

World Journal of *Gastroenterology*

World J Gastroenterol 2022 February 7; 28(5): 502-607



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INDEXING/ABSTRACTING

The WJG is now indexed in Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central, and Scopus. The 2021 edition of Journal Citation Report® cites the 2020 impact factor (IF) for WJG as 5.742; Journal Citation Indicator: 0.79; IF without journal self cites: 5.590; 5-year IF: 5.044; Ranking: 28 among 92 journals in gastroenterology and hepatology; and Quartile category: Q2. The WJG's CiteScore for 2020 is 6.9 and Scopus CiteScore rank 2020: Gastroenterology is 19/136.

RESPONSIBLE EDITORS FOR THIS ISSUE

Production Editor: *Ying-Yi Yuan*, Production Department Director: *Xiang Li*, Editorial Office Director: *Ze-Mao Gong*.

NAME OF JOURNAL

World Journal of Gastroenterology

ISSN

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

LAUNCH DATE

October 1, 1995

FREQUENCY

Weekly

EDITORS-IN-CHIEF

Andrzej S Tarnawski

EDITORIAL BOARD MEMBERS

<http://www.wjgnet.com/1007-9327/editorialboard.htm>

PUBLICATION DATE

February 7, 2022

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INSTRUCTIONS TO AUTHORS

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PUBLICATION MISCONDUCT

<https://www.wjgnet.com/bpg/gerinfo/208>

ARTICLE PROCESSING CHARGE

<https://www.wjgnet.com/bpg/gerinfo/242>

STEPS FOR SUBMITTING MANUSCRIPTS

<https://www.wjgnet.com/bpg/GerInfo/239>

ONLINE SUBMISSION

<https://www.f6publishing.com>

Basic Study

Effect of *Bacillus subtilis*, *Enterococcus faecium*, and *Enterococcus faecalis* supernatants on serotonin transporter expression in cells and tissues

Yi-Ming Chen, Ying Li, Xin Wang, Ze-Lan Wang, Jun-Jie Hou, Shuai Su, Wei-Long Zhong, Xin Xu, Jie Zhang, Bang-Mao Wang, Yu-Ming Wang

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Author contributions: Wang YM, Wang BM and Zhang J conceived and designed the study; Chen YM and Wang ZL conducted the experiments; Li Y and Wang X wrote the manuscript; Su S, Hou JJ, Zhong WL and Xu X analyzed data; Chen YM and Li Y contributed equally to this work; all authors read and approved the manuscript.

Institutional review board statement: This study was reviewed and approved by the Ethics Committee of Tianjin Medical University General Hospital (IRB2021-WZ-100).

Institutional animal care and use committee statement: All procedures involving animals were reviewed and approved by the

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Abstract

BACKGROUND

Bacillus subtilis (*B. subtilis*), *Enterococcus faecium* (*E. faecium*), and *Enterococcus faecalis* (*E. faecalis*) are probiotics that are widely used in the clinical treatment of irritable bowel syndrome (IBS). Whether the supernatants of these three probiotics can improve gastrointestinal sensation and movement by regulating the serotonin transporter (SERT) expression needs to be clarified.

AIM

To investigate whether *B. subtilis*, *E. faecium*, and *E. faecalis* supernatants can upregulate SERT expression *in vitro* and *in vivo*.

METHODS

Caco-2 and HT-29 cells were stimulated with probiotic culture supernatants for 12 and 24 h, respectively. A male Sprague-Dawley rat model of post-infectious irritable bowel syndrome (PI-IBS) was established and the rats were treated with phosphate-buffered saline (group A) and three probiotics culture supernatants (groups B, C, and D) for 4 wk. The levels of SERT were detected by quantitative PCR and western blotting.

RESULTS

The levels of SERT at post-treatment 12 and 24 h were significantly elevated in Caco-2 cells treated with *B. subtilis* supernatant compared with those in the control group ($^aP < 0.05$). Those levels were markedly upregulated in Caco-2 cells stimulated with *E. faecium* and *E. faecalis* supernatants at 24 h ($^aP < 0.05$). In addition, SERT expression in groups B, C, and D was significantly higher than

Institutional Animal Care and Use Committee of the Institute of Radiation Medicine, Chinese Academy of Medical Sciences (Tianjin, China) (IACUC Protocol No. IRM-DWLL-2021142).

Conflict-of-interest statement: The authors have no conflicts of interest to declare.

Data sharing statement: No additional data are available.

Supported by the National Natural Science Foundation of China, No. 81570489; and the Youth Project of National Natural Science Foundation of China, No. 81900487.

Country/Territory of origin: China

Specialty type: Gastroenterology and hepatology

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): A
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

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Received: July 21, 2021

Peer-review started: July 21, 2021

First decision: October 3, 2021

Revised: October 16, 2021

Accepted: January 6, 2022

that in group A in the 2nd wk ($^aP < 0.05$). Increased SERT expression was only found in group D in the 3rd wk ($^aP < 0.05$). However, there was no significant difference in SERT expression between the groups in the last week ($P > 0.05$).

CONCLUSION

The supernatants of *B. subtilis*, *E. faecium*, and *E. faecalis* can upregulate SERT expression in intestinal epithelial cells and the intestinal tissues in the rat model of PI-IBS.

Key Words: *Bacillus subtilis* supernatant; *Enterococcus faecalis* supernatant; *Enterococcus faecium* supernatant; Serotonin transporter expression; Irritable bowel syndrome

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Core Tip: Irritable bowel syndrome (IBS) is a functional gastrointestinal disorder that can cause chronic symptoms and changes in bowel habits. Serotonin and serotonin transporter (SERT) play significant roles in the development of IBS. *Bacillus subtilis* (*B. subtilis*), *Enterococcus faecium* (*E. faecium*), and *Enterococcus faecalis* (*E. faecalis*) are probiotics that are widely used in the clinical treatment of IBS. Here, we explored the effects of *B. subtilis*, *E. faecium*, and *E. faecalis* supernatants on the mRNA and protein expression of SERT *in vitro* and *in vivo*, highlighting the significance of the regulation of SERT expression in the treatment of IBS patients.

Citation: Chen YM, Li Y, Wang X, Wang ZL, Hou JJ, Su S, Zhong WL, Xu X, Zhang J, Wang BM, Wang YM. Effect of *Bacillus subtilis*, *Enterococcus faecium*, and *Enterococcus faecalis* supernatants on serotonin transporter expression in cells and tissues. *World J Gastroenterol* 2022; 28(5): 532-546

URL: <https://www.wjgnet.com/1007-9327/full/v28/i5/532.htm>

DOI: <https://dx.doi.org/10.3748/wjg.v28.i5.532>

INTRODUCTION

Irritable bowel syndrome (IBS) is a functional gastrointestinal disorder that can cause chronic symptoms, such as abdominal pain and changes in bowel habits[1]. According to the Rome IV criteria, IBS is divided into four subtypes: IBS with predominant constipation (IBSC), IBS with predominant diarrhea (IBSD), IBS with mixed bowel habits (IBSM), and unclassified IBS (IBSU)[2]. With a prevalence varying between 5% and 10% worldwide, IBS can reduce the health-related quality of life and lower work productivity. Some symptoms of IBS such as an abnormal psychological state, anxiety, and depression may impose an economic burden on individuals, healthcare systems, and society[3,4]. The etiology of IBS is complex and still elusive. The widely recognized pathogenic mechanisms include gastrointestinal dysmotility, varied visceral sensitivity, brain-gut axis disorder, gut microenvironment, and psychosocial/psychosomatic behaviors[3]. For decades, the significant decrease in expression of the serotonin transporter (SERT) in the intestinal mucosa has been considered one of the most important pathophysiology in the development of IBS, leading to the gastrointestinal motility disorders[5-7]. SERT terminates serotonin or 5-hydroxytryptamine (5-HT) activity by combining with it from the interstitial space. Reduced SERT expression and function may result in excess 5-HT, along with motor, sensory, and secretory dysfunctions of the gut, which in turn leads to the development of diarrhea and abdominal pain associated with the pathogenesis of IBS[8,9]. In addition, alterations in 5-HT metabolism have been postulated to play a role in the pathogenesis of IBS[10,11]. Overall, 5-HT and SERT contribute significantly to the development of IBS[12].

