghai, China), PVDF membranes (Millipore, MA, USA), chemiluminescent HRP substrate reagent (Bio-Rad Laboratories, CA, USA), HE staining kit (Beyotime biotechnology, Shanghai, China).

2.2. Animals and treatments

Male Sprague-Dawley rats (42 to 55 days, Zhejiang Vital River Laboratory Animal Technology Co. Ltd.) were used. The animals were allowed to acclimatize the animal facilities for 7 days before the experiments. They were housed in an animal laboratory with a temperature of 20–26 °C, 40–70% humidity, a 12 h light/dark cycle, and free access to water and food. All animal protocols were approved by the Institutional Animal Care and Use Committee of Lunan Pharmaceutical Group Corporation.

The rats were randomly divided into 2 groups. 10 rats were randomly selected and raised under normal conditions, which was set as the normal group. The other rats underwent CUMS procedures. The CUMS procedures which was based on the practice [9] were as follows: The rats were housed in single cages, and randomly stressed by factors once a day for 28 days. The factors included: 1) water deprivation for 24 h; 2) food deprivation for 24 h; 3) day-night reversal; 4) ice-water swimming for 5 min; 5) heat stress for 10 min; 6) wet cage for 24 h; 7) empty cage for 24 h (i.e., without cushion material). After 28 d of CUMS procedures, The CUMS model rats were randomly divided into different groups and treatments with different drugs for 28 days. The groups and treatments were set as follows: (1) IBS model group (0.9% saline); (2) Linaclotide Capsules group (positive drug group); (3) DXL-A-24 L group (2 mg/kg); (3) DXL-A-24 M group (6 mg/kg); (3) DXL-A-24 H group (12 mg/kg). The doses of DXL-A-24 were selected on the basis of previous reports [5,7,8]. All drugs were dissolved in distilled water immediately before use and were administered in a volume of 10 ml/kg by intragastric injection (i.g.).

2.3. Assessments of visceral sensitivity

The visceral pain threshold was determined by colorectal distension (CRD). Rats were deprived of food but had free access to water 24 h before the assessments. The latex balloon with a diameter of 5 mm after inflation was inserted into the descending colon and rectum under anesthesia and the balloon catheter was fixed at the root of the tail. Then the rats were placed into a transparent plastic box that could not escape or turn around for 30 minutes until recovered from the anesthesia before testing. To measure the pressure threshold of CRD, the colonic balloon was gradually inflated until a visible contraction of the abdominal wall was observed. Five abdominal withdraw reflex (AWR) scores (AWR 0 to AWR 4) were used to assess the visceral sensitivity: 0 no remarkable behavior changes; 1 immobility of the rat body or occasionally swing of the head; 2 mild abdominal muscle contraction; 3 abdominal muscle contraction and lifting of abdominal wall; 4 body arching and lifting of pelvic structures [10]. The pain threshold was defined as the minimal pressure inside the balloon when the rat showed lifting of abdominal wall (AWR 3). All the measurements were manipulated by two blinded observers, repeated three times with at least 5 min recovery intervals.

2.4. Hematoxylin-eosin staining

Rats were anesthetized with Tiletamine hydrochloride and zolazepam hydrochloride for injection and xylazine hydrochloride injection (10 mg/kg), colon tissue was excised and fixed with 4% paraformaldehyde solution for 24 h at room temperature, then refer to the method [11] for paraffin embedding, alcohol dehydration, tableting, neutral gum sealing and other operations. The morphological changes of the colonic mucosa were observed and photographed under the 200 objectives of the digital camera system.

2.5. ELISA

Rat blood samples were collected, centrifuged at 3000 rpm for 10 min to collect serum, and frozen at −80 °C for later use. The contents of DAO, D-lactic acid and TNF-α in serum were detected by ELISA, according to the manufacturer's instructions.

2.6. Immunohistochemistry

The tissue sections were subjected to the following operations: (1) dewaxing: the sections were performed in xylene and graded alcohol, respectively. (2) Antigen retrieval: antigen retrieval was performed in citrate buffer (PH6.0); (3) Serum blocking: dropped 3% BSA on the sections block at 37 °C for 30 min. (4) Incubation antibody: dropped the primary antibody on the sections at 4 °C overnight, then dropped the secondary antibody at 37 °C for 50 min; PBS was used for washing tissue sections after each step. Finally, slices were visualized using DAB. Each tissue section was observed with an optical microscope, and light yellow to brownish-yellow particles under the microscope were regarded as positive expression, and three 200× fields of view were randomly selected to collect pictures. Analysis of mean densitometric values was performed with IPP (Image Pro Plus) image analysis software.

The mean optical density value was calculated as follows: positive area ×OD/total area

2.7. Western blot analysis

Western blot was performed as follows. Colonic tissue were lysed in RIPA buffer supplemented with 1 mM PMSF and were kept on ice for 10 min. The protein concentration was determined using the BCA method. After that, protein was denatured with SDS-loading buffer in a 100 °C metal bath for 10 min, Equal amounts of the protein from each group were loaded onto 10% SDS-PAGE gels, and were then transferred to PVDF membranes. The membranes were blocked in TBST supplemented with 5% BSA for 1h and incubated with primary antibodies at 4 °C overnight. After three washes in TBST, the membranes were incubated with HRP-conjugated goat anti-rabbit IgG for 1.5 h at room temperature. The membranes were washed three times in TBST, the blots were visualized with a chemiluminescent HRP substrate reagent, and images were acquired using a chemiluminescence system Tanon-5200 (Tanon Science & Technology Co. Ltd, Shanghai, China).

