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YINDARA-4 relieves visceral hypersensitivity in irritable bowel syndrome rats *via* regulation of gut microbiota and serotonin levels

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Abstract

Objective: The present study aims to evaluate the *in vivo* efficacy of YINDARA-4 in improving the symptoms of irritable bowel syndrome (IBS) in a rat model and investigate the impact of YINDARA-4 on potential targets of IBS management, such as the serotonin level in intestinal tissues and the structure and composition of the gut microbiota.

Methods: We developed an IBS rat model by combining stress from maternal separation, acetic acid administration, and restraint. We administered YINDARA-4 water extract to the IBS rat model for 10 consecutive days. The fecal water content, visceral sensitivity, gut microbiota, and serotonin levels in the colonic tissue were then analyzed and compared between the control group, IBS model group, and YINDARA-4–treated groups.

Results: Treatment with YINDARA-4 reversed visceral hypersensitivity in a dose-dependent manner in the experimental rat model of IBS. The relief of visceral hypersensitivity upon treatment with YINDARA-4 involved regulation of the gut microbiota structure and composition, and normalization of elevated serotonin levels in the colon. The decrease in colonic serotonin levels with YINDARA-4 treatment might be associated with a reduction in the abundance of *Helicobacter* and enrichment of *Butyricimonas*.

Conclusions: Treatment with YINDARA-4 was beneficial against visceral hypersensitivity in a rat model of IBS. The improved symptoms exhibited in IBS rats were associated with favorably altered gut microbiota and normalization of serotonin levels in the colon.

Keywords: 5-Hydroxytryptamine, Gut microbiota, Irritable bowel syndrome, Traditional Mongolian medicine, Visceral hypersensitivity, YINDARA-4

Introduction

Irritable bowel syndrome (IBS) is a commonly chronic, relapsing functional gastrointestinal disorder. IBS prevalence ranges from 10% to 20% of the global population, and occurs in 15% to 20% of Chinese adults[1–2]. IBS significantly worsens the quality of life of patients and places a heavy burden on both the patients themselves and society[3]. The etiology and pathophysiology of IBS are not fully understood. Symptoms of IBS are believed to result from visceral hypersensitivity, abnormal motility, inflammation and infection, neurotransmitter imbalance, psychosocial and genetic factors, and altered gut microbiota[4]. Among these, the role of the gut microbiota has attracted increasing attention in recent years. Patients with IBS have an altered gut microbiota compared with healthy controls[5], and animal studies have shown that perturbation of the intestinal microbiota induces typical symptoms of IBS, such as visceral hypersensitivity and altered gastrointestinal motility[6–7]. These findings suggest a critical role of the gut microbiota in the pathogenesis of IBS. In addition, some studies have reported that the gut microbiota and microbial metabolites regulate visceral hypersensitivity and gastrointestinal motility *via* serotonin synthesis[8–9].

Serotonin plays a significant role in visceral sensitivity, gastrointestinal motility, and immune function. Serotonin is synthesized from tryptophan by tryptophan hydroxylase 1 (TPH1) in enterochromaffin (EC) cells within the gastrointestinal tract. It is stored in secretory granules and released into the lumen or lamina propria when EC cells are activated by various physiological and pathological luminal stimuli[9]. Many studies have shown that serotonin levels are increased in the intestinal tissues and plasma of patients with IBS[4,10]. Several drugs targeting the serotonin system are currently being used for the treatment of IBS. Although these drugs attenuate the symptoms of IBS, they also have adverse side effects such as ischemic colitis and arrhythmia[11–12]. Thus, treatment options for IBS remain limited.

YINDARA-4 is a classical formula in traditional Mongolian medicine that has been used to treat

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gastrointestinal disorders for hundreds of years. It is currently used as an in-hospital preparation for the treatment of IBS in many traditional medical hospitals in Inner Mongolia, and has been demonstrated to be effective in ameliorating the symptoms of IBS[13–16]. YINDARA-4 comprises dry fruit of *Cynanchum thesioides* (Freyn) K. Schum., *Polygonum bistorta* L., *Clematis armandii* Franch., and *Ophiopogon japonicus* (Thunb.) Ker Gawl. Pharmacological research on these four herbs has shown that they exhibit various antimicrobial and/or anti-inflammatory activities[17–21], suggesting that YINDARA-4 may be able to regulate the intestinal microbiota. Therefore, we hypothesized that YINDARA-4 may ameliorate visceral hypersensitivity in a rat model of IBS by manipulating the gut microbiota.

In the present study, the effects of YINDARA-4 on gut pathophysiology and microbiota were investigated using a rat model of IBS developed using multiple stimuli. The objectives of this study were to (1) evaluate the *in vivo* efficacy of YINDARA-4 in improving symptoms in rats undergoing experimental IBS, and (2) investigate the impact of YINDARA-4 on potential targets of IBS management, such as serotonin levels in intestinal tissues and the structure and composition of the gut microbiota.

Materials and methods

Preparation of YINDARA-4

The herbal formula YINDARA-4 is a combination of four medicinal herbs, including *Cynanchum thesioides* (Freyn) K. Schum. (*Vincetoxicum sibiricum* (L.) Decne., (*Temeen-Huh* in Mongolian and *Di-Shao-Gua* in Chinese), *Polygonum bistorta* L. (Bistorta *officinalis* Delarbre [Polygonaceae], *Meher* in Mongolian, and *Quan-Shen* in Chinese), *Clematis armandii* Franch. (*Balega* in Mongolian and *Chuan-Mu-Tong* in Chinese), and *Ophiopogon japonicus* (Thunb.) Ker Gawl. (*Chagan-Bong-a* in Mongolian and *Mai-Dong* in Chinese), at a ratio of 9:7:7:5 (w/w/w/w), respectively. *Cynanchum thesioides* (Freyn) K. Schum, also called Yindara (spelled as Yindara or Yindari in Mongolian) or Dugemonong in traditional Mongolian medicine, was collected from the vicinity of Barun Akta Gacha, Horqin Left Wing Rear Banner of Inner Mongolia, China (GPS coordinates: 122.18, 43.02). Fresh fruits were collected in July and August, dried in the shade, and then broken open to remove fly floes. The remaining three herbs were purchased from Tong-Ren-Tang (Beijing, China). All the herbs were identified by two experienced pharmacists. Voucher specimens (DSGgan201807, CMTtrt, QStrt, and MDtrt) were deposited at the School of Pharmacy, Minzu University of China. The names of the plants were crosschecked using the Plant List database (http://www.theplantlist.org) on July 3, 2020. For the preparation of YINDARA-4, the four herbs were first crushed and mixed at the above ratios and then boiled in water (ratio of material to liquid 1:8) for 1 hour, filtered through a gauze, and then boiled again in water for 1 h. The supernatant from two runs was then collected and freeze-dried to make the dried YINDARA-4 extract. Dry YINDARA-4 extract (227 g) was obtained from 1 kg of raw YINDARA-4.