Bacillus subtilis (*B. subtilis*), *Enterococcus faecium* (*E. faecium*), and *Enterococcus faecalis* (*E. faecalis*) are probiotics that are widely used in the clinical treatment of IBS[13-16]. These probiotics can alleviate IBS symptoms such as decreasing the intensity of abdominal pain and discomfort and reducing stool frequency. Our previous study revealed that *Lactobacillus rhamnosus* GG supernatants can upregulate the expression

Article in press: January 6, 2022

Published online: February 7, 2022

P-Reviewer: Nakaji K, Saraiva MM

S-Editor: Wang LL

L-Editor: Filipodia

P-Editor: Wang LYT



level of SERT in epithelial cells and colon tissues in rats with post-infectious IBS (PI-IBS)[7,17]. We also demonstrated that *Lactobacillus acidophilus* and *Bifidobacterium longum* supernatants have similar effects on SERT expression in intestinal epithelial cells[18]. However, whether the supernatants of *B. subtilis*, *E. faecium*, and *E. faecalis* can improve gastrointestinal sensation and movement by regulating SERT expression still needs to be clarified.

The current research explored the effects of *B. subtilis*, *E. faecium*, and *E. faecalis* supernatants on the mRNA and protein expression of SERT *in vitro* and *in vivo*.

MATERIALS AND METHODS

Bacterial culture

B. subtilis (CGMCC 1.3358), *E. faecium* (CGMCC 1.2136), and *E. faecalis* (CGMCC 1.2135) were obtained from China General Microbiological Culture Collection Center (Beijing, China). These bacteria were respectively inoculated into the corresponding liquid medium and cultured at 37 °C for 24 h (*B. subtilis*: nutrient broth medium, HB0105, Hope Biotechnology Co., Ltd., Qingdao, China; *E. faecium*: TSB, HB4114, Hope Biotechnology Co., Ltd., Qingdao, China; *E. faecalis*: TSB, HB4114, Hope Biotechnology Co., Ltd., Qingdao, China). Then they were diluted in the corresponding media, and continued to be cultured to reach the logarithmic stage with an optical density (OD) of 0.5 detected at a wavelength of 600 nm. The supernatants were collected by centrifugation (5000 g, 10 min) and filtered twice through a 0.22 µm filter.

Campylobacter jejuni 81-176 (BAA-2151; ATCC, Manassas, VA, United States) was grown on Skirrow's selective medium (Columbia Agar Base, Oxoid CM0331) at 42 °C for 24 h. Bacterial colonies were obtained with an inoculating loop and diluted with culture medium until reaching a concentration of 10¹⁰ CFU/mL. Determination of bacterial concentration was carried out by the conventional plate-counting method.

Cell culture

Caco-2, a human epithelial colorectal adenocarcinoma cell line, was grown in Eagle's minimal essential medium (MEM; Gibco, New York, NY, United States) supplemented with 20% fetal bovine serum (FBS; Gibco) and 1% nonessential amino acids at 37 °C. The human colonic epithelial carcinoma cell line, HT-29, was grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, New York, NY, United States) supplemented with 10% FBS and 1% nonessential amino acids at 37 °C. The cells were incubated with the culture supernatants of *B. subtilis*, *E. faecium*, and *E. faecalis* diluted in MEM/DMEM (dilution ratios: 1:100, 1:50, and 1:20, respectively) for 12 and 24 h and classified as the 1:100 group, 1:50 group, and 1:20 group, respectively.

Animal studies

Sixty-six male Sprague-Dawley rats (270-310 g) were maintained in a room at 22 ± 1 °C under a 12-h light:12-h dark cycle in the Institute of Radiation Medicine, Chinese Academy of Medical Sciences (Tianjin, China). Rats in all groups were given the same housing conditions and diet. All animals were euthanized by barbiturate overdose (intravenous injection, 150 mg/kg pentobarbital sodium) for tissue collection. The animal protocol was designed to minimize pain or discomfort to the animals. All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Institute of Radiation Medicine, Chinese Academy of Medical Sciences (IACUC Protocol No. IRM-DWLL-2021142).

Intervention and evaluation of a rat model of PI-IBS

Rats were randomly divided into a PI-IBS group (*n* = 52) and control group (*n* = 14). Rats in the PI-IBS group were given 10¹⁰ CFU/mL *C. jejuni* for 7 days, while those in the control group were given phosphate-buffered saline (PBS) for 7 days. The rats in both groups were fed separately. *C. jejuni* stool culture and biochemical tests were carried out to evaluate the infection phase of rats in the PI-IBS group. Four rats were randomly chosen from the PI-IBS and control group to perform the intestinal motility test with carbon solution gavage. The specific experimental process was previously described by Wang *et al*[19]. After evaluation of the rat model of PI-IBS, the remaining rats were randomly assigned to four different experimental groups (*n* = 12 for each group): Group A, PBS (i.g); Group B, *B. subtilis* supernatant (i.g); Group C, *E. faecalis* supernatant (i.g); Group D, mixed supernatant of *B. subtilis* and *E. faecalis* with a dilution of 1:1 (i.g). These rats were treated for 4 wk, and changes in general and fecal

states were observed and recorded. Besides, three rats from each group were sacrificed, and protein expression levels of SERT in colonic tissues were detected weekly. The rats were fed 10% activated carbon suspension after fasting for 24 h and were killed 1 h later. The intestinal section from pyloric to terminal rectum was removed immediately. The total length of intestinal tract and the propelling distance of activated carbon were measured. Intestinal transit rate = propelling distance of activated carbon/total length of intestinal tract.

quantitative PCR

Total RNA was extracted from Caco-2 and HT-29 cells with Trizol reagent (Thermo Fisher Scientific, Waltham, MA, United States). cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Inc., Hercules, CA, United States). The raw materials for PCR synthesis included cDNA, 2x iQSYBR Green Supermix (Thermo Fisher Scientific), primers, and double-distilled water. The primers used for quantitative PCR (qPCR) are listed in Table 1. Relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

Proteins were extracted from HT-29 cells, Caco-2 cells, and colonic tissues of rats using radio-immunoprecipitation assay lysis buffer (Solarbio Science & Technology Co., Ltd., Beijing, China) according to the manufacturer's instructions. Protein concentrations were quantified using a bicinchoninic acid protein assay kit (Solarbio Science & Technology Co., Ltd., Beijing, China). A total of 40 g protein samples were isolated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The isolated protein was transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked in 5% skimmed milk powder at room temperature for 1 h, and incubated with primary rabbit polyclonal antibodies (SERT, Cat. No. ab102048, Abcam, Cambridge, UK; β -actin, Cat No. KM9001, Tianjin Sungene Biotech Co., Ltd., Tianjin, China) at 4 °C overnight. The PVDF membranes were incubated with diluted secondary antibody (LK2001, Tianjin Sungene Biotech Co.) at room temperature for 1 h. Then the membranes were washed with Tris-buffered saline with 0.1% Tween® 20 Detergent and detected using an enhanced chemiluminescence kit (BB-3501; Amersham, Buckinghamshire, UK). The Bio-Rad Image Analysis System (Bio-Rad Laboratories) was utilized to visualize the protein bands. The protein quantitative analysis was undertaken using ImageJ software. The relative expression was calculated using the ratio of the gray value of protein band to that of glyceraldehyde 3-phosphate dehydrogenase.