2.8. Collection of fresh feces

Fresh feces of rats were quickly collected in 10 mL sterile frozen storage tube and placed in liquid nitrogen for 2 h, then stored at −80 °C refrigerator for subsequent fecal 16S rRNA gene sequencing analysis.

2.9. Microbiota sequencing

Microbial DNA was extracted from samples using the PowerSoil DNA Isolation Kit (MO BIO Laboratories) according to manufacturer's protocol. The concentration of DNA was determined by testing 1 μl extracted fecal DNA. The DNA was stored at −80 °C refrigerator until further processing.

The V3–V4 regions of 16S rRNA gene were amplified by PCR with the common primers (forward: 5′-ACTCCTACGGGAGGCAGCA-3′ and reverse: 5′-GGACTACHVGGGTWTCTAAT-3′). PCR followed the basic principles in that the low cycle number amplification was adopted and the circulation number of the single sample amplification was consistent. Then they formed a sequencing library and High-throughput sequencing analysis of bacterial rRNA genes was performed on the purified, pooled sample using the Illumina Hiseq 2000/2500 platform at Yuewei Gene Technologies Corporation, Beijing, China.

2.10. Bioinformatic analyses

The raw low-quality reads were removed and filtered to obtain effective-quality reads using FSATQC. Then chimeric reads were

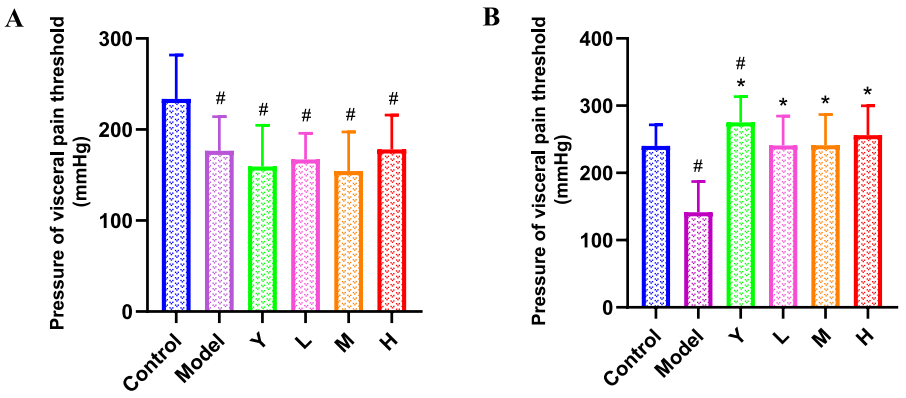

Fig. 1. Pressure threshold for colorectal distension. Measurements after CUMS (A) and administration of treatment (B). Model: irritable bowel syndrome. Y: linaclotide capsules. Results are expressed as the mean ± SD (n = 10/group). #p < 0.05 vs control by one-way ANOVA followed by LSD(L) test(A). *p < 0.05 vs model, #p < 0.05 vs control by two-way ANOVA followed by LSD(L) test(B).

trimmed using Mothur software to obtain the final usable reads. According 97% similarity. The final reads were categorized OTUs following usearch61 of QIIME (V1.9.1) software. The OTU representative sequence was compared with the microbial reference database to obtain the species classification information corresponding to each OTU, and then the composition of each sample was counted at each level (i.e. phylum, class, order, family, genus, species). Alpha diversity indicators (richness, diversity index, rarefaction curve and Rank Abundance curve) and Beta diversity analysis (PCA, PCoA) were based on the OUTs using Bray-Curtis algorithm of QIIME and R software to compare the similarity of species diversity in different samples. Finally, Species at the genus level were analyzed for differences in all samples.

2.11. Statistical analysis

Data were expressed as mean ± SD and were visualized using GraphPad Prism 8.0.1 software. Comparisons between multiple groups were conducted using one-way or two-way analysis of variance (ANOVA) followed by LSD (L) test. All statistical analysis was performed using SPSS.20 software. $p < 0.05$ was considered significant difference.

3. Results

3.1. Effect of DXL-A-24 on CUMS-induced visceral hyperalgesia

At the end of 28d of CUMS and intragastric administration, the pressure threshold of CRD was respectively measured. After modeling, the pressure threshold of CRD was significantly lower than control ($p < 0.05$) (Fig. 1A). These results showed that visceral hyperalgesia was successfully induced by CUMS. DXL-A-24 and Y were reversed the lower pressure threshold in IBS model rats ($p < 0.05$) (Fig. 1B).

3.2. Body weight variation of rats

During the CUMS and the drug treatment period, the body weight of rats was measured, we found that body weight increased gradually during the treatment period in all groups, with the DXL-A-24 groups significantly higher than the IBS model group ($p < 0.05$) (Fig. 2A). There were respectively 1.24% (model), 4.14% (Y), 4.45% (DXL-A-24 L), 6.61% (DXL-A-24 M), 4.29% (DXL-A-24 H) weight growth rates in all groups. However, these had no significant difference compared with IBS model group (1.24%) (Fig. 2B).

3.3. Effects of DXL-A-24 on DAO, D-LA and TNF-α level in serum

DAO and D-LA are associated with intestinal barrier. ELISA results indicated that DAO and D-LA concentration in all groups decreased after drug administration, and DXL-A-24 M and H lower significantly than IBS model group ($p < 0.05$) (Fig. 3A,B). TNF-α concentration did not decrease significantly ($p > 0.05$) (Fig. 3C). However, in western blot tests we found TNF-α were significant decreased compared with IBS model group ($p < 0.05$) (Fig. 3D).

