Original Article

# *Lactobacillus acidophilus* and *Bifidobacterium longum* supernatants upregulate the serotonin transporter expression in intestinal epithelial cells

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## Abstract

**Background/Aims:** Probiotics play a role in relieving irritable bowel syndrome (IBS); however, the underlying mechanism is yet unclear. The aim of the study was to investigate the effects of the supernatants of *Lactobacillus acidophilus* and *Bifidobacterium longum* on the expression of serotonin transporter (SERT) messenger ribonucleic acid (mRNA) and protein.

**Materials and Methods:** HT-29 and Caco-2 cells were treated with different concentrations of *L. acidophilus* and *B. longum* supernatants for 12 h and 24 h, respectively. SERT mRNA and proteins levels were detected by real-time polymerase chain reaction (real-time PCR) and Western-blotting.

**Results:** The mRNA levels of SERT in HT-29 and Caco-2 cells treated with different concentrations of *L. acidophilus* or *B. longum* supernatants for 12 h and 24 h, each, were higher than that in the control groups. In addition, the expression of the protein in both cells was also upregulated, which was approximately similar to that of the corresponding mRNA.

**Conclusions:** *L. acidophilus* and *B. longum* supernatants can upregulate SERT mRNA and protein levels in intestinal epithelial cells.

**Keywords:** *Bifidobacterium longum* supernatant, intestinal epithelial cells, *Lactobacillus acidophilus* supernatant, serotonin transporter

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## INTRODUCTION

Irritable bowel syndrome (IBS) is a functional bowel disorder; the abdominal pain or discomfort is associated with defecation and/or a change in bowel habit. According to the Rome IV criteria,[1] IBS may be sub-classified as IBS with predominant constipation (IBS-C), IBS with predominant diarrhea (IBS-D), IBS with mixed bowel habits (IBS-M), and IBS unclassified (IBS-U). The level of severity of IBS is modulated by various factors, such as chronic immunity reactions after intestinal microbiome alteration, visceral hypersensitivity associated with gut-brain pathways, and impaired bowel permeability.[2] As a signal transducer and a neurotransmitter, serotonin (5-hydroxytryptamine, 5-HT)

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mediates the intercellular signaling transmission in the gut, which occurs maximally in the enterochromaffin cells of the gut. The levels of 5-HT decrease in patients with IBS-C and increase in patients with IBS-D.[3] The inactivation of 5-HT is equally crucial as its release for the maintenance of the dynamic equilibrium. Similar to the number of neurotransmitter sodium symporters or the solute carrier superfamily 6, the serotonin transporter (SERT) plays a unique role in the inactivation of 5-HT by its removal from the interstitial space in the lamina propria into mucosal enterocytes and presynaptic neurons that are responsible for catabolism. Coates *et al.* first characterized a significantly decreased level of SERT in IBS.[4] However, another conflicting finding showed increased SERT expression in IBS.[5,6] Despite the lack of consensus on the wide range of roles of potential factors, immunity activation, inflammatory response, gut microbiota, and their relationships have been suggested to regulate the expression of SERT in post-infectious IBS (PI-IBS).[7]

*Lactobacillus acidophilus* and *Bifidobacterium longum* are two probiotics that have been used in the clinical treatment of IBS. The previous study demonstrated that *Lactobacillus rhamnosus* GG (LGG) supernatant could upregulate the SERT messenger ribonucleic acid (mRNA) and protein levels in intestinal epithelial cells and mice intestinal tissues.[8] However, the widely used probiotics for IBS include *L. acidophilus* and *B. longum*. Whether the supernatants of these two species could also improve intestinal motility and gastrointestinal sensation by regulating SERT expression is yet to be elucidated. Thus, the present study investigated the effects of the supernatants of *L. acidophilus* and *B. longum* on the expression of SERT mRNA and protein.

## MATERIALS AND METHODS

### Bacterial culture, *L. acidophilus* and *B. longum* supernatants

*L. acidophilus* (ATCC 4356) and *B. longum* (ATCC 15707) were obtained from China General Microbiological Culture Collection Center.