Statistical analysis

The Student's *t*-test was used to assess differences between two groups. All statistical analyses were performed using SPSS 20.0 software (IBM, Armonk, NY, United States). $P < 0.05$ was considered statistically significant.

RESULTS

Effects of *B. subtilis* supernatant on the mRNA and protein expression levels of SERT in Caco-2 and HT-29 cells

Caco-2 and HT-29 cells were treated with supernatants of *B. subtilis* from 1:100, 1:50, and 1:20 groups. SERT mRNA and protein expression levels in Caco-2 and HT-29 cells were detected by qPCR and western blotting, respectively. SERT mRNA levels at both 12 and 24 h after treatment were significantly increased in the 1:50 and 1:20 groups in Caco-2 cells (Figure 1 Caco-2 A&B, all $^aP < 0.05$) compared with those in the control group. Increased SERT protein expression was found in the 1:20 group at 12 h after treatment (Figure 1 Caco-2C, $^aP < 0.05$). Both SERT mRNA and protein levels increased at 24 h (Figure 1 Caco-2B&D, $^aP < 0.05$). The mRNA and protein expression levels of SERT in the 1:20 group were significantly higher than those in the 1:100 group at 12 and 24 h ($^aP < 0.05$), which indicated that the effect of *B. Subtilis* supernatant on the mRNA and protein levels of SERT was concentration-dependent.

SERT protein expression in HT-29 cells was markedly elevated in the 1:50 and 1:20 groups at 12 h compared with that in the control group, and the level of SERT proteins in the 1:20 group was higher than that of the 1:100 and 1:50 group (Figure 1 HT-29C, $^aP < 0.05$). However, there was no significant difference in SERT mRNA level at 12 h among the treatment and control groups (Figure 1 HT-29A, $P > 0.05$). By contrast,

Table 1 Primer sequences for RT-PCR

Gene	Sequence (5'-3')
rGAPDH	Forward: 5'-CCATCAACGACCCCTTCATT-3'; Reverse: 5'-GACCAGCTTCCCATTCTCAG-3'
rSERT	Forward: 5'-ACTGTTACCAAGATGCCCTIG-3'; Reverse: 5'-ATCTTCATTCCTCATCTCCGC-3'
hGAPDH	Forward: 5'-ACA GCA ACT CCC ATT CTT-3'; Reverse: 5'-TCC AGG GTT TCT TAC TCC-3'
hSERT	Reverse: 5'-AAT GGG TAC TCA GCA GTT CC-3'; Reverse: 5'-CCA CAG CAT AGC CAA TCA C-3'

hGAPDH: Human glyceraldehyde-3-phosphate dehydrogenase; hSERT: Human serotonin transporter; rGAPDH: Rat glyceraldehyde-3-phosphate dehydrogenase; rSERT: Rat serotonin transporter.

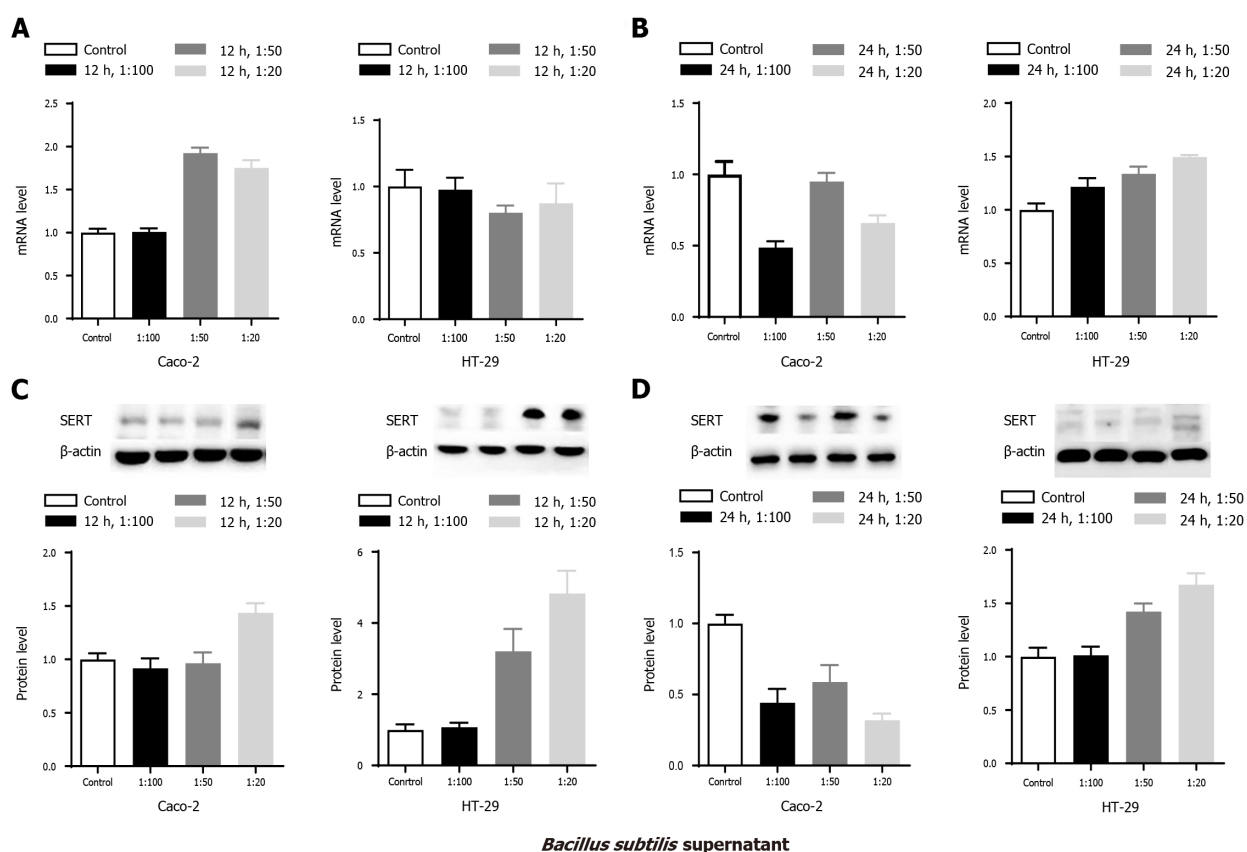


Figure 1 Effects of *Bacillus subtilis* supernatant on serotonin transporter mRNA and protein expression in Caco-2 and HT-29 cells. A: Serotonin transporter (SERT) mRNA levels at 12 h; B: SERT mRNA levels at 24 h; C: Quantitative analysis of SERT protein levels at 12 h; D: Quantitative analysis of SERT protein levels at 24 h.

significant reductions in the mRNA and protein levels of SERT were observed among the treatment groups at 24 h (Figure 1 HT-29-B&D, all $^aP < 0.05$).