Analysis of the chemical components of YINDARA-4

The main chemical components in the water extract of YINDARA-4 were detected using ultra-high performance liquid chromatography-quadrupole time-of-flight-tandem mass spectrometry (UHPLC-QTOF-MS/MS). UHPLC-QTOF-MS/MS detection was conducted on a Triple TOFTM6600 system with a Duo Spray source in positive ion mode (AB SCIEX, Foster City, CA, USA). Electrospray ionization was applied in the positive ion mode with the following parameters: ion spray voltage, 5,500 V; ion source temperature, 500°C; curtain gas, 35 psi; nebulizer gas (GS1), 50 psi; heater gas (GS2), 50 psi; and declustering potential (DP), 80 V. The mass ranges were set at m/z 100 to 1,000 Da for the time-of-flight mass spectrometry (TOF-MS) scan and 100 to 1,000 Da for the TOF MS/MS experiments. In the information-dependent acquisition (IDA)–mediated MS/MS experiment, the collision energy (CE) was set at 40 ev, and the collision energy spread (CES) was (±)20 ev for UHPLC-QTOF-MS/MS detection. The most intensive five ions from each TOF-MS scan were selected for MS/MS fragmentation. Dynamic background subtraction (DBS) was applied to match the IDA tests for UHPLC-QTOF-MS/MS detection. LC-MS/MS data were analyzed using PeakView 1.2 software (AB SCIEX, Foster City, CA, USA).

Animals

Specific pathogen-free (SPF) Sprague Dawley rats were used in the present study. Pregnant rats were purchased from SPF Biotechnology Co., Ltd. (Beijing, China) on day 15 of the pregnancy. Rats were housed in hardwood chip bedding cages (42 cm × 20.5 cm × 20 cm) in a designated room at 22 ± 2°C with a 12/12 h light/dark cycle in a controlled environment. The rats had *ad libitum* access to water and standard chow. They were checked daily for delivery and the day after birth was defined as postnatal day (PND) 1. The pups were housed with the dam until weaning on PND22. After weaning, three animals were housed in a cage, and all female rats were excluded from the study to avoid the effects of sex hormones. The study design was approved by the Ethics Committee of Minzu University of China (ECMUC2019009CA). The experiments were performed with good laboratory practices (GLP) in a GLP-accredited laboratory.

Study design

The rats were randomly assigned to 5 groups (n = 7 per group): (1) control (CTRL), (2) IBS model (IBS), (3) YINDARA-4 high-dosage intervention (YDRh), (4) YINDARA-4 middle-dosage intervention (YDRm), and (5) YINDARA-4 low-dosage intervention (YDRl). The rats in the CRTL group were not manipulated. The rats in the other four groups were exposed to multiple adverse stresses, including maternal separation, acetic acid instillation, and restraint during PND1 to PND42. Maternal separation was conducted once daily from 09:00 am to 12:00 am during PND1 to PND21 by removing the litter from the dam's cage into separate cages on top of heating pads (Suzhou Guofei Laboratory Instrument

Co., Ltd., Suzhou, China) set at 30°C to 33°C in a separate room. Acetic acid instillation was conducted once daily at 09:00 am from PND15 to PND28. Acetic acid at a concentration of 0.5% (Sangon Biotech Co., Ltd., Shanghai, China) was instilled into the colon with a tube (Head Biotechnology Co., Ltd., Beijing, China) with a diameter of 1 mm from the anus. Considering that young rats may not be able to withstand excessive stimulation, the volume of the enemas was 0.2 mL initially and was increased by 0.1 mL per day until the dose was 0.5 mL per day, which was then maintained throughout. Restraint stress was conducted once daily from 09:00 am to 12:00 am during PND29 to PND42. The rats were kept in a handcrafted plastic restraint cylinder for 3 h each day for restraint stress. The dimensions of the restraint cylinder could be manually adjusted to a suitable size such that the rat could not turn around freely.

All rats were fed normally from PND43 to PND68. From PND69 to PND78, freeze-dried extracts of YINDARA-4 were re-dissolved in water and administered by oral gavage at approximately 11:00 am daily to the rats in the YDRh, YDRm, and YDRl groups, while the rats in the IBS group received normal saline (1 mL). The dose of YINDARA-4 used was based on the conversion of the recommended adult dosage (10 g raw YINDARA-4/day). Rats in the YDRh, YDRm, and YDRl groups were administered YINDARA-4 at a dose of 1.8, 0.9, and 0.45 g/kg, respectively, which corresponds to 2 times, 1 time, and 1/2 times the human equivalent dose, respectively.

All rats were anesthetized using isoflurane (Shuilantai Chemical Co., Ltd., Zaozhuang, China) for collection of colorectal fecal contents and tissue samples, and were then euthanized by exsanguination on PND84 as described previously[22].