*L. acidophilus* was incubated in lactic acid bacteria culture medium (MRS) broth (Thermo Fisher Oxoid, UK) at 37°C for 24 h, followed by dilution in the MRS broth and incubation to reach the exponential phase with the density of 0.5 at optical density (OD) 600. On the other hand, *B. longum* was incubated in brain heart infusion (BHI) as described above. The culture suspensions were centrifuged at 5,000 × g for 10 min at 4°C. The supernatant was filter-sterilized through 0.22 μm filters.

### Cell culture and cell treatment

HT-29, a human colonic epithelial carcinoma cell line, was grown in Dulbecco's Modified Eagle Medium (DMEM) media supplemented with 10% fetal bovine serum and 1.0% nonessential amino acids at 37°C. Caco-2, a continuous heterogeneous human epithelial colorectal adenocarcinoma cell line, was grown in Minimum Essential Medium (MEM) media supplemented with 20% fetal bovine serum and 1.0% nonessential amino acids at 37°C. The cells were serum starved (0.5%) at 37°C for approximately 24 h before the experiments and then treated with the supernatants of *L. acidophilus* and *B. longum*, respectively (supernatant-to-cell media ratios: 1:100, 1:50, and 1:20) for 12 h and 24 h. The control cells were treated with MRS and BHI broth, respectively.

### Real-time polymerase chain reaction

The total RNA from HT-29 and Caco-2 cells was extracted using TRIzol reagent® (Life, Hilden, Germany) after the cells were treated with the supernatants of *L. acidophilus* and *B. longum*, respectively for 12 h and 24 h. The complementary deoxyribonucleic acid (cDNA) was synthesized with a two-step reverse transcription kit (BioRad Laboratories Inc., Hercules, CA, USA), followed by real-time PCR conducted on an Applied Biosystems (ABI) One plus setup PCR thermocycler using the SYBR Green PCR Master Mix (Roche Applied Science, Mannheim, Germany) in a 96-well plate. The PCR reactions were set up in a volume of 20 μL containing 2 μL cDNA, 10 μL 2 × iQ™ SYBR® Green Supermix (Roche Applied Science, Mannheim, Germany), 1 μL each of the forward and reverse primers, and filled the remaining with the RNase-Free Water. The PCR cycle parameters were as follows: initial denaturation at 95°C for 10 min, 40 cycles of 95°C for 10 s and 60°C for 30 s, followed by a dissociation stage for recording the melting curve. The cycle threshold (Ct) values for all genes were obtained, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was measured as an internal control for normalization. Data were analyzed according to the relative expression using the $2^{-\Delta\Delta Ct}$ method. Each sample was analyzed in triplicate, and the mean values were presented. Primers used for the PCR were: SERT forward, 5'-AAT GGG TAC TCA GCA GTT CC-3' and reverse, 5'-CCA CAG CAT AGC CAA TCA C- 3'; GAPDH forward, 5'-CCCTTCATTGACCTCAACTACATGG-3' and reverse, 5'- CATGGTGGTGAAGACGCCAG-3'.

### Western blotting

Proteins were extracted from HT-29 and Caco-2 cells after treating with *L. acidophilus* and *B. longum* supernatants, respectively for 12 h and 24 h; the concentrations were determined by a Bicinchoninic Acid (BCA) protein

assay kit (Beijing Solarbio Science and Technology Co., Ltd., Beijing, China). The remaining supernatant (60 μL) was combined (3:1) with 4 × Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer, and boiled for 10 min. An equivalent of 20 μg protein from each sample was analyzed on a 10% SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% (w/v) nonfat milk, and SERT protein was detected using rabbit anti-SERT polyclonal antibody (ab181034, 1:500; Abcam, Cambridge, UK). Mouse anti-β-actin (Abcam) monoclonal antibody was used to the reference protein. The membranes were incubated with either horseradish peroxidase-labeled goat anti-rabbit (EarthOx Life Sciences, Millbrae, CA, USA) or goat anti-mouse secondary antibodies (EarthOx Life Sciences). All the antibodies were diluted with 5% (w/v) nonfat milk. The SERT/β-actin ratio was calculated from the films with the Quantity One Analysis Software (Bio-Rad, Hercules, CA, USA), and the results expressed in densitometric units.