Effects of *E. faecium* supernatant on the mRNA and protein levels of SERT in Caco-2 and HT-29 cells

To explore the effects of *E. faecium* supernatant on the mRNA and protein levels of SERT in Caco-2 and HT-29 cells, similar experiments were performed. The experimental results showed that there were no significant differences in the mRNA and protein levels of SERT at 12 h among the mentioned groups (Figure 2 Caco-2-A&-C, $^aP > 0.05$). SERT mRNA and protein levels were significantly upregulated in the 1:50 and 1:20 groups compared with the control group at 24 h (Figure 2 Caco-2-B&-D, $^aP < 0.05$), indicating that the effects of *E. faecium* supernatant on SERT mRNA and protein expression were time-dependent. Besides, SERT protein level in the 1:20 group was higher than that in the 1:50 and 1:100 group at 24 h (Figure 2 Caco-2D, $^aP < 0.05$), demonstrating that the effect of *E. faecium* supernatant was concentration-dependent.

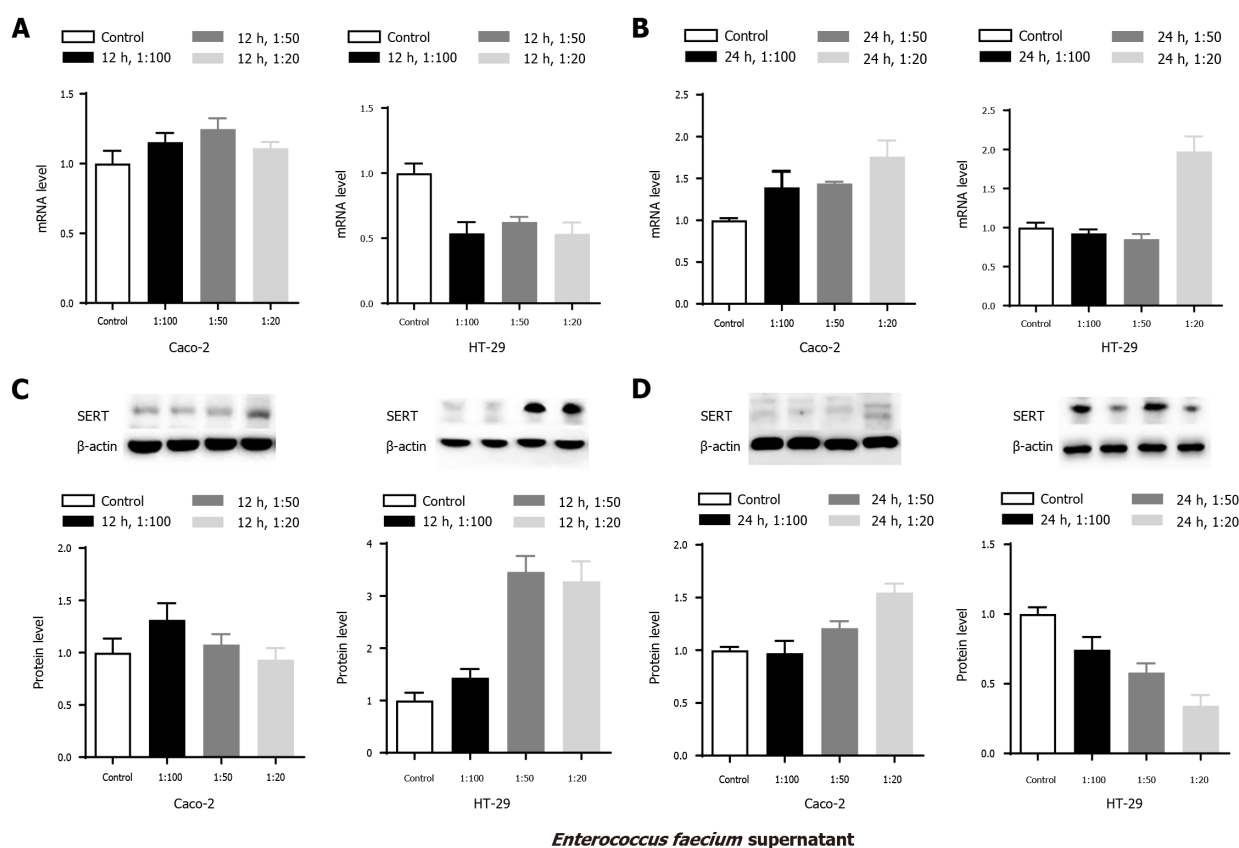


Figure 2 Effects of *Enterococcus faecium* supernatant on serotonin transporter mRNA and protein expression in Caco-2 and HT-29 cells. A: Serotonin transporter (SERT) mRNA levels at 12 h; B: SERT mRNA levels at 24 h; C: Quantitative analysis of SERT protein levels at 12 h; D: Quantitative analysis of SERT protein levels at 24 h.

SERT protein levels in the 1:50 and 1:20 groups were markedly higher than those in the control group in HT-29 cells at 12 h (Figure 2 HT-29C, $^aP < 0.05$). However, SERT mRNA levels in the three treatment groups were dramatically reduced at 12 h (Figure 2 HT-29A, $^aP < 0.05$). Furthermore, compared to the control group, SERT mRNA level was notably elevated in the 1:20 group at 24 h (Figure 2 HT-29B, $^aP < 0.05$), while SERT protein level was significantly reduced in the 1:50 and 1:20 groups at 24 h (Figure 2 HT-29D, $^aP < 0.05$).

Effects of mixed supernatant of *B. subtilis* and *E. faecium* on the mRNA and protein levels of SERT in Caco-2 and HT-29 cells

To further investigate the abovementioned results, Caco-2 and HT-29 cells were stimulated with the mixed supernatants of *B. subtilis* and *E. faecium* in the 1:1 group. Compared with the control group, SERT mRNA level was upregulated in the 1:20 group in Caco-2 cells at 12 h (Figure 3 Caco-2 A, $^aP < 0.05$), and SERT protein levels were significantly elevated in the three groups at 12 h (Figure 3 Caco-2 C, $^aP < 0.05$). However, there were no significant differences in the mRNA and protein levels of SERT among these groups at 24 h (Figure 3 Caco-2 B&D, $P > 0.05$).

The expression of SERT mRNA in HT-29 cells was upregulated in the 1:100 and 1:20 groups at 12 h (Figure 3 HT-29A, $^aP < 0.05$) compared to the control group. An increased level of SERT protein was found in the 1:20 group at 12 h, whereas a significantly decreased level was observed in the 1:50 group compared with the control group (Figure 3 HT-29C, $^aP < 0.05$). Furthermore, compared with the control group, SERT mRNA levels were attenuated in the 1:100 and 1:20 groups at 24 h, whereas SERT protein levels were decreased in all the three treatment groups (Figure 3 HT-29 B&D, $^aP < 0.05$).

Effects of *E. faecalis* supernatant on mRNA and protein levels of SERT in Caco-2 and HT-29 cells

To explore the effects of *E. faecalis* supernatant on the mRNA and protein levels of SERT in Caco-2 and HT-29 cells, similar experiments were conducted. SERT mRNA