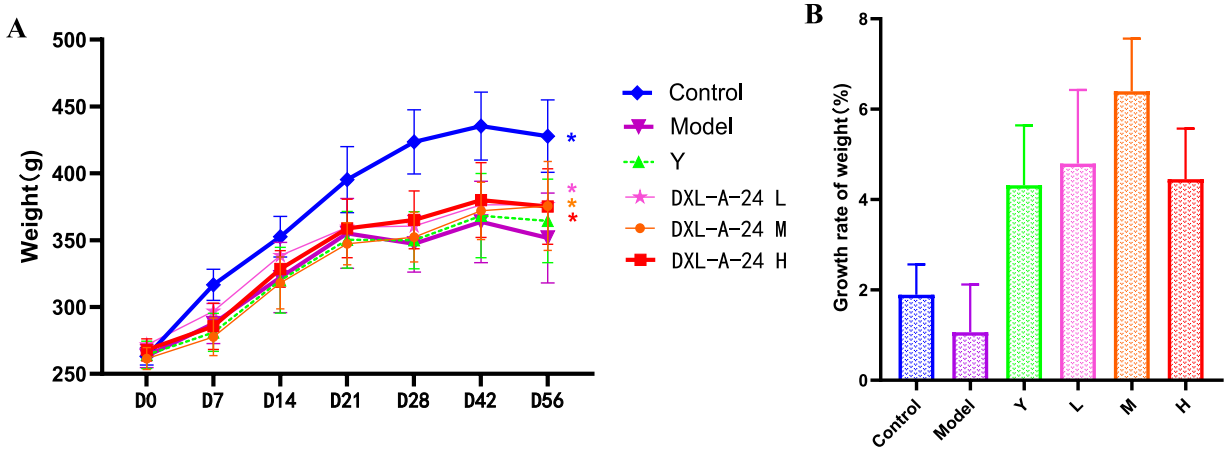

Fig. 2. Body weight of rats during CUMS (D0-28) and treatment (D29-56) period (A). Growth rate of weight after treatment. Model: irritable bowel syndrome. Y: linaclotide capsules. Results are expressed as the mean ± SD (n = 10/group). *p < 0.05 vs model by two-way ANOVA followed by LSD (L) test.

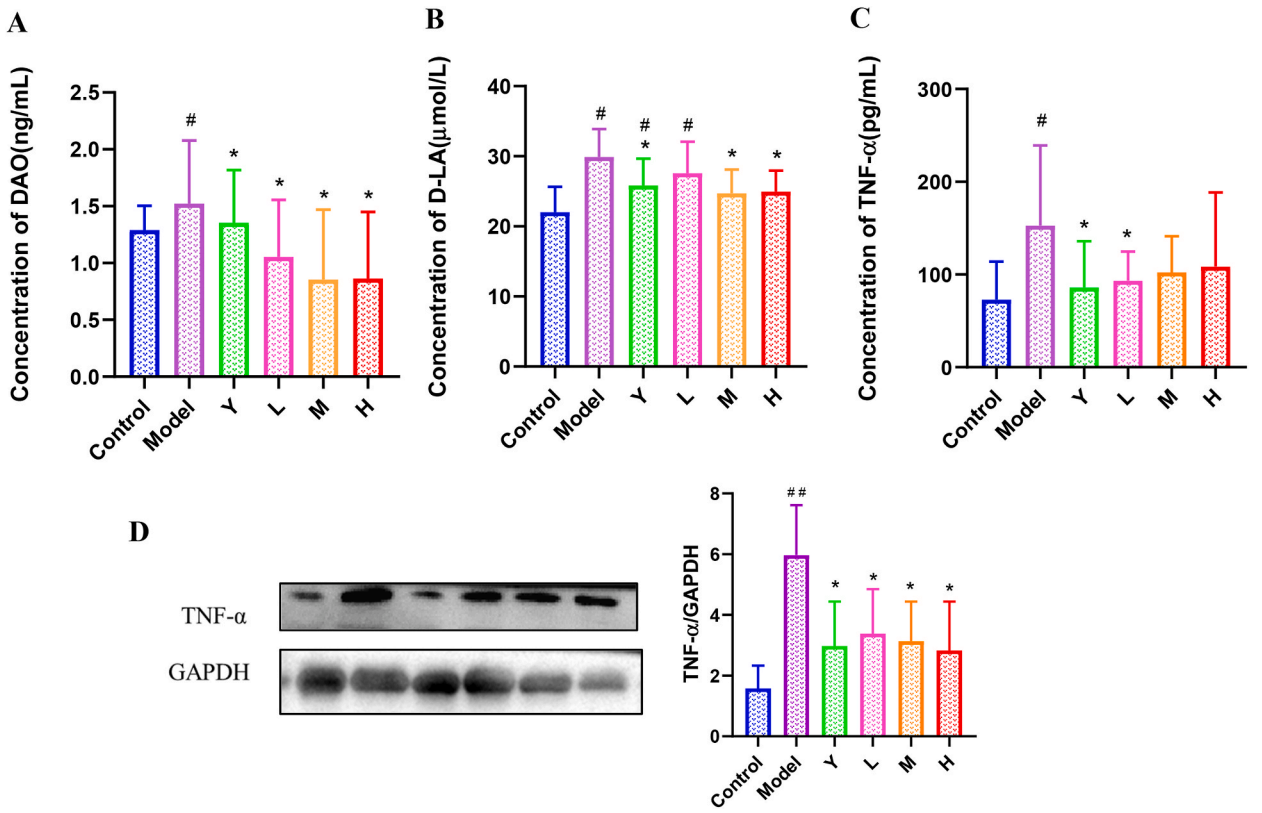

Fig. 3. Changes of serum DAO (A), D-LA (B) and TNF-α (C) levels in rats by ELISA (n = 10/group). The levels of TNF-α (D) by western blotting (n = 3/group). Model: irritable bowel syndrome. Y: linaclotide capsules. Results are expressed as the mean ± SD. *p < 0.05 vs model, #p < 0.05 vs control by two-way ANOVA followed by LSD(L) test.