### Statistical analyses

The statistical analysis was carried out using SPSS 19.0 (SPSS, Chicago, IL, USA). One-way analysis of variance (ANOVA) and posthoc tests (Dunnett's T3 and Dunnett's C) were used to compare the values of RT-PCR. For all analyses, $P = 0.05$ was defined as statistically significant.

## RESULTS

### Effects of *L. acidophilus* supernatant on SERT mRNA and protein expression in HT-29 and Caco-2 cells

The mRNA levels of SERT in HT-29 cells treated with 1:100, 1:50, and 1:20 dilutions of *L. acidophilus* supernatant for 12 h were 1.80-, 2.24-, and 2.28-fold higher than that in the control group, respectively ($P < 0.05$, $P < 0.05$, $P < 0.05$). On the other hand, the levels after 24 h treatment were 2.04, 2.30, and 2.80-fold higher than that in the control group, respectively ($P < 0.05$, $P < 0.05$, $P < 0.05$). The upregulated of SERT mRNA stimulated by 1:20 dilution of *L. acidophilus* supernatant for 24 h was significantly higher than that by 1:100 ($P < 0.05$). Similarly, the protein levels were increased significantly in response to 12 h and 24 h incubation of HT-29 cells at 1:100, 1:50, and 1:20 of *L. acidophilus* supernatant [Figure 1: SERT in HT-29 cells treated with *L. acidophilus* supernatant].

SERT mRNA levels in Caco-2 cells treated with 1:100, 1:50, and 1:20 dilutions of *L. acidophilus* supernatant for