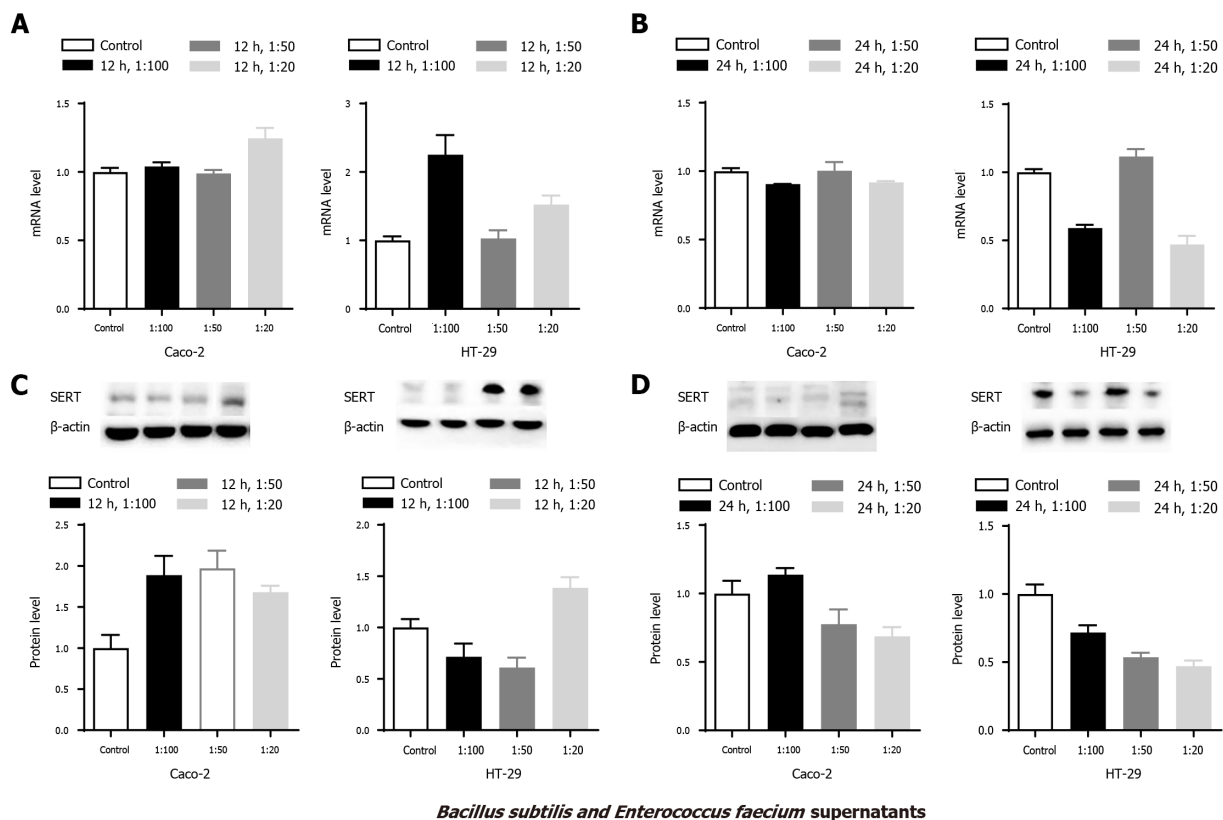


Figure 3 Effects of *Bacillus subtilis* and *Enterococcus faecium* supernatants on serotonin transporter mRNA and protein expression in Caco2 and HT-29 cells. A: Serotonin transporter (SERT) mRNA levels at 12 h; B: SERT mRNA levels at 24 h; C: Quantitative analysis of SERT protein levels at 12 h; D: Quantitative analysis of SERT protein levels at 24 h.

levels in Caco-2 cells in the three treatment groups were significantly increased at 12 h (Figure 4 Caco-2 A, $^aP < 0.05$). Western blot analysis showed that there were no significant differences in SERT protein levels among the three treatment groups at 24 h, whereas a decreased level was observed in the 1:20 group at 12 h compared to the other two groups (Figure 4 Caco-2 C, $^aP < 0.05$). In addition, increased SERT mRNA level was only found in the 1:50 group at 24 h (Figure 4 Caco-2-B, $^aP < 0.05$). Similarly, the expression level of SERT protein was markedly upregulated in the 1:50 and 1:20 groups at 24 h (Figure 4 Caco-2 D, $^aP < 0.05$). SERT protein level in 1:20 group was significantly higher than that in the 1:50 and 1:100 groups at 24 h (Figure 4 Caco-2 D, $^aP < 0.05$). Besides, SERT protein level in the 1:50 group was also higher than that in the 1:100 group at 24 h (Figure 4 Caco-2 D, $^aP < 0.05$), indicating that the effect of *E. faecalis* supernatant on the mRNA and protein levels of SERT was concentration-dependent.

SERT mRNA level was in steady-state in HT-29 cells at 12 h among the treatment groups (Figure 4 HT-29A, $P > 0.05$), while western blot analysis showed that the SERT mRNA level was significantly elevated in all three groups (Figure 4 HT-29-C, $^aP < 0.05$) compared to the control group. Additionally, the expression level of SERT protein in the 1:50 group was markedly higher than that in the 1:20 group at 12 h (Figure 4 HT-29C, $^aP < 0.05$). SERT mRNA level was markedly increased in the 1:20 group at 24 h compared to the 1:100 group (Figure 4 HT-29B, $^aP < 0.05$). Similarly, the levels of SERT protein were significantly elevated in the 1:20 and 1:50 groups at 24 h. Meanwhile, the increase in SERT mRNA expression in the 1:20 and 1:50 groups was noticeably higher than that in the 1:100 group at 24 h (Figure 4 HT-29-D, $^aP < 0.05$), confirming that the effect of *E. faecalis* supernatant on the mRNA and protein levels of SERT was concentration-dependent in HT-29 cells.

The construction and evaluation of a rat model of PI-IBS

To further verify the effect of culture supernatant of probiotics in treating IBS, we constructed a rat model of PI-IBS. Before intervention, all rats were negative for *C. jejuni* stool culture and biochemical tests. Fifty-two of the rats were used to establish a rat model of PI-IBS as the PI-IBS group. The results of fecal bacteriological culture and