3.4. Effects of DXL-A-24 on the expression of SP and CGRP in colon tissue

DXL-A-24 H and Y reversed the high expression of SP and CGRP in the colon of rats with CUMS-induced IBS (Fig. 4). The ratio of SP positive cells to the positive staining area of the specimen is 4.57% (control), 9.56% (model), 6.18% (Y), 8.13% (DXL-A-24 L), 7.51% (DXL-A-24 M), 6.09% (DXL-A-24 H), respectively. DXL-A-24 H and Y groups were significantly lower than IBS model group (Fig. 4A,C). Similar to the expression of SP, CGRP is 3.55% (control), 7.76% (model), 4.57% (Y), 4.82% (DXL-A-24 L), 4.11% (DXL-A-24 M), 4.09% (DXL-A-24 H), DXL-A-24 and Y groups were significantly lower than IBS model group ($p < 0.05$) (Fig. 4B,D). Besides, in western blot tests we found SP and CGRP were significant decreased compared with IBS model group ($p < 0.05$) (Fig. 4E,F).

3.5. Pathological changes in colon tissue

By HE staining, we observed that the morphology of colon mucosa and villi of the control group were intact, the epithelial cells and glandular were arranged in conventional manner, no red blood cells or inflammatory cells were found in the submucosa. Besides, the muscular layer was also normal in morphology and structure. The results of the model and others groups were similar to the control (Fig. 5).

3.6. Sequencing data quality assessment and alpha diversity analysis

A total of 8525785 paired-end 16SrRNA gene sequence reads covering the V4 hypervariable regions from 48 samples, 8476945 clean reads were obtained after merging and filtering. 8350406 effective reads were obtained after removing chimeric sequences. A total of 5516 zero-radius operational taxonomic units (ZOTUs) were ultimately generated at a similar level of 97% and further used for data analysis.

In order to prove the number of the sequencing samples was reasonable and included enough the microbial species, we conducted alpha diversity analysis based on the sequencing results of 16SrRNA, and found that with the increase of the number of sequences sampled, exponential dilution curve and substance accumulation of Chao1 and Shannon tended to be flat, indicating that the amount of sequencing data was large enough to cover most of the microbiome (Supplementary Fig. S1). The Shannon and Chao1 indices respectively represent the diversity and richness of flora. We found that the Shannon and Chao1 indices of the DXL-A-24 L, M and H