Visceral sensitivity assessment

Visceral hypersensitivity was assessed using the abdominal withdrawal reflex (AWR) score at PND78 from 09:00 am to 12:00 am, as described previously[23–24]. In brief, the rats were moved into a self-made plastic cubicle and allowed to acclimate to the container for 2 min. A self-made flexible balloon attached to Tygon tubing with a diameter of 2 mm (Head Biotechnology Co., Ltd., Beijing, China) was inserted into the descending colon (8 cm from the anus). The balloon was rapidly inflated to various pressures (40, 50, 60, 70, 80 and 90 mmHg) for a 10-second colorectal distension stimulation, and the behavioral responses of the rats to each pressure were assessed by two blinded observers using the AWR score system, where 0 represents normal behavior without any response, 1 represents light response motion without visible abdominal muscle contraction, 2 represents visible abdominal muscle contraction, 3 represents visible abdominal wall lifting, and 4 represents visible pelvic structure lifting and body arching.

Fecal water content

The fecal water content of each rat was measured on PND82 at 09:00 am as an objective measure of stool consistency. Fresh feces were collected, weighed immediately, dried in an oven at 100°C for 10 min, and weighed again. The fecal water content was calculated using the following formula:

$$\text{fecal water content } (\%) = (\text{wet weight of fecesdried weight of feces}] / \text{wet weight of feces} \times 100\%.$$

Collection of the colorectal fecal contents

Colorectal fecal contents were collected on PND84 just after euthanasia. Fresh fecal pellets from the distal colon were collected and preserved in a solution containing 4 M guanidine thiocyanate (Solarbio Life Sciences Co., Ltd. Beijing, China) at –20°C. All fecal samples were then transported to the laboratory and stored at –80°C for a day.

Histopathological examination

Distal colon tissues were fixed in neutral buffered 10% formalin for 12 h, dehydrated, and embedded in paraffin. For hematoxylin-eosin staining, two sequential 5 μm thick sections were cut and processed. Slides were de-identified and observed by a pathologist.

For immunohistochemistry, two sequential 5 μm thick sections were cut for each sample, pretreated with 3% hydrogen peroxide and normal horse serum, and incubated with an antibody against serotonin (1:400, ab66047, Abcam, Cambridge, UK) for 8 h at 4°C. The sections were then incubated with a secondary antibody and developed using 3,3-diaminobenzidine. The sections were then incubated with hematoxylin for nuclear counterstaining. A total of five random fields (40× objective magnification) per section were observed using Image-Pro Plus software (version 5.0; Media Cybernetics, Silver Spring, MD, USA) by an investigator blinded to the group settings. Optical density was calibrated before measuring the area sum, density mean, and integrated optical density (IOD). The areas of interest were set as follows: hue (0–200), saturation (0–255), and intensity (0–90). The reagents used for histopathological examination were purchased from Servicebio Technology Co. Ltd., Wuhan, China.

Microbiota sequencing

Total bacterial DNA was extracted using the Power Soil DNA Isolation Kit (MO Bio Laboratories, Inc. Carlsbad, CA, USA). After checking the quality and quantity of the DNA sample using OD ratios at 260 nm/280 nm and 260 nm/230 nm, the V3–V4 hypervariable region of the bacterial 16S rRNA gene was amplified with the common primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′), combined with adapter sequences and barcode sequences. Second-generation sequencing was conducted on the purified, pooled PCR product sample using the HiSeq 2500 platform (Illumina, Inc. San Diego, CA, USA) (2 × 250 paired ends) at the Biomarker Technologies Corporation Beijing, China.

Bioinformatic analyses

Raw sequence reads were processed using the UNOISE pipeline in the Usearch v11.0.667linux32 program (www.drive5.com/usearch/). High-quality sequences were classified into zero-radius operational taxonomic units (ZOTUs). The Ribosomal Database Project (RDP) classifier was used to annotate the taxonomic information of each ZOTU sequence with an 80% confidence threshold. The Chao1, Shannon, and Simpson indices were calculated using the QIIME 1.91 pipeline[25]. Principal coordinates analysis (PCoA), distance-based redundancy analysis (db-RDA), and adonis tests were performed to evaluate differences among bacterial communities based on Bray–Curtis distance metrics using "vegan" package in R[26].

Statistical analyses

R software (version 3.52, R Foundation for Statistical Computing, Vienna, Austria) was used for the statistical analyses. The Shapiro–Wilk test was used to test for normal distribution, and the analysis of variance (ANOVA) test or Kruskal–Wallis test was used to evaluate the differences in the measured variables among the groups. Spearman's correlation analysis was performed using "psych" package in R to determine associations between different variables. Finally, the linear discriminant analysis (LDA) effect size (LEfSe) method (threshold of ±2) was used to explore significantly different bacteria among the treatment groups.

Results

Phytochemical characterization of YINDARA-4

Twenty-six compounds were identified in the water extract of YINDARA-4 (Figure 1). Molecular information of the compounds is presented in Table 1. Among the 26 compounds, there were seven flavonoids (including baicalin, bavachinin A, rocyanidin B2, salvianolic acid B, catechin, rutin, and quercetin), seven alkaloids (including betaine, trigonelline, stachydrine, arecoline, atropine, irinotecan, and norisoboldine), four phenylpropanoid compounds (including praeruptorin C +Na, phenprobamate, fraxin, and easculetin), three organic acids (succinic acid, quinic acid, and 4-O-Caffeoyl Quinic acid), two terpenoids (ginkgolide B +NH3 and resveratrol), two quinones (diacerein and tanshinone IIA), and one adenosine analog (cordycepin).

Effect of YINDARA-4 on fecal water content

The fecal water content of the IBS model group was lower than that of the CTRL group (11.6% ± 3.9% vs. 20.1% ± 4.4%, n = 7), and increased to varying degrees after YDR treatment (15.2% ± 3.7% for the YDRl group, 17.3% ± 6.8% for the YDRm group, and 15.7% ± 4.8% for the YDRh group, n = 7). However, none of the differences were statistically significant (P = 0.11; Figure 2A).

Effect of YINDARA-4 on visceral hypersensitivity

Our results showed that 30 mmHg of pressure was the non-nociceptive intracolonic pressure that did not evoke any differences in the abdominal muscle contractility of rats. When the colorectal distention pressure reached 40 mmHg, the AWR scores differed significantly between the groups. The AWR scores of the IBS model group were significantly higher than those of the CTRL group at 40 to 80 mmHg pressure. After the intervention, the AWR scores of all three YINDARA-4–treated groups were lower than those of the IBS model group. The difference in AWR scores was significant for the high dose group at pressures of 40 to 90 mmHg, whereas for the middle dosage and low dosage groups, the difference in AWR scores was significant at pressures of 50 and 40 to 70 mmHg, respectively (Figure 2B).