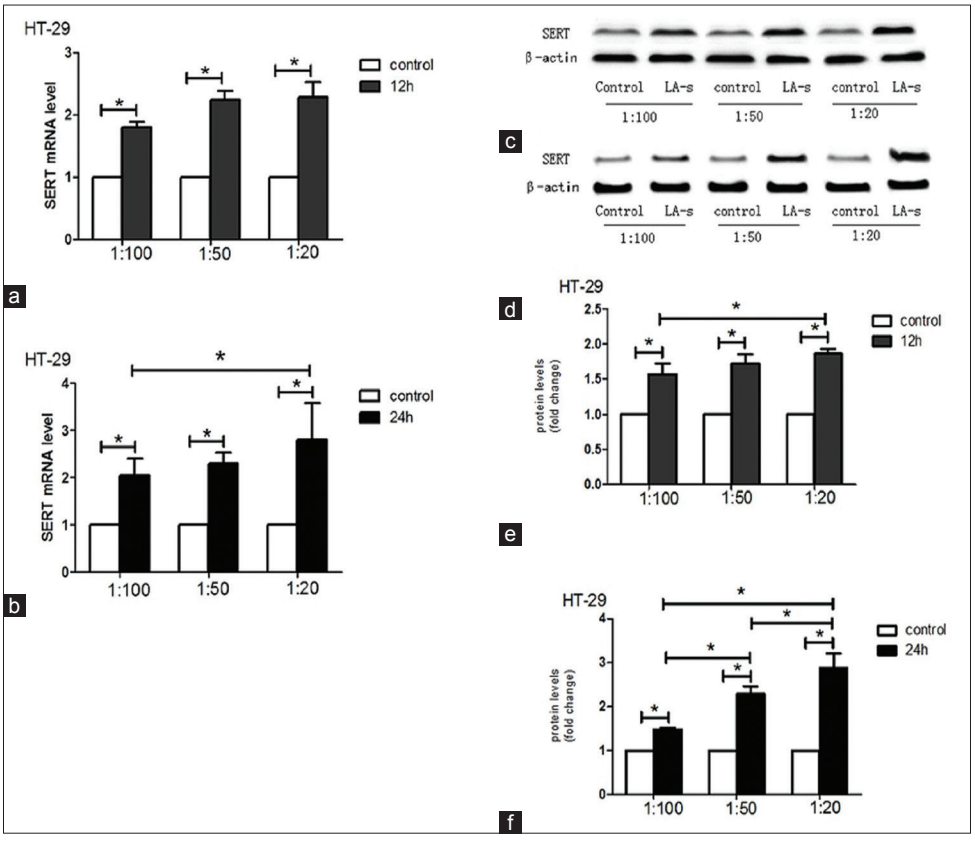


**Figure 1:** SERT in HT-29 cells treated with L. acidophilus supernatant. The levels of mRNA of 12h (a), 24h (b); the protein of 12h (c and e), 24h (d and f). *: $P<0.05$, a significant difference

12 h were 1.20-, 1.22-, and 1.61-fold higher than that in the control group, respectively ($P > 0.05$, $P > 0.05$, $P < 0.05$). SERT mRNA levels in Caco-2 cells treated with 1:100, 1:50, and 1:20 dilutions of *L. acidophilus* supernatant for 24 h were 1.16-, 1.50-, and 2.13-fold higher than that in the control group, respectively ($P > 0.05$, $P < 0.05$, $P < 0.05$, respectively). The upregulated of SERT mRNA stimulated by 1:20 dilution of *L. acidophilus* supernatant for 12 h and 24 h was significantly higher than that by 1:100 and 1:50 ($P < 0.05$, $P < 0.05$, respectively). The upregulated level of SERT mRNA stimulated by 1:50 of *L. acidophilus* supernatant for 24 h was also significantly higher than that by 1:100 ($P < 0.05$). The increase in protein levels in Caco-2 cells in response to 12 h and 24 h incubation in different concentrations of *L. acidophilus* supernatant were similar to that of SERT mRNA [Figure 2: SERT in Caco-2 cells treated with *L. acidophilus* supernatant].

### Effects of *B. longum* supernatant on SERT mRNA and protein expression in HT-29 and Caco-2 cells

The SERT mRNA levels in HT-29 cells treated with dilutions 1:100, 1:50, and 1:20 of *B. longum* supernatant for 12 h were 0.96-, 1.90-, and 2.50-fold higher than that in the control group, respectively ($P > 0.05$, $P < 0.05$, $P < 0.05$, respectively). The mRNA levels after 24 h treatment were 2.58-, 2.19-, and 3.21-fold higher than that in the control group, respectively ($P < 0.05$, $P < 0.05$, $P < 0.05$, respectively). The upregulated level of SERT mRNA stimulated by 1:20 concentration of *B. longum* supernatant for 12 h was significantly higher than that by 1:100 ($P < 0.05$). Similar to the mRNA levels, the protein levels were significantly increased in response to 12 h and 24 h incubation of HT-29 cells at 1:50 and 1:20 concentrations of *B. longum* supernatant [Figure 3: SERT in HT-29 cells treated with *B. longum* supernatant].

The SERT mRNA levels in Caco-2 cells treated with 1:100, 1:50, and 1:20 dilutions of *B. longum* supernatant for 12 h were 1.25-, 2.06-, and 1.83-fold higher than that in the control group, respectively ($P > 0.05$, $P < 0.05$, $P < 0.05$, respectively). SERT mRNA levels in Caco-2 cells treated with1:100, 1:50, and 1:20 concentrations of *B. longum* supernatant for 24 h were 1.84-, 2.23-, and 2.13-fold higher than that in the control group, respectively ($P < 0.05$, $P < 0.05$, $P < 0.05$). The upregulated levels of SERT mRNA stimulated by 1:20 and 1:50 concentrations of *B. longum* supernatant for 12 h were significantly higher