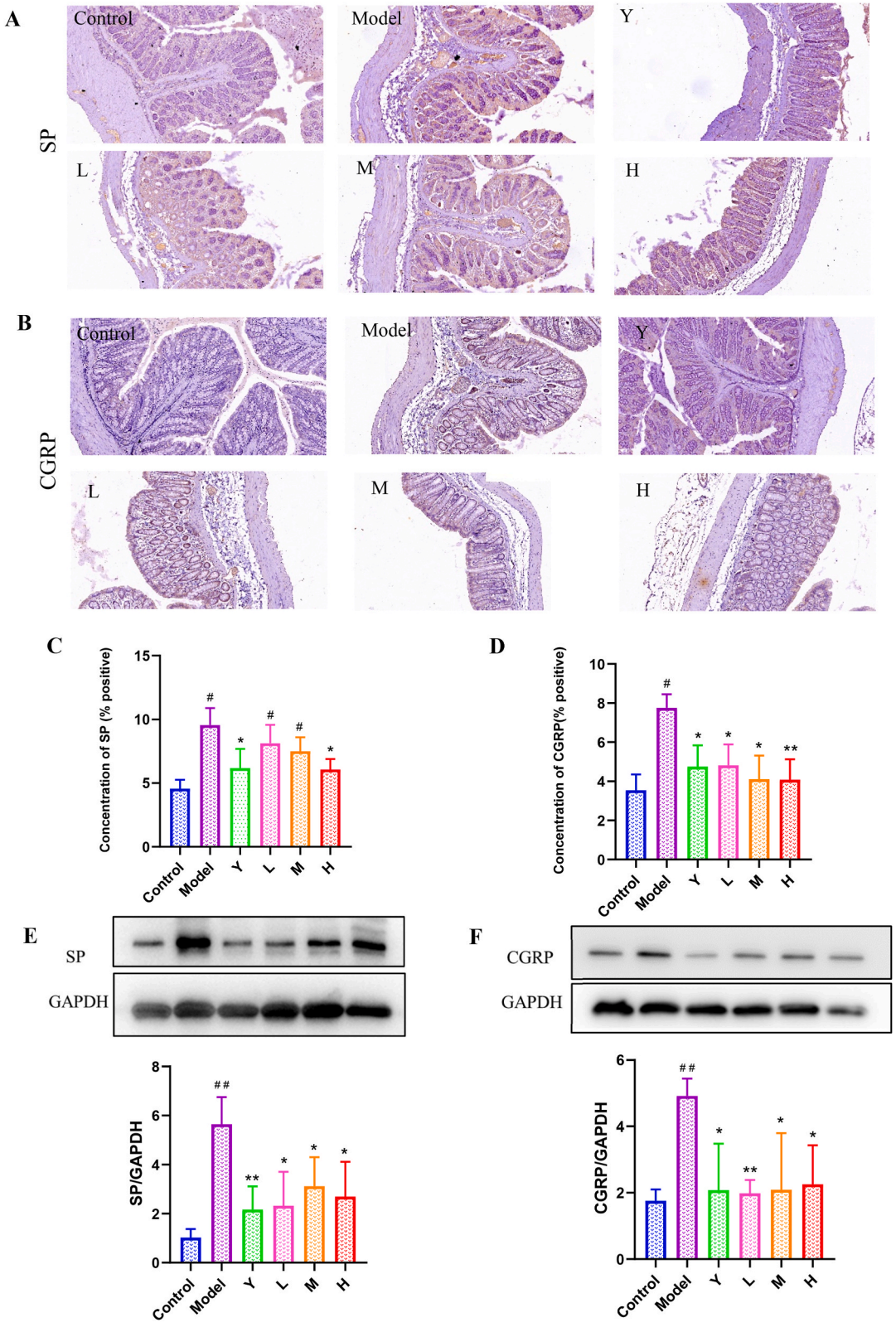

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Fig. 4. The expression of SP and CGRP in the colon. Representative images for SP (A, C) and CGRP (B, D) by immunohistochemistry (200× magnification). The levels of SP (E) and CGRP (F) by western blotting. Model: irritable bowel syndrome. Y: linaclotide capsules. Results are expressed as the mean ± SD (n = 3/group). *p < 0.05 vs model, **p < 0.01 vs model, #p < 0.05 vs control, ##p < 0.01 vs control by two-way ANOVA followed by LSD(L) test.

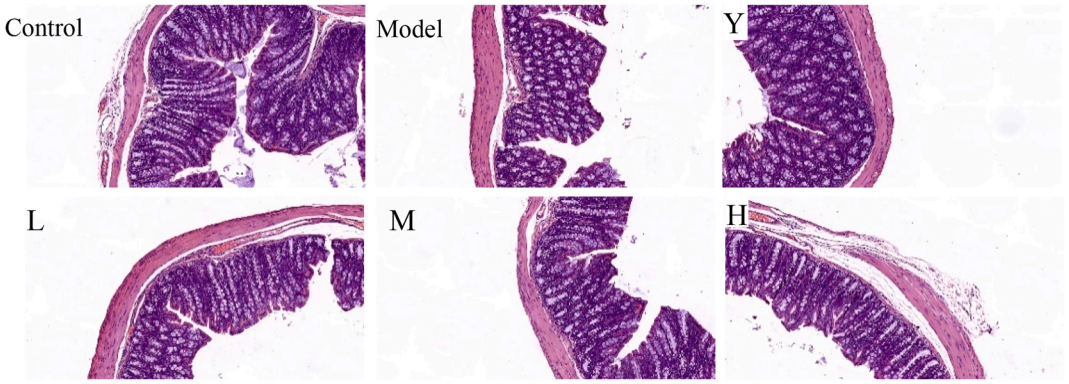

Fig. 5. Pathological changes of colon tissue. Representative H&E-stained images. Model: irritable bowel syndrome. Y: linaclotide capsules.

groups was similar to the control group, they were all significantly higher than the model group ($p < 0.05$) (Fig. 6A,B).

3.7. Comparison of Microbial Community Structures and Beta diversity analysis

We analyzed species annotations of gut microbiota at the phylum level (Fig. 7A). The top 5 were *Firmicutes*, *Bacteroidetes*, *Tenericutes*, *TM7 Bacteria* and *Proteobacteria*, they made up 90% of all gut microbes, of which *Firmicutes* were 72.9% (control), 81.4% (model), 69.9% (Y), 65.7% (DXL-A-24 L), 76.7% (DXL-A-24 M) and 70.4% (DXL-A-24 H), Bacteroidetes were 18.4% (control), 9.5% (model), 23.7% (Y), 25.6% (DXL-A-24 L), 14.3% (DXL-A-24 M) and 19.9% (DXL-A-24 H). Compared with the model group, *Firmicutes* decreased and *Bacteroides* increased after intragastric administration. As shown in the Fig. 7B was the species annotations at the gene level, gut microbiota included *Lactobacillus, Oscillospira, Bacteroidetes* and *Ruminococcus* et al. The PCoA principal coordinate analysis based on Unifrac distance metric was used to evaluate the Beta diversity of the gut microbiota. There were obvious differences between the model group and other groups. Likewise, the result of Anosim analysis (Fig. 8B) is similar to PCoA (Fig. 8A) ($p < 0.05$). These results indicated that gut microbiota varied between DXL-A-24 groups and the model group.

3.8. Analysis of Significantly Different Microbiota

Comparison of all the groups indicated the presence of 18 (control vs model), 17 (Y vs model), 5 (DXL-A-24 L vs model), 0 (DXL-A-24 M vs model), 9 (DXL-A-24 H vs model) taxa with significant differences. Specifically, *Actinobacteria, Alcaligenaceae, Bacilli,*