The minimal distention pressure that evoked behavioral responses corresponding to each of the AWR scores was defined as the distension threshold (DT). A comparison of the DT values among the three treatment groups is shown in Figure 2C. The DT for AWR scores of 2, 3, and 4 showed an increasing trend with the increase in the YINDARA-4 intervention dose. Among them, the DT of AWR score 4 was significantly different among the different dose groups. Spearman's rho correlation analysis was performed on the association between the YINDARA-4 intervention dose and the DT of the rats, and it was found that there was a statistically significant positive correlation between the YINDARA-4 intervention dose and the DT of AWR score 4 (rho = 0.506, P = 0.019), showing that treatment with YINDARA-4 significantly decreased visceral hypersensitivity in rats undergoing experimental IBS in a dose-dependent manner.

Histopathology of colonic tissues

In our observations, the structure of the intestinal mucosa was intact, with a normal epithelium. The simple columnar epithelium within the colonic mucosa was arranged in

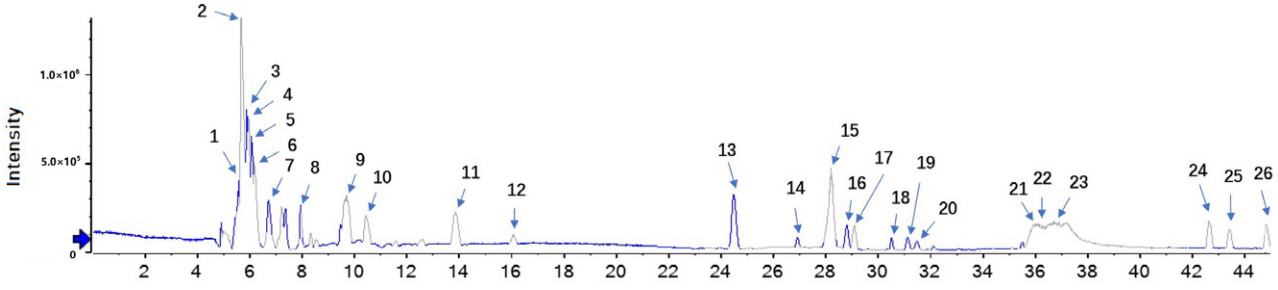

Figure 1. Ultra-high performance liquid chromatography-quadrupole time-of-flight-tandem mass spectrometry chromatograms of YINDARA-4 extract.

Table 1

Compounds of water extract from YINDARA-4

No.	Found at retention time (min)	Name	Formula	Mass (Da)	Found at mass (Da)	Extraction mass (Da)	Error (ppm)
1	5.54	Baicalin	$C_{21}H_{18}O_{11}$	446.0849	447.0900	447.0922	−4.8
2	5.70	Cordycepin	$C_{10}H_{13}N_5O_3$	251.1018	252.1083	252.1091	−3.3
3	5.81	Betaine	$C_5H_{11}NO_2$	117.0790	118.0862	118.0863	−0.7
4	5.89	Trigonelline	$C_7H_7NO_2$	137.0477	138.0549	138.0550	−0.6
5	6.09	Ginkgolide B +NH_3	$C_{20}H_{24}O_{10}.NH_3$	441.1635	442.1693	442.1708	−3.2
6	6.15	Stachydrine	$C_7H_{13}NO_2$	143.0946	144.1018	144.1019	−0.8
7	6.75	Arecoline	$C_8H_{13}NO_2$	155.0946	156.1015	156.1019	−2.4
8	7.95	Succinic acid	$C_4H_6O_4$	118.0266	119.0348	119.0339	7.9
9	9.58	Bavachinin A	$C_{21}H_{22}O_4$	338.1518	339.1571	339.1591	−5.8
10	10.55	Praeruptorin C +Na	$C_{24}H_{28}O_7.Na$	451.1733	452.1788	452.1806	−3.8
11	13.86	Quinic acid	$C_7H_{12}O_6$	192.0634	193.0689	193.0707	−9.1
12	16.09	Phenprobamate	$C_9H_{11}NO_2$	165.0790	166.0862	166.0863	−0.3
13	24.63	Atropine	$C_{17}H_{23}NO_3$	289.1678	290.1724	290.1751	−9.2
14	26.92	Rocyanidin B2	$C_{30}H_{26}O_{12}$	578.1425	579.1501	579.1497	0.6
15	28.20	4-O-Caffeoyl quinic acid	$C_{16}H_{18}O_9$	354.0951	355.1028	355.1024	1.2
16	28.76	Salvianolic acid B	$C_{36}H_{30}O_{16}$	718.1534	719.1560	719.1607	−6.5
17	29.09	Catechin	$C_{15}H_{14}O_6$	290.0790	291.0866	291.0863	1.0
18	30.51	Fraxin	$C_{16}H_{18}O_{10}$	370.0900	371.1009	371.0973	9.7
19	31.32	Esculetin	$C_9H_6O_4$	178.0266	179.0339	179.0339	0.0
20	31.50	Diacerein	$C_{19}H_{12}O_8$	368.0532	369.0631	369.0605	6.9
21	35.83	Rutin	$C_{27}H_{30}O_{16}$	610.1534	611.1667	611.1607	9.8
22	36.36	Quercetin	$C_{15}H_{10}O_7$	302.0427	303.0503	303.0499	1.1
23	36.80	Resveratrol	$C_{14}H_{12}O_3$	228.0786	229.0861	229.0859	1.0
24	42.65	Irinotecan	$C_{33}H_{38}N_4O_6$	586.2791	587.2909	587.2864	7.7
25	43.42	Tanshinone IIA	$C_{19}H_{18}O_3$	294.1256	295.1327	295.1329	−0.6
26	44.74	Norisoboldine	$C_{18}H_{19}NO_4$	313.1314	314.1396	314.1387	2.9

an orderly fashion, and goblet cells were rich in the CTRL and IBS model groups. After the intervention, there was no significant difference in the histological features among the three treatment groups and IBS model group.