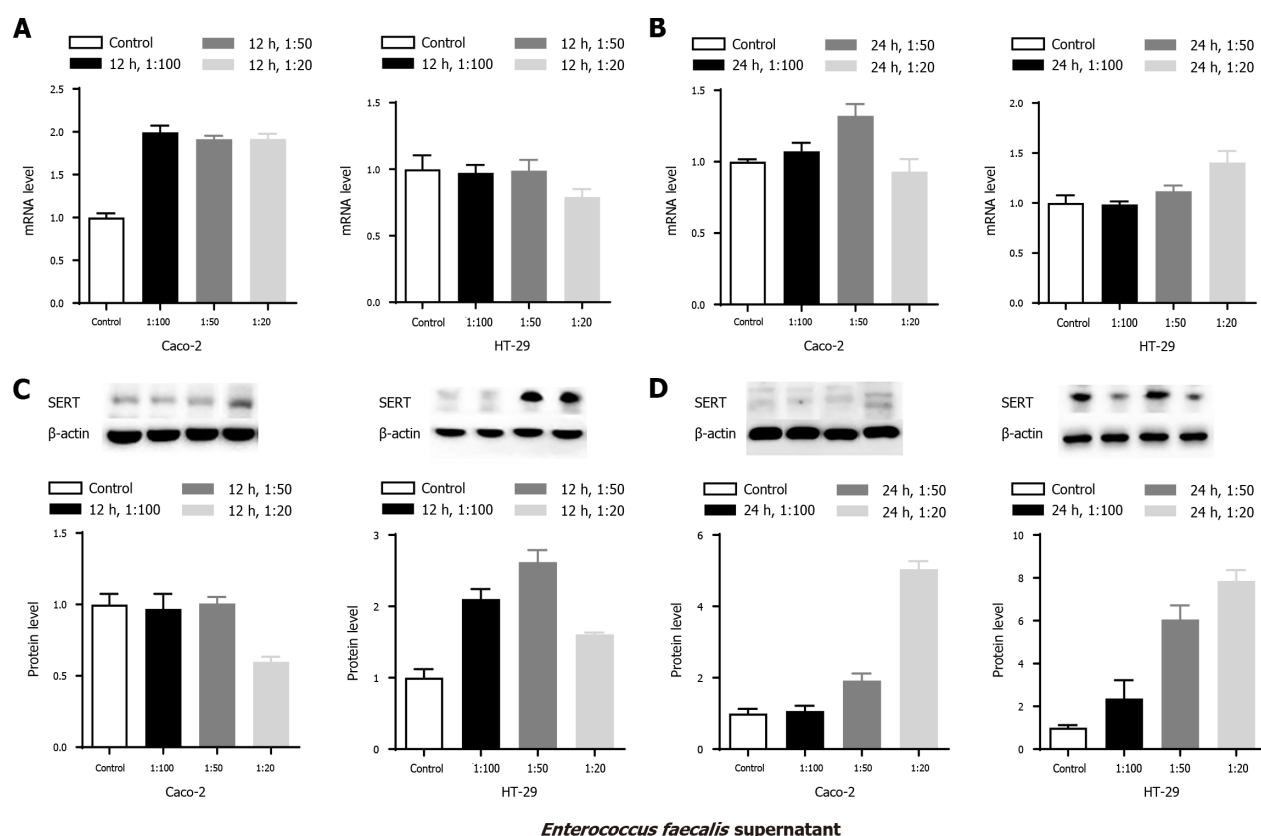


Figure 4 Effects of *Enterococcus faecalis* supernatant on serotonin transporter mRNA and protein expression in Caco-2 and HT-29 cells. A: Serotonin transporter (SERT) mRNA levels at 12 h; B: SERT mRNA levels at 24 h; C: Quantitative analysis of SERT protein levels at 12 h; D: Quantitative analysis of SERT protein levels at 24 h.

biochemical tests were positive on the 7th d after gavage in the PI-IBS group. Twenty-four rats in the control group had lively spirits, quick reactions, bright hair, normal diet, and normal urine and feces. The activities of rats in the PI-IBS group were reduced, lack of luster was observed in the hair of rats, and their appearance was thinner compared with the control group (Figure 5A). However, there were no significant differences between the PI-IBS and control group from 5th wk to 7th wk after gavage. The negative rates of *C. jejuni* fecal culture and biochemical tests (Figure 5B) in the PI-IBS group were higher than 95% at the 7th wk and 9th wk. After 8 wk of *C. jejuni* gavage, four rats from each group were randomly selected for model's evaluation, and the rat model of PI-IBS was confirmed to be successfully constructed. The intestinal transport speed in the PI-IBS group were higher than those in the control group and the expression level of SERT protein in the PI-IBS group were lower than those in the control group (Figure 5C and 5D, $^aP < 0.05$).

The efficacy of probiotic therapy on the rat model of PI-IBS

After administration of several probiotic supernatants (group B: *B. subtilis* supernatant; group C: *E. faecalis* supernatant; group D: mixed supernatants of *B. subtilis* and *E. faecalis* with dilution ratio of 1:1), the expression level of SERT protein was further detected to evaluate the intestinal motility of rats.

SERT protein expression in groups C and D were elevated compared with that in the model group (group A) during the 1st wk, and the efficacy of group C was superior than that of group B (Figure 6 w₁, $^aP < 0.05$), demonstrating that the efficacy of *E. faecalis* supernatant was superior than *B. Subtilis* supernatant in the 1st wk. The expression levels of SERT protein in groups B, C, and D were markedly higher than that in group A in the 2nd wk (Figure 6 w₂, $^aP < 0.05$), and the increase was only observed in the group D in the 3rd wk (Figure 6 w₃, $^aP < 0.05$). Besides, the expression level of SERT protein in group D was greater than that in groups B and C in the 2nd wk and 3rd wk (Figure 6 w₂, w₃, $^aP < 0.05$), reflecting that the efficacy of mixed supernatant was more significant than single supernatant in the 2nd wk and 3rd wk. Meanwhile, the ratio of expression level of SERT protein in group B to that of group A was elevated from the 1st wk to the 2nd wk, indicating that the efficacy of *B. Subtilis*

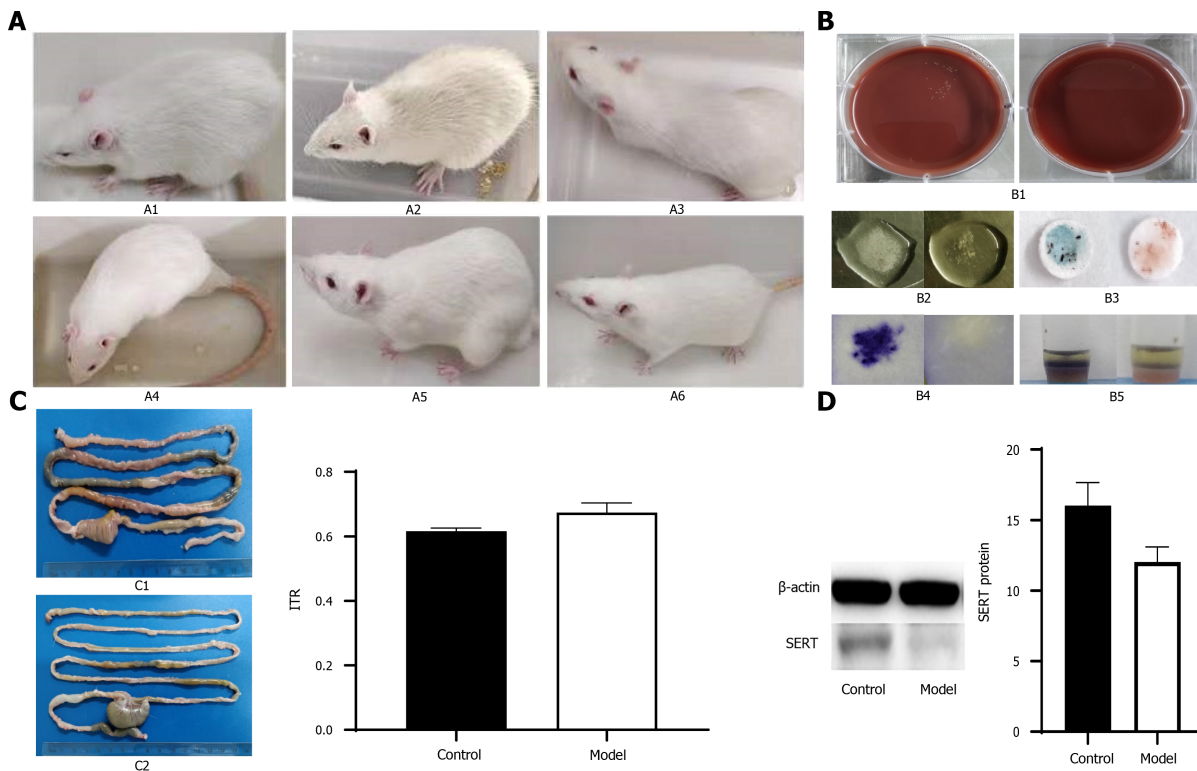


Figure 5 Rat model of post-infectious irritable bowel syndrome. A: Appearance changes of experimental rats; A1: model rats on the 7th day after gavage; A2: the 14th day after gavage; A3: The 28th d after gavage; A4: the 42nd d after gavage; A5: the 56th day after gavage; A6: the normal control group; B: Fecal culture and biochemical detection of *Campylobacter jejuni* B1: *Campylobacter jejuni* culture; B2: catalase test; B3: indole acetate test; B4: oxidase test; e: sodium hippurate hydrolysis test (a-e pictures show that the left picture is positive, the right picture is negative); C: Intestinal transport (ITR) experiment C1: Pylorus to rectal segment in model group; C2: Pyloric to rectal segment in control group; C3: Comparison of ITR histogram ($^aP < 0.05$); D: Expression of serotonin transporter (SERT) protein in the colon during the evaluation period after model evaluation and measurement of ITR experiment. Colon tissue was taken for western blotting to measure SERT level, and quantitative analysis of protein band gray level was used ($^aP < 0.05$).

supernatant was time-dependent. Besides, the ratio of expression level of SERT protein in group C to that of group A was reduced from the 2nd wk to the 3rd wk, representing that the efficacy of *E. faecalis* supernatant was not significant for long-term. There was no significant increase in the expression levels of SERT protein among the treatment groups at the end of the last week (Figure 6 w₄), demonstrating that the long-term efficacy of probiotic therapy was insignificant.