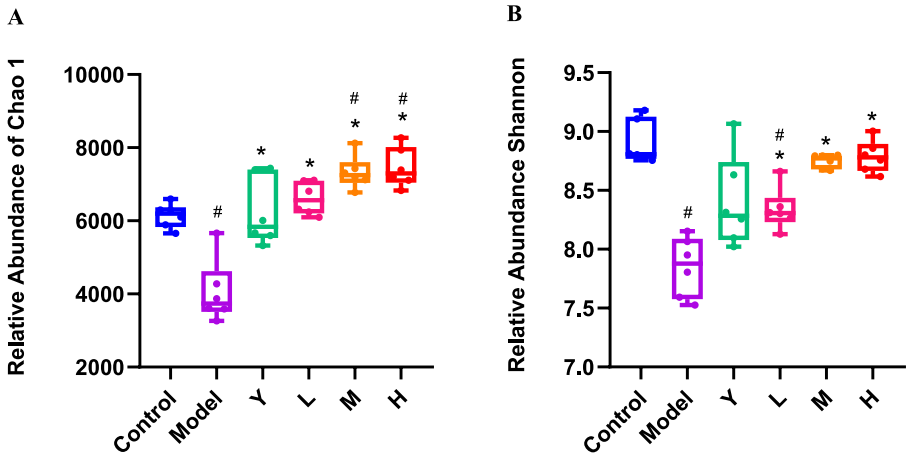

Fig. 6. Alpha diversity values of all groups. The Chao 1 index (A). The Shannon index (B). (n = 8/group). * p < 0.05 vs model, # p < 0.05 vs control by two-way ANOVA followed by LSD(L) test.

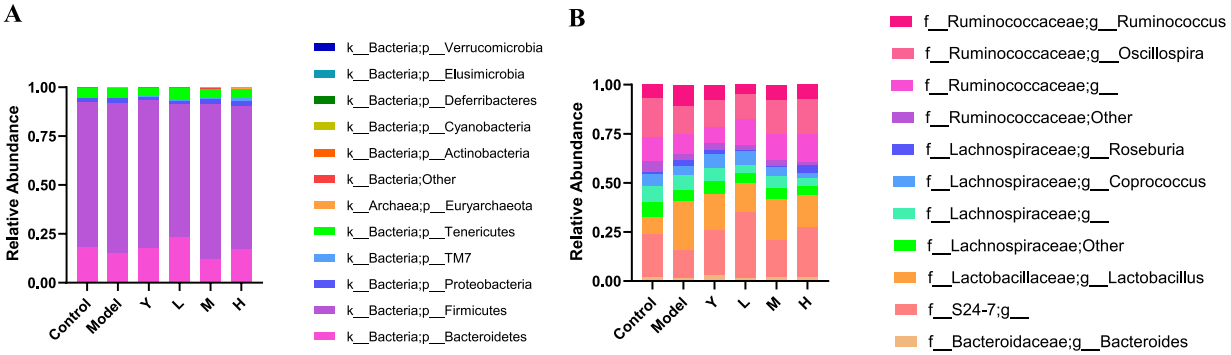

Fig. 7. The histogram of species distribution at the phylum (A) and genus (B) levels revealed by 16S rRNA sequencing.