Immunohistochemistry for the expression of serotonin in the colon

Immunohistochemistry (Figure 3A–C) revealed that the number of serotonin-positive areas and the IOD of serotonin (brown particles) in the colon were significantly higher in the IBS model group than in the CTRL group ($P < 0.001$). The number of serotonin-positive areas and IOD of serotonin were significantly lower in the YDRh group than in the IBS model group ($P < 0.001$).

Effect of YINDARA-4 on microbiota community structure and composition in colon content

Among the three YINDARA-4 dosage groups, the YDRh group showed the most significant changes in visceral hypersensitivity. To investigate the potential interrelationships between the alteration of the gut microbiota and the improvement of IBS symptoms, we investigated the microbiota in the colon contents of rats in the CTRL, IBS, and YDRh groups. A total of 978,728 clean reads were collected from 21 samples (seven samples each from the CTRL, IBS, and YDRh groups) after trimming and filtering. A ZOTU table with 4,139 ZOTUs was generated and used for data analysis. Using the RDP classifier, 4,139, 3,966, 3,910, 3,743, and 1,738 ZOTUs were annotated at the phylum, class, order, family, and genus levels, respectively, comprising seven phyla, 12 classes, 13 orders, 20 families, and 33 genera.

In the comparison of alpha diversity among different groups, no significant differences were observed for any of the three diversity indices (data not shown). However, a PCoA based on the Bray–Curtis distance matrix revealed a trend toward a differential distribution for the three groups (Figure 4). Adonis tests and db-RDA analyses confirmed the significance of the associations between the different groups and the structure of the bacterial communities (Table 2).

For the microbiota composition, LEfSe analysis in the comparison of the IBS model group and the YDRh group showed that the abundance of *Butyricimonas* increased significantly after YDRh treatment, while those of *Helicobacter* and its family, order, and class were significantly reduced in the YDRh group (shown in Figure 5A). Further comparison of the abundance of these two genera within the three groups (Figure 5B) showed that the abundance of the genus *Butyricimonas* was significantly lower in the IBS model group than in the CTRL and YDRh groups, whereas there was no difference between the CTRL and YDRh groups. The abundance of *Helicobacter* was significantly higher in the IBS model group than in the CTRL group and was decreased after treatment with YDRh. No significant difference was found in the abundance of *Helicobacter* between the CTRL and YDRh groups.

Analysis of correlation between the differential microbiota composition and gut pathophysiological parameters

To further assess potential correlations between gut pathophysiological parameters and certain microbiota composition, we conducted Spearman's correlation tests