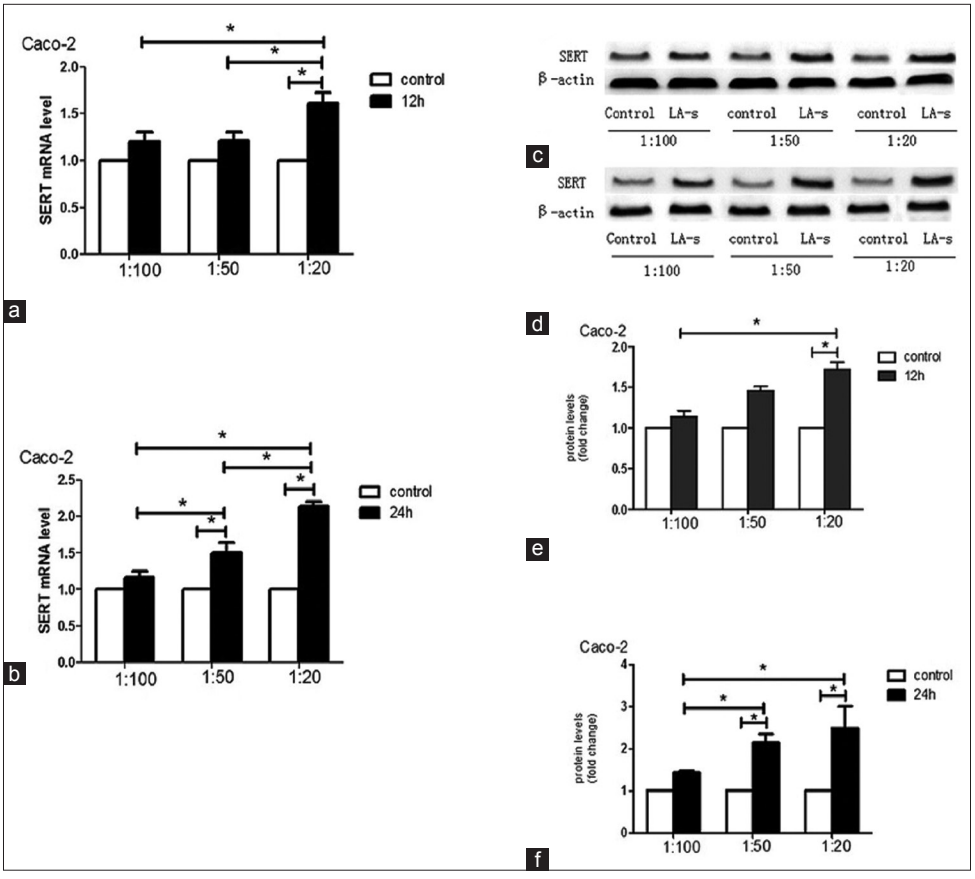


**Figure 2:** SERT in Caco-2 cells treated with L. acidophilus supernatant. The levels of mRNA of 12h (a), 24h (b); the protein of 12h (c and e), 24h (d and f). *: $P<0.05$, a significant difference