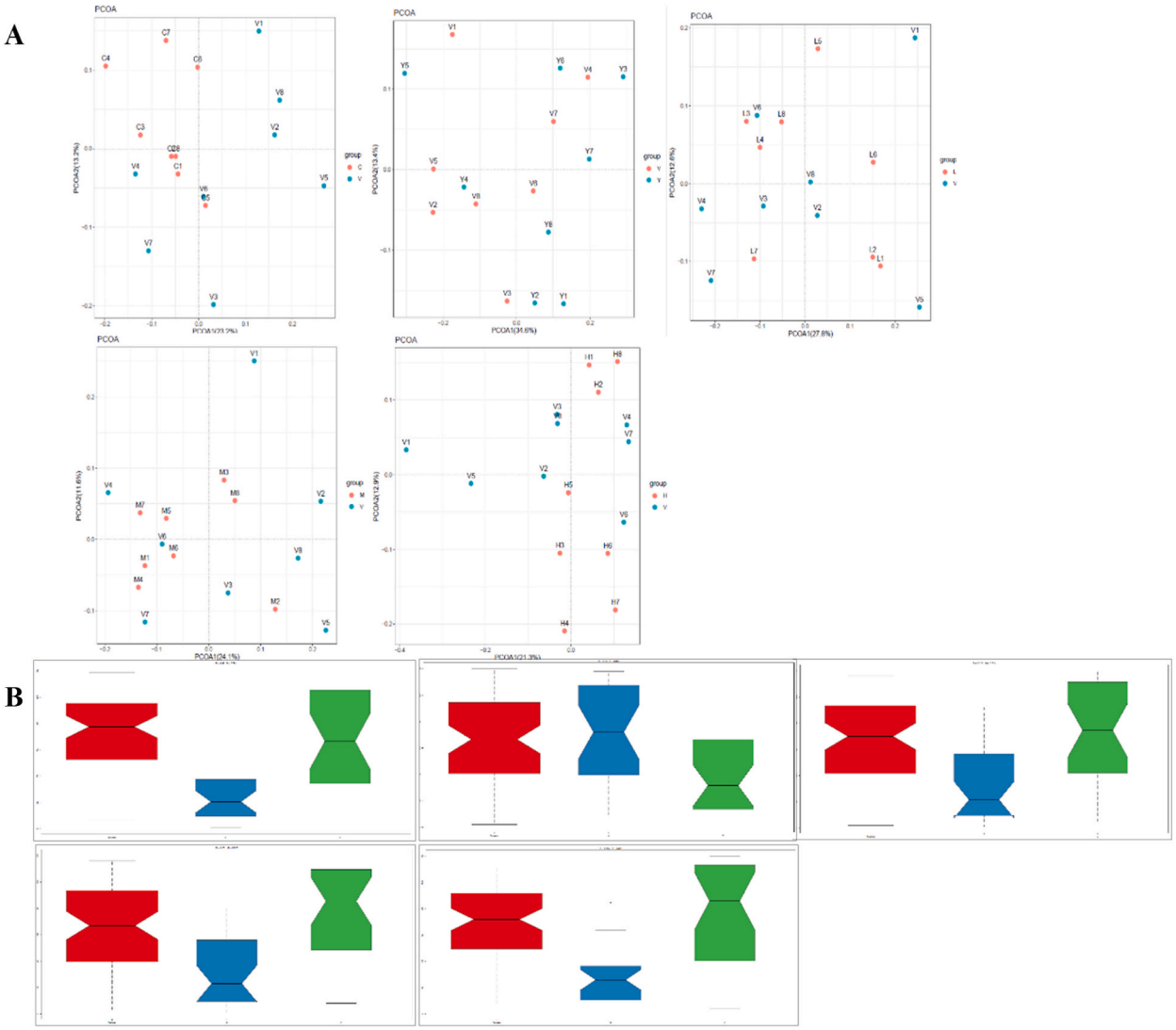

Fig. 8. Beta diversity analysis. PCoA (A) and Anosim (B) analysis among all groups. C, Y, L, M, H vs V from left to right. V: irritable bowel syndrome. Y: linaclotide capsules.

Fig. 9. The differences in gut microbiota taxa among all groups. The phylogenic relationship of the taxon with differences were shown in the cladogram (A) and histogram (B). V: irritable bowel syndrome. Y: linaclotide capsules.

Burkholderiales, Clostridiaceae, Erysipelotrichaceae, Fusobacteriaceae, Gammaproteobacteria, Lactobacillaceae, Mogibacteriaceae and Peptococcaceae were more abundant in the model group. The *Anaeroplasmataceae, Turicibacteraceae, Deferribacteraceae* and their orders were more abundant in the control group. *Anaeroplasmatanceae* and its order, *Antinomycetacea* were more plentiful in the Y group. *Butyricicoccus, Clostridium* and *Paraprevotellaceae* were more abundant in the DXL-A-24 groups. Besides, *Ruminococcaceae, Oscillospira, Cerasicoccaceae, Opitutae* and *Faecalibacterium* were more plentiful in the DXL-A-24 H group (Fig. 9).

4. Discussion

In the present study, the treatment group administered with DXL-A-24 blocked visceral allodynia and increased colonic microbiota diversity of rats at 28 days. In addition, by measuring serum DAO, we proved that it also prevented the damage of intestinal mucosa. It is currently believed that IBS is induced by a variety of factors but the molecular mechanism of IBS has not been elucidated. Stress-induced stimulation could cause symptom flare-ups in IBS [12]. In our research, rats were stimulated by 7 unpredictable ways which was consistent with related literature [9]. The CRD demonstrated that CUMS caused visceral pain and decreased the pain threshold in rats. DXL-A-24 was previously used for analgesia studies, which was probably attributed to the activation of peripheral α7 nAChR and M4 mAChR, the subsequent inhibition of the CaMKIIα/CREB signalling pathway, and finally the inhibition of TNF-α and CGRP expression [7,8]. On this basis, we speculated that DXL-A-24 may be used to irritable bowel syndrome by analgesia. After 28 days of administration, DXL-A-24 improved pain threshold, decreased visceral pain in rats, so we examined the expression of calcitonin gene related peptide (CGRP) and substance P(SP) in colon. CGRP is a neuropeptide composed of 37 amino acids that activates mast cells to release proinflammatory cytokines leading to inflammation and pain hypersensitivity [13,14]. Research has proven anti-calcitonin gene related peptide antibody decreases stress-induced colonic hypersensitivity [15]. SP is a neuropeptide consisting of 11 amino acids that is highly expressed in the gastrointestinal tract and central nervous system [16]. Its release is increased when the gut is damaged or inflamed, and acts similarly to CGRP, activating mast cells and causing visceral pain [17]. In addition, SP exerts biological effects on target cells by binding to its high-affinity receptor (NK1), such as regulating gastrointestinal motility, glandular secretion, vascular permeability, and sensitivity to pain stimuli [18]. We found that DXL-A-24 displayed obvious effects on reduced the expression of CGRP and SP in contrast to the model group. Otherwise, DXL-A-24 had been shown to reduce TNF-α in chronic constriction injury and spinal nerve ligation model [7,8]. Similarly, we found a significant reduction in TNF-α in colon tissue after DXL-A-24 treatment compared to the IBS model group.

In addition to visceral pain, IBS also manifests as disruption of the intestinal barrier and microecological disorders [2]. We found that DXL-A-24 reduced the levels of Diamine oxidase (DAO) and D-lactic acid (D-LA) in serum. DAO is a highly active enzyme distributed in the intestinal mucosal villi, it mainly reflects the damage and repair of intestinal mucosal epithelium, which is released into the blood or into the intestinal cavity after the intestinal mucosa is damaged and necrotic. D-lactic acid is a metabolite of intestinal bacteria, there is a lot of D-lactic acid produced when bacteria multiply in the gut [19]. Serum DAO and D-LA levels are very low under normal circumstances, but their levels have risen in IBS patients, as they are released into the blood in large quantities [20].

The diversity and richness of microbiota are crucial for gut microecosystems homeostasis. We further examined changes in gut microbiota diversity after CUMS and DXL-A-24 treatment, some studies have reported alterations in gut microbial composition and richness in patients. Some of these have proved that gut microbiota richness but not diversity was decreased in IBS-D patients and intestinal microbiota have changed at different taxonomic levels [21]. Other results indicted the composition of the intestinal microbiota differed between IBS and the healthy [22]. Stressful stimuli in animals caused by adverse early life events affect gut microbiota, characterized by reduced gut microbial diversity and richness [23]. However, there were also different views about IBS. IBS rats maternal separation alone and multiple-early-adversity modeling were established and compared the alpha diversity, the results showed between different models had no differences [24]. Consistent with most studies, we found that the gut microbial diversity and richness were reduced and inter individual variation within the host population also increased after CUMS in rats, which was reversed by DXL-A-24.