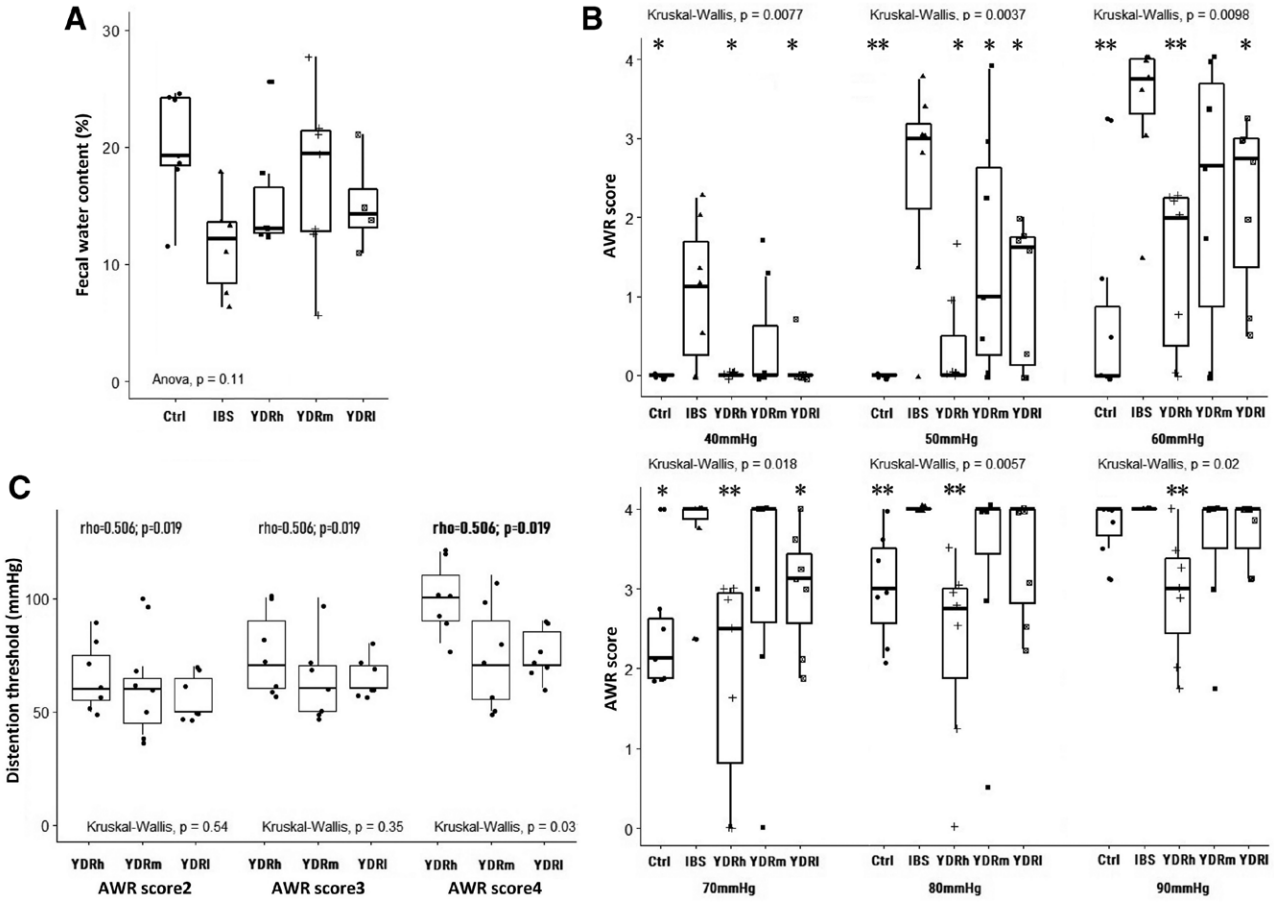

Figure 2. Comparison of the fecal water content and visceral sensitivity in rats from different groups. (A) Comparison of the fecal water content in rats after YINDARA-4 treatment (n = 7) with the IBS model group. (B) Comparison of the AWR scores in rats from different groups (n = 7) in response to graded colorectal distension with different pressures. (C) Correlations between the YINDARA-4 intervention dose and the DT of rats. Minimal distention pressures that evoked behavioral responses corresponding to each of the AWR scores were defined as the DT. *P < 0.05 as compared with the IBS model group. **P < 0.01 as compared with the IBS model group. AWR: Abdominal withdrawal reaction; Ctrl: Control; DT: Distension threshold; IBS: Irritable bowel syndrome; YDRh: YINDARA-4 high-dosage intervention; YDRl: YINDARA-4 low-dosage intervention; YDRm: YINDARA-4 middle-dosage intervention.

on relative abundance data of the genera *Butyricimonas* and *Helicobacter* using the psych package in R. The genus *Butyricimonas* was positively correlated with the DT for AWR scores 2 and 3 and the fecal water content, and was negatively correlated with the two indicators of serotonin expression in the colon, the number of serotonin-positive areas and the IOD of serotonin. The genus *Helicobacter* was positively correlated with the number of serotonin-positive areas and the IOD of serotonin (Figure 6).

Discussion

In the present study, a rat model of IBS was developed by mimicking multiple etiological factors of IBS through the combination of maternal separation, acetic acid instillation, and restraint stress. Rats undergoing experimental IBS exhibited symptoms of visceral hypersensitivity and abnormal fecal water content, with no organ-level changes found upon pathological examination, indicating that the model was successfully constructed. A 10-day administration of YINDARA-4 significantly decreased the AWR score and increased the pain threshold in rats undergoing experimental IBS in a dose-dependent manner, suggesting that YINDARA-4 attenuated visceral hypersensitivity in IBS model rats.

Serotonin is an important neurotransmitter involved in mutual communication between the brain and the gut[27]. It has been reported that the abundance of serotonin is increased in the blood of IBS patients, and alterations in the gastrointestinal serotonin signaling pathway are considered to be associated with disrupted visceral sensation and intestinal motility in patients with IBS[28–30]. In the present study, we observed that the expression level of serotonin in the colon was significantly increased in the IBS model group compared with that in the CTRL group and was restored to CTRL levels in the YDRh group. This suggests that the serotonin level in the colon might play a key role in the amelioration of visceral sensitivity upon treatment with YINDARA-4.

Increasing evidence has demonstrated the pivotal role of the gut microbiota in the pathogenesis of IBS; thus, manipulation of the gut microbiota is emerging as an attractive therapeutic strategy for this disease[31]. Various microbiota-manipulating interventions, such as fecal microbiota transplantation, administration of prebiotics, probiotics, synbiotics, and certain non-absorbable antibiotics, have been reported to alter the ecological structure of the intestinal microbial community, selectively suppress harmful bacteria, and stimulate the activity or growth of beneficial bacteria, thereby ameliorating the symptoms

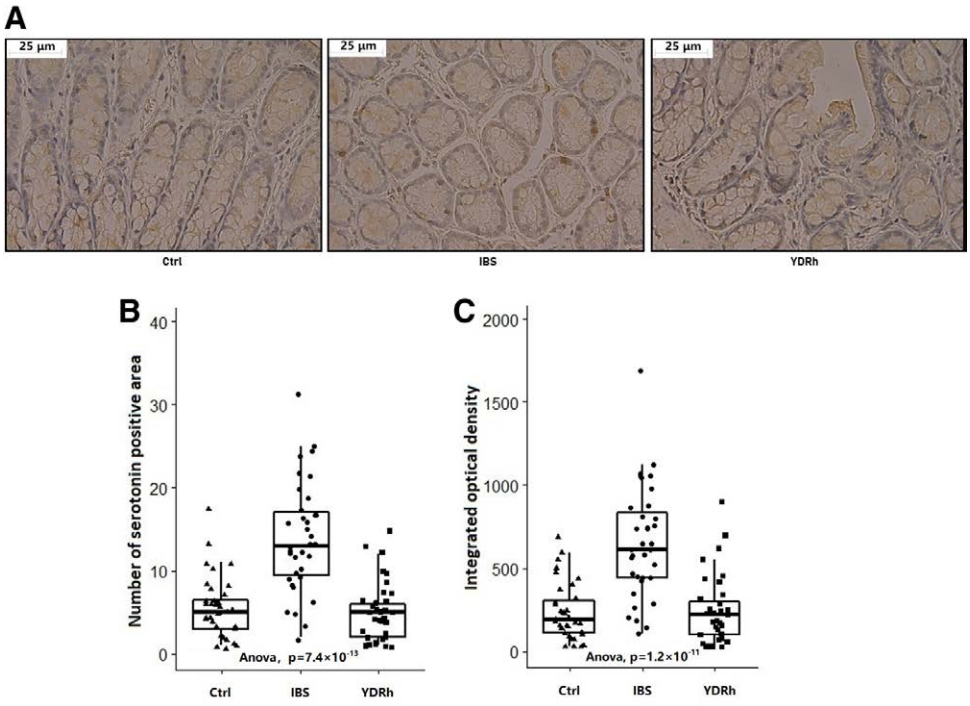

Figure 3. Comparison of the colonic serotonin level between different groups. (A) Representative immunohistochemistry images taken at 40× magnification. (B) Comparison of the number of serotonin-positive areas between the different groups. (C) Comparison of the integrated optical density of serotonin between the different groups. Ctrl: Control; IBS: Irritable bowel syndrome; YDRh: YINDARA-4 high-dosage intervention.

of IBS[32–35]. Studies have also shown that enteric resident microbiota can regulate the synthesis of serotonin in intestinal EC cells and host serotonergic signaling[9]. Our results demonstrated that YINDARA-4 treatment exerted similar microbiota-manipulating effects in IBS model rats. The microbial community structure in the colon contents of IBS model rats was significantly altered after treatment with YINDARA-4. The abundance of the harmful bacterial genus *Helicobacter* was significantly lower in the YDRh group than in the IBS group, whereas the beneficial bacterial genus *Butyricimonas* was significantly enriched in the YDRh group.