DISCUSSION

IBS is characterized by recurrent abdominal pain, and is associated with a change in stool frequency[3]. At present, there is no effective medical treatment for relieving the symptoms of IBS. Probiotic therapy is extensively applied in supporting the treatment of a broad range of gastrointestinal diseases, and has shown a modest effect on ameliorating the symptoms of IBS[20-23]. *L. rhamnosus* GG, *L. acidophilus*, *Bifidobacterium animalis* subspecies *lactis*, and *B. longum* are commonly used probiotics that can alleviate the symptoms of IBS[24-36]. A review of four clinical studies, which enrolled 214 IBS patients to evaluate the efficacy of a combination of *B. subtilis* and *E. faecium*, concluded that the combined therapy had a more significant effect on relieving abdominal pain without adverse events[16].

Previous animal studies have demonstrated that the combination of *E. faecium* and *B. subtilis* enhanced intestinal barrier function, increased the expression levels of zonula occludens-1 protein and occludin, and improved gut microbiota with a higher amount of *Lactobacilli*[37]. The expression level of ileal mucin 2 gene and bacteria population were increased in chickens intervened with *B. subtilis* in the 1st wk[38]. Besides, heat-killed *E. faecalis* EF-2001 has shown protective effects on a mouse model of colitis[39]. However, the underlying mechanism of therapeutic effects on IBS remains elusive and requires additional research.

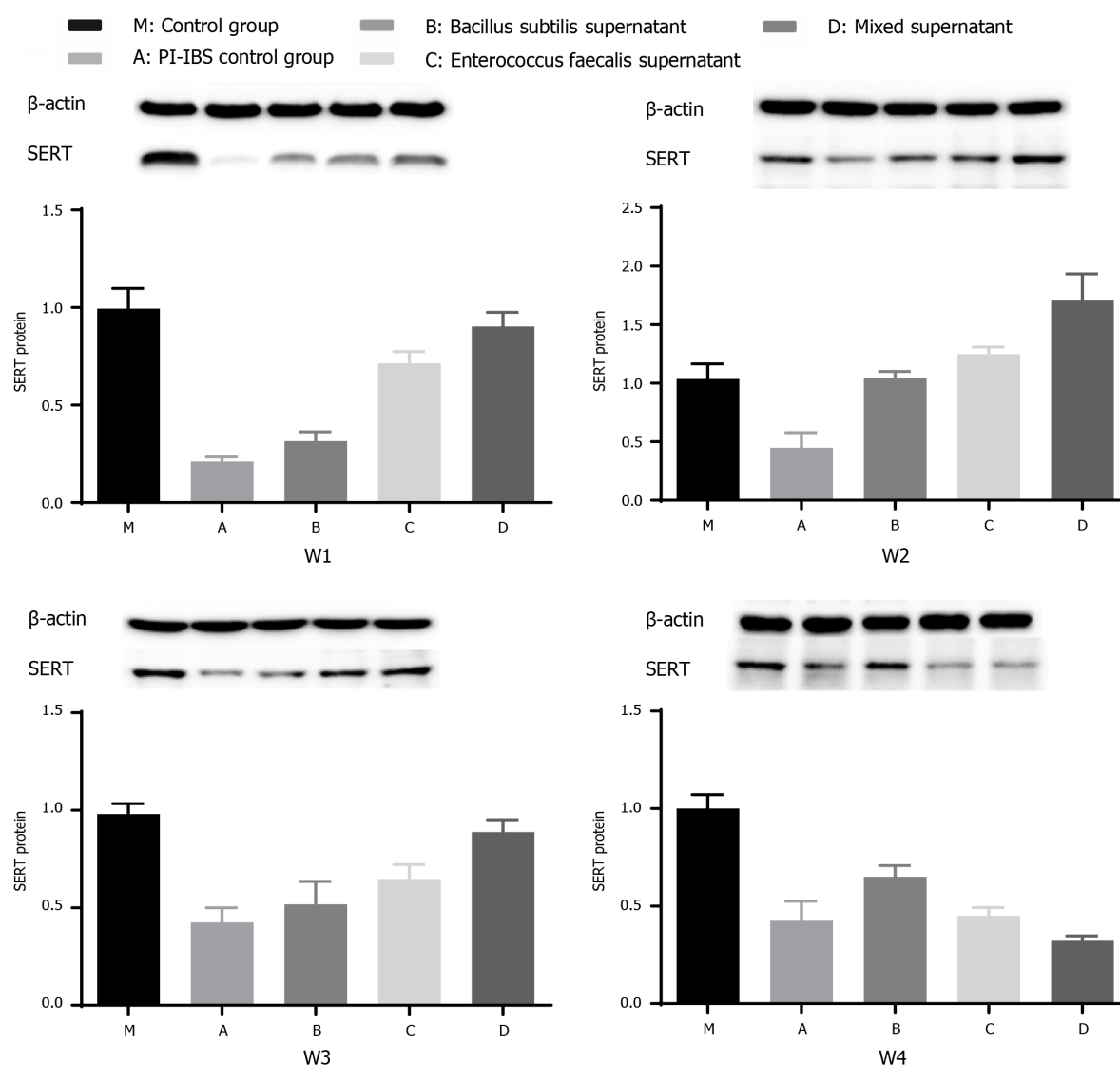


Figure 6 Effects of different supernatants of probiotics on serotonin transporter protein expression in rat intestinal tissues. Quantitative analysis of serotonin transporter (SERT) protein levels in the 1st wk (W1), 2nd wk (W2), 3rd wk (W3), and 4th wk (W4) analyzed by western blotting.

Previous studies have demonstrated that the supernatants of *L. rhamnosus* GG, *L. acidophilus*, and *B. longum* can upregulate the expression of SERT protein in epithelial cells and PI-IBS rats [7,17,18]. SERT, a regulator of the level of 5-HT, plays a significant role in the pathogenesis of IBS and can serve as a novel therapeutic target for IBS. In the present study, we found that the supernatants of *B. subtilis*, *E. faecium*, and *E. faecalis* upregulated the mRNA and protein levels of SERT in intestinal epithelial cells, and the trend of mRNA and protein expression changes were similar. The expression level of SERT protein was upregulated in the intestinal tissues in a rat model of PI-IBS receiving probiotic therapy. Meanwhile, the efficacy of combined supernatants of *B. subtilis* and *E. faecalis* was superior to a single supernatant.