To further explore the effect of DXL-A-24 on the composition of gut microbial community, we compared the composition and different species among the model and other groups. Most bacteria belong to *Firmicutes* and *Bacteroides* in the human gut, and *Actinomycetes* and *Proteobacteria* are also abundant [25]. Similar to human gut microbiota [21], the rat gut microbes were dominated by *Firmicutes* and *Bacteroides*, in addition, the former increased and latter decreased after CUMS, while there is a certain reversal after DXL-A- 24 treatment.

The model group showed obvious changes in taxa abundance compared with other groups and the bacterial taxonomic differences unique to the model group involved *Actinobacteria, Alcaligenaceae, Bacilli, Burkholderiales, Erysipelotrichaceae, Fusobacteriaceae, Gammaproteobacteria, Lactobacillaceae* and so on. Among them, the abundance of *Actinobacteria*, *Erysipelotrichaceae*, *Gammaproteobacteria Lactobacillaceae* and *Fusobacteriaceae* in the model group was significantly higher than that in other groups. *Fusobacterium* is a mucosa-adhering organism that invades human epithelial cells and endothelial cells [26,27]. Studies have found that the metabolites of *Fusobacterium* contained butyric acid, which could promote visceral hypersensitivity via enteric glial cell-derived nerve growth factor in an IBS-like model [28]. *Gammaproteobacteria* is a type of *Proteobacteria*. The report pointed out that the abundance of *Proteobacteria* in the intestinal tract of the health is not high, and its large proliferation produces inflammatory factors due to certain pathogenic factors [29]. *Erysipelotrichaceae* was reported in IBS rat model maternal separation with significantly elevated abundance, similar to our results [24]. *Actinomycetes* include *Bifidobacterium*, *Collinsella* and other beneficial bacteria. In general, *Bifidobacterium* is beneficial to the integrity of the intestinal mucosal barrier in gastrointestinal diseases [30]. However, some reports have found that the abundance of *Bifidobacterium* was inconsistent in IBS patients, as Moratalla A et al. found increased abundance [31,32,33], while Malinen E et al. demonstrated a decrease in bifidobacterial abundance [34,35,36]. *Lactobacillus* is generally considered to be a

requisite part of its probiotics, which has anti-inflammatory effects by regulating and interacting with epithelial cells, macrophages, dendritic cells and T cells and improves intestinal barrier function by remodeling tight junction [37]. Our results found that the abundance of *Lactobacillus* in the model group was higher than control. Kassinen A found that the abundance of *Lactobacillus* in the gut of IBS patients was significantly increased [34,38]. Interestingly, the abundance of *Lactobacillus* was also increased in Y and DXL-A-24 groups than control group (Supplementary Fig. S2). There are also reports of unchanged [32,33] or decreased [39]. The overall inconsistency suggests that the beneficial properties of these probiotics may be attenuated, possibly due to different causes of irritable bowel syndrome.

The DXL-A-24 groups showed obvious changes in taxa abundance compared with the model group and the bacterial taxonomic differences unique to the DXL-A-24 groups involved *Paraprevotellaceae*, *Ruminococcaceae, Clostridium*, *Faecalibacterium* and *Cerasicoccaceae*.

Among them, *Ruminococcus* and *Butyricicoccus* had a low level in related patients [40,41]. Besides, *Butyricicoccus, Clostridium* and *Ruminococcus* are all short-chain fatty acid-producing genera such as butyrate, which is considered beneficial for colonic epithelial cells to obtain energy [42,43,44].

Rapat Pittaynon summarized many studies and found that *Faecalibacterium* decreased in IBS patients compared with healthy controls [45]. We proved that DXL-A-24 could increase the abundance of *Faecalibacterium*. The biomarker here between *Alcaligenaceae, Burkholderiales* and *Paraprevotellaceae* with CUMS has not been reported elsewhere. Therefore, their effects in irritable bowel syndrome require further studies to confirm.

Our study proved that DXL-A-24 reduced visceral sensitivity, improved intestinal barrier, increased the diversity and richness of gut microbes, and inhibited the reproduction of harmful microbes in rats with IBS. However, there are certain limitations, the molecular mechanism of DXL-A-24 and the relationship between visceral sensitivity, intestinal barrier and microbiota needs to be further elucidated. In addition, this was an animal study, the side effects of the drug have not been clearly identified, and it will be a long time before the drug DXL-A-24 can be used in human clinical trials. Despite the limitations above, our study suggests that DXL-A-24 may be effective in IBS, as visceral hypersensitivity is associated with pain, and the role of DXL-A-24 in pain relief has been demonstrated. In the future, more pharmacological effects of DXL-A-24 need to be further studied.

Author contribution statement

Yan Xu: Conceived and designed the experiments.
Ru Yao: Analyzed and interpreted the data; Wrote the paper.
Wenxue Zhao, Jianguo Zhu: Performed the experiments.
Jingchun Yao, Guimin Zhang, Dongguang Liu: Contributed reagents, materials, analysis tools or data.

Data availability statement

No data was used for the research described in the article.

Institutional Review Board Statement

The animal-related protocols were approved by the Institutional Animal Care and Use Committee of Lunan Pharmaceutical Group Corporation (ethics approval number: AN-IACUC-2021-078).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e16544.

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