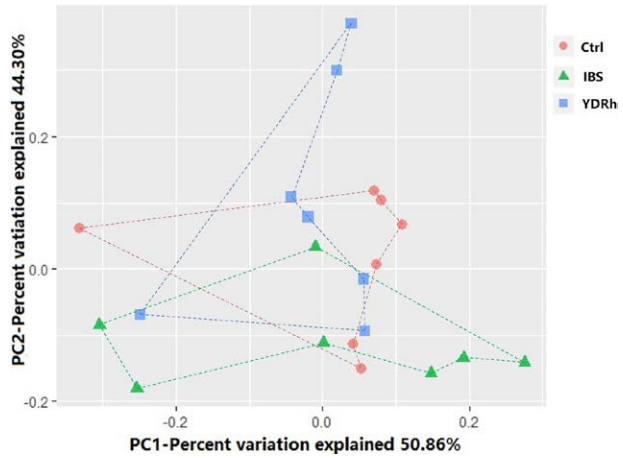

Figure 4. Two dimensions PCoA plots showing differences in the gut microbiota of rats from the different groups based on the Bray–Curtis distance metric (n = 7). Ctrl: Control; IBS: Irritable bowel syndrome; PCoA: Principal coordinates analysis; YDRh: YINDARA-4 high-dosage intervention.

The genus *Helicobacter* is a highly prevalent bacterium and is associated with various chronic gastrointestinal diseases in both humans and animals, such as chronic gastritis, peptic ulcer, and gastric carcinoma[36]. Several studies have proposed that *Helicobacter* may play a role in IBS[37–38]. *Helicobacter* infection can induce systemic inflammatory responses, which could lead to the stimulation of mast cells and EC cells and increase the secretion of proinflammatory neurotransmitters, such as serotonin, substance P, and calcitonin gene–related peptide, all of which have an intimate relationship with the symptoms of IBS[39–40]. Our results are consistent with the above findings, showing that the abundance of *Helicobacter* was positively associated with the expression of serotonin in the colon.

On the other hand, the genus *Butyricimonas* is a group of obligate anaerobic bacteria, which can produce butyrate. Butyrate is one of the most common short-chain fatty acids in the intestinal tract, and previous studies have shown that it can induce regulatory T cells, reduce inflammation, and maintain healthy intestinal function.

Table 2

Adonis and db-RDA analysis results investigating the association between the gut microbiota and the three groups based on the Bray–Curtis distance metrics

	Adonis test		db-RDA analysis	
Variants	***P***	***r*²**	***P***	***r*²**
Model groups (Ctrl, IBS, and YDRh)	0.009	15.69%	0.003	6.43%

CTRL: control group; IBS: IBS model group; YDRh: YINDARA-4 high dosage intervention group; db-RDA: Distance-based redundancy analysis (db-RDA)