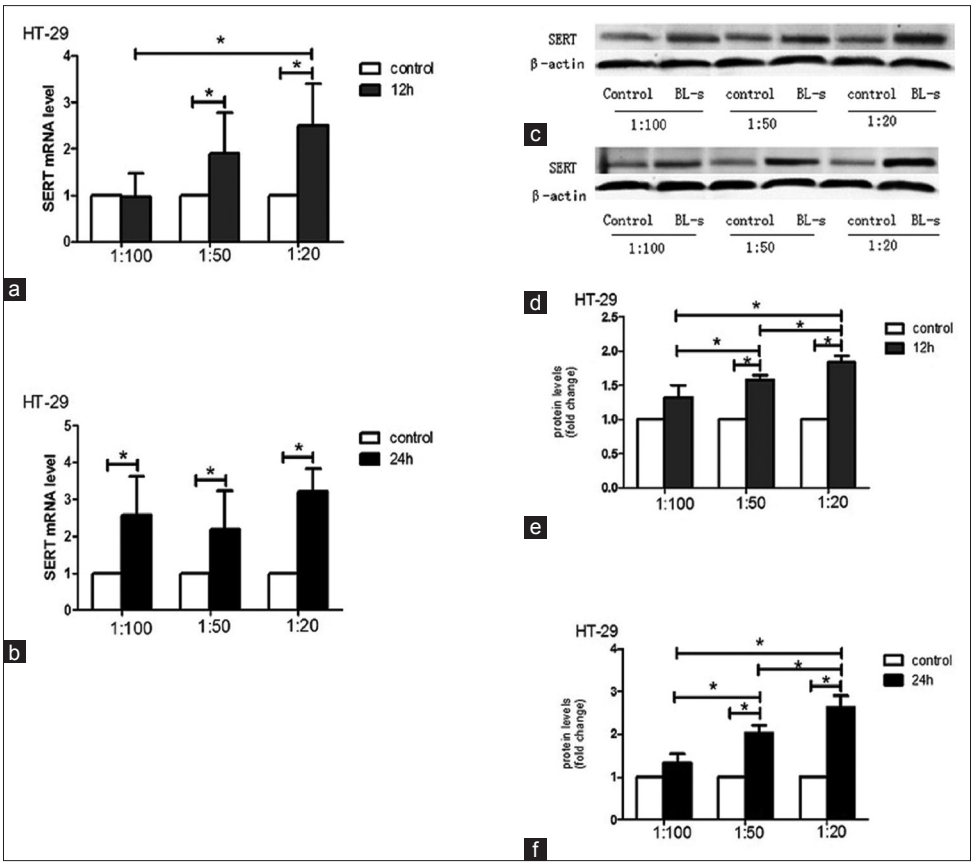


**Figure 3:** SERT in HT-29 cells treated with B. longum supernatant. The levels of mRNA of 12h (a), 24h (b); the protein of 12h (c and e), 24h (d and f). *: *P*<0.05, a significant difference

than that by 1:100 ($P < 0.05$, $P < 0.05$). The increase in the protein levels in Caco-2 cells in response to 24 h incubation at different concentrations of *B. longum* supernatant were similar to that of SERT mRNA [Figure 4: SERT in Caco-2 cells treated with *B. longum* supernatant].

## DISCUSSION

IBS is a chronic functional bowel disease with a prevalence of 10–15% in the industrialized world.[9] As a multifactorial functional disorder, the pathophysiology of IBS is not completely understood; however, the genetic factors, visceral hypersensitivity, gastrointestinal motility abnormalities, and other factors might be involved in the pathogenesis of IBS.[10,11]

Probiotics are defined as living microorganisms, which exert beneficial effects on human health upon ingestion. The most commonly administered probiotics belong to genera lactobacillus or bifidobacterium and can be applied alone (monospecies) or in combination with several other species (multispecies). A meta-analysis showed that probiotics improved the pain scores if they contained *B. breve*, *B. longum*, or *L. acidophilus*. Distension scores were improved by probiotics containing *B. breve*, *B. infantis*, *L. casei*, or *L. plantarum* species.[12] Yoon *et al.* found that multispecies probiotics (a mixture of *B. longum*, *B. bifidum*, *B. lactis*, *L. acidophilus*, *L. rhamnosus*, and Streptococcus thermophiles) are effective in IBS patients that can alter the composition of intestinal microbiota.[13]

*L. acidophilus* is a Gram-positive bacilli belonging to the genus Lactobacillus and can release lactic acid, acetic acid, and some antibiotics primarily in the small intestine.[14,15] Bifidobacterium is an anaerobic Gram-positive bacillus isolated from the feces of breast-fed infants by Tissier *et al.*[16] Bifidobacterium genus of bacteria harbors more than 30 species, of which, nine are present in the human intestinal tract.[17-20] *L. acidophilus* and *B. longum* are used clinically and have been widely used in probiotic research. Current studies have shown that *L. acidophilus* and *B. longum* can improve the clinical symptoms in IBS patients; however, the underlying mechanism is not clear. The previous studies have shown that LGG supernatants can upregulate the levels of SERT expression in Caco-2, HT-29, and mouse colon tissues.[8] Moreover, we hypothesize that *L. acidophilus* and *B. longum* can regulate the expression level of SERT in intestinal epithelium cells.