The mRNA and protein levels of SERT at 12 and 24 h were significantly increased in Caco-2 cells treated with supernatant of *B. subtilis*. *E. faecium* supernatant upregulated the SERT mRNA level in Caco2 cells at 24 h, and the trend of SERT protein level alteration was similar to that of SERT mRNA level. The results were similar in HT-29 cells stimulated with *B. subtilis* supernatant or *E. faecium* supernatant. SERT protein level was elevated at 12 h and reduced at 24 h. Thus, we speculated that upregulation of SERT expression in HT-29 cells intervened with *B. subtilis* or *E. faecium* supernatant was significant in the short-term. Moreover, we explored the efficacy of combination of *B. subtilis* and *E. faecium* supernatants on SERT expression. We found that the mRNA and protein levels of SERT were elevated at 12 h in Caco-2 and HT-29 cells treated with mixed supernatants of *B. Subtilis* and *E. faecium*, and the trend of SERT protein expression was similar to that of mRNA expression. Furthermore, our results confirmed the efficacy of mixed supernatants in relieving the symptoms of IBS, which

was consistent with the findings of a previous study[14].

Besides, increased mRNA and protein levels of SERT were observed in Caco-2 and HT-29 cells treated with *E. faecalis* supernatant for 12 and 24 h. The upregulation of SERT expression treated with *E. faecalis* supernatant could decrease intestinal motility, confirming that ProSymbioflor containing *E. faecalis* and *E. coli* was effective on relieving the symptoms of IBS patients[15].

We also assessed the effects of mixed supernatants of *B. subtilis* and *E. faecalis* with dilution of 1:1 on the expression level of SERT in intestinal tissues in the rat model of PI-IBS. Western blot analysis revealed that SERT protein expression was upregulated in the three therapeutic groups except for the last week. The efficacy of mixed supernatants was superior than that of single supernatant in the first 3 wk. The abovementioned results indicated that the *in vivo* regulatory mechanisms might be more complicated. Due to the acidic environment in the stomach, the function of active ingredients in the supernatants might be attenuated, which might justify the weak efficacy of the supernatants in the last week. Thus, establishing a rat model of PI-IBS motivated us to explore a new approach for protecting important organs from possible damages.

The change in trend of mRNA and protein expression was similar, and the difference in SERT expression level between Caco-2 cells and HT-29 cells might be attributed to different types of cells. Occasionally, SERT protein level alterations in the stimulated groups were not identical to that of the mRNA level in the following conditions. First, the difference between reductions or no changes in the SERT mRNA level and significantly increased SERT protein expression could be explained by the fact that the process of mRNA transcription occurred earlier than protein translation, mRNA was easier degraded than protein, and the status of mRNA was less stable than protein. Second, the inconsistent results regarding an increased SERT mRNA level and a reduced SERT protein could be interpreted by unknown factors of supernatants that might be involved in the process of translation from mRNA to protein. Third, the non-change trend of SERT protein expression was in contrast with the increased SERT mRNA level, which was illustrated by the fact that the expression process of SERT protein was slower and more complicated.

The efficacy of the supernatants of *B. subtilis*, *E. faecium*, and *E. faecalis* was found time-dependent, which might help guide physicians to provide continuous treatment for patients with IBS in clinical practice. Moreover, the SERT expression level, which was regulated by the supernatants in a concentration-dependent manner, could be assessed by possible substances, such as bacterial components or small-molecule proteins regulating the SERT expression level. Such a soluble protein, p40, derived from LGG supernatant, could transactivate epidermal growth factor receptor (EGFR) signaling to reduce cytokine-induced apoptosis and epithelial barrier dysfunction *in vivo* and *in vitro*[40]. Benmansour *et al*[41] showed that EGF could upregulate the expression level of SERT in intestinal epithelial cells by activating the activator protein-1 (AP-1), and further interfering with the uptake of 5-HT. Cui *et al*[42] found that EGF could upregulate the expression level of SERT *via* EGFR signaling pathway. Other factors influencing the expression level of SERT include SERT gene polymorphisms, immune cells, inflammatory cytokines, microRNA 16 (MiR-16), and MiR-545[12]. *B. subtilis* has a highly adaptable metabolism and efficient protein expression and secretion system[43], including alkaline phosphatase and thermostable β -galactosidase[43]. A number of studies have demonstrated that *B. subtilis* and its conditioned media induced cytoprotective heat shock proteins in Caco-2 cells to protect colonic epithelial cells from damages[44-46]. Thus, it deserves to explore which specific factors in the supernatants of *B. subtilis*, *E. faecalis*, and *E. faecium* could regulate the expression level of SERT. The expression level of SERT was upregulated after stimulation with *B. subtilis* or *E. faecalis* supernatants for 12 and 24 h. However, the increase on the expression level of SERT was only observed in the cells stimulated with *E. faecium* supernatant at 24 h, and further research indicated that different potential active ingredients from probiotic supernatants might be effective at different time points or *via* different signaling pathways.

CONCLUSION

In conclusion, we found that the supernatants of *B. subtilis*, *E. faecium*, and *E. faecalis* could upregulate the expression level of SERT in intestinal cells, and the effect of combined supernatants of *B. subtilis* and *E. faecalis* was superior than that of single supernatant on the expression level of SERT in colonic tissues in the rat model of PI-

IBS. Additionally, 5-HT and SERT showed to play important roles in the development of IBS, thus, these proteins were significant to treat IBS patients with lower expression level of SERT or with symptoms of diarrhea. In the next research, we will attempt to explore which effective ingredients in the probiotic supernatants can regulate the SERT expression, and will also find out signaling pathways modulating the SERT expression.

ARTICLE HIGHLIGHTS

Research background

It is worthwhile to explore which effective ingredients in the supernatants of *Bacillus subtilis*, *Enterococcus faecium*, and *Enterococcus faecalis* and which signaling pathways are regulating serotonin transporter (SERT) expression further.

Research motivation

The supernatants of *B. subtilis*, *E. faecium*, and *E. faecalis* can upregulate SERT expression in intestinal epithelial cells and the intestinal tissues in the rat model of PI-IBS. And combined supernatants of *B. subtilis* and *E. faecalis* was more efficacious than single supernatant.

Research objectives

The levels of SERT (at post-treatment 12 and 24 h) were significantly elevated in Caco-2 cells treated with *B. subtilis* supernatant compared with those in the control group ($^aP < 0.05$). Those levels were markedly upregulated in Caco-2 cells stimulated with *E. faecium* and *E. faecalis* supernatants at 24 h ($^aP < 0.05$). In addition, the SERT expression in groups B, C and D was significantly higher than that in group A in the 2nd wk ($^aP < 0.05$). Increased SERT expression was found only in group D in the 3rd wk ($^aP < 0.05$). However, there was no significant difference in the SERT expression between the groups in the last week ($P > 0.05$).

Research methods

Caco-2 and HT-29 cells were stimulated with probiotic culture supernatants for 12 and 24 h, respectively. A rat (male Sprague-Dawley rat) model of post-infectious irritable bowel syndrome (PI-IBS) was constructed and the rats were treated with PBS (group A) and three probiotics culture supernatants (groups B, C, and D) for 4 wk. The levels of SERT were detected by quantitative PCR and western blotting.

Research results

The present study aimed to explore whether *B. subtilis*, *E. faecium*, and *E. faecalis* supernatants could upregulate SERT expression *in vitro* and *in vivo*.

Research conclusions

5-HT and SERT contribute significantly to the development of IBS. Whether the supernatants of *B. subtilis*, *E. faecium*, and *E. faecalis* can improve gastrointestinal sensation and movement by regulating SERT expression needs to be clarified. The research is significant to the treatment of IBS patients with lower expression level of SERT or with symptoms of diarrhea.

Research perspectives

IBS is a functional gastrointestinal disorder, of which the onset and development are associated with serotonin and SERT. Recent studies have shown that *B. subtilis*, *E. faecium*, and *E. faecalis* play important roles in the clinical treatment of IBS. However, the underlying mechanism of therapeutic effects on IBS remains elusive and requires additional research.

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