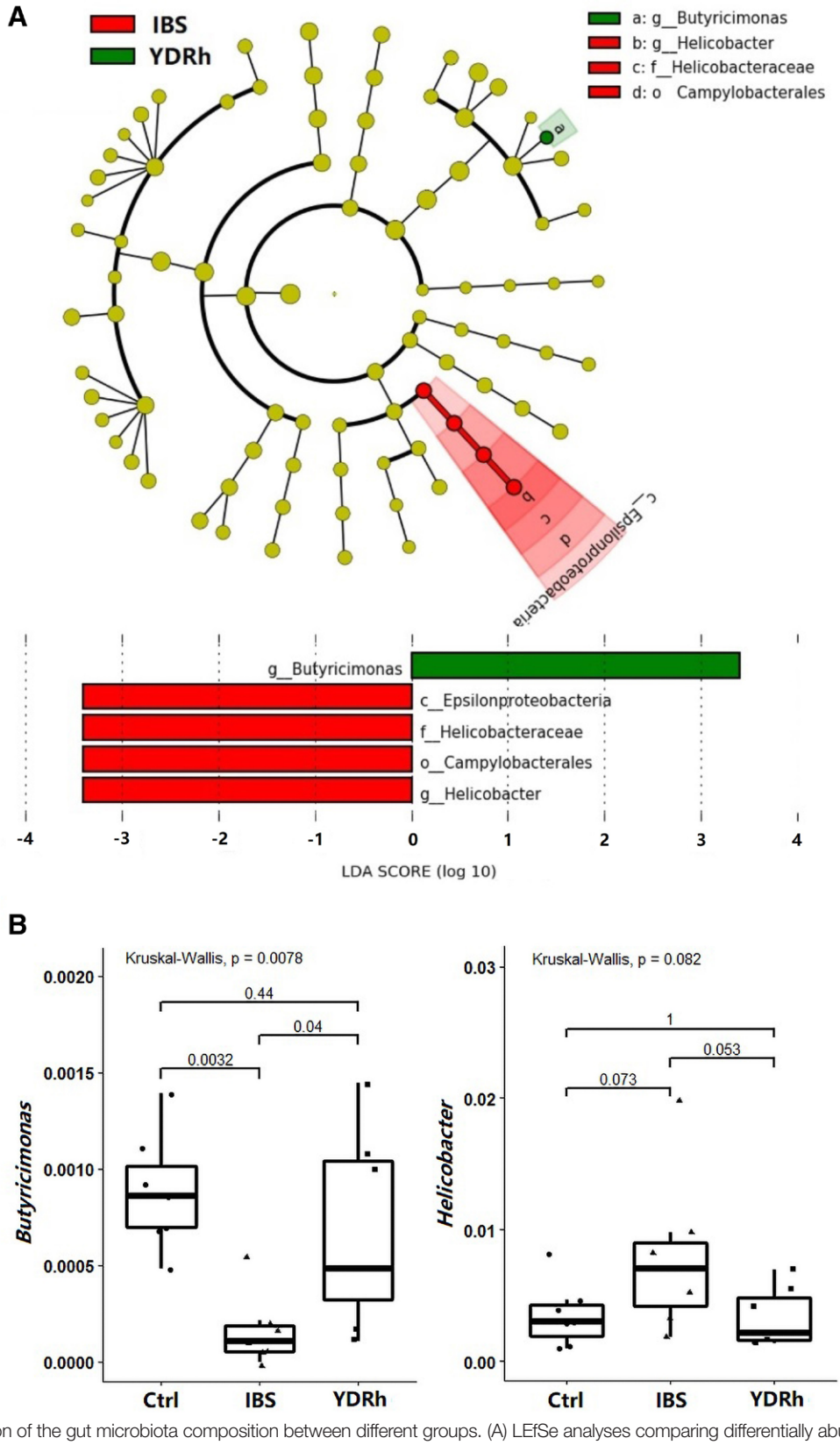

Figure 5. Comparison of the gut microbiota composition between different groups. (A) LEfSe analyses comparing differentially abundant taxa between the communities in the IBS and YDRh groups. (B) Kruskal–Wallis non-parametric test comparing differentially abundant taxa among the communities in the control, IBS, and YDRh groups. IBS: Irritable bowel syndrome; LEfSe: Linear discriminant effect size; YDRh: YINDARA-4 high-dosage intervention.

Hence, decreased *Butyricimonas* within the intestine is thought to play a role in the inflammation associated with the pathogenesis of IBS[41–42]. In addition, the genus *Butyricimonas* is also associated with IBS, as butyrate production has been shown to regulate colonic serotonin production[43]. Using a human EC cell model, Reigstad et al.[44] demonstrated that treatment with a high concentration of butyrate significantly reduced the secretion of serotonin by suppressing the mRNA expression of *TPH1*, which is the key rate-limiting enzyme during the

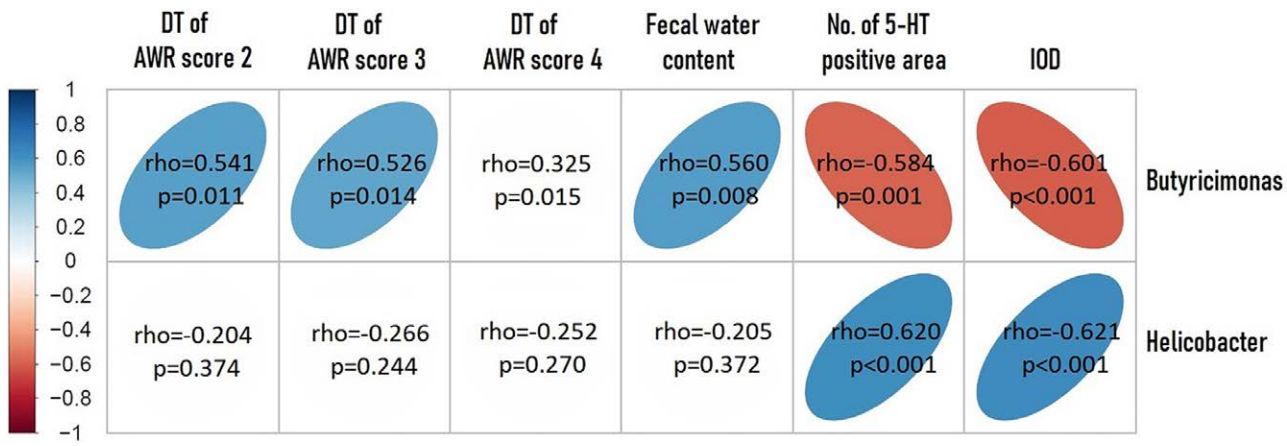

Figure 6. Spearman's correlation between the differential microbiota composition and gut pathophysiological parameters. Blue = positive correlation; red = negative correlation. 5-HT, serotonin; DT, distension threshold; IOD, integrated optical density.

synthesis of serotonin in the gut. Consistent with the literature, the present study found that the abundance of *Butyricimonas* was negatively associated with serotonin expression in the colon.

Conclusion

Taken together, our research provides experimental evidence that YINDARA-4 treatment attenuates visceral hypersensitivity in a dose-dependent manner in an IBS rat model. The relief of visceral hypersensitivity following treatment with YINDARA-4 involved normalization of elevated serotonin levels in the colon and regulation of the gut microbiota structure and composition. The decrease in the levels of serotonin in the colon upon YINDARA-4 treatment may be associated with a reduction in *Helicobacter* and enrichment in *Butyricimonas*. To help expand clinical practice using YINDARA-4 to treat IBS, the specific mechanism(s) of YINDARA-4 involved in regulating serotonin secretion and managing the abundance of specific bacterial genera in the intestinal tract should be investigated in future studies.

Conflict of interest statement

The authors declare no conflict of interest.

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Author contributions

Yaqin Ling and Enqi Wu performed the experiments. Ling Ding, Zhigang Tian, and Enqi Wu analyzed the data. Enqi Wu, Lingpeng Pei, and Yaqin Ling obtained funding for this project and planned the experiments. Lingpeng Pei and Yaqin Ling drafted the manuscript. All the authors have read and approved the final manuscript.

Ethical approval of studies and informed consent

This study was approved by the Ethics Committee of the Minzu University of China (ECMUC2019009CA). The experiments were performed within a Good Laboratory Practice (GLP)–accredited laboratory and followed the ARRIVE guidelines.

Availability of data and materials

The datasets generated and/or analyzed during the current study are available in the SRA database repository (Accession No: PRJNA609270).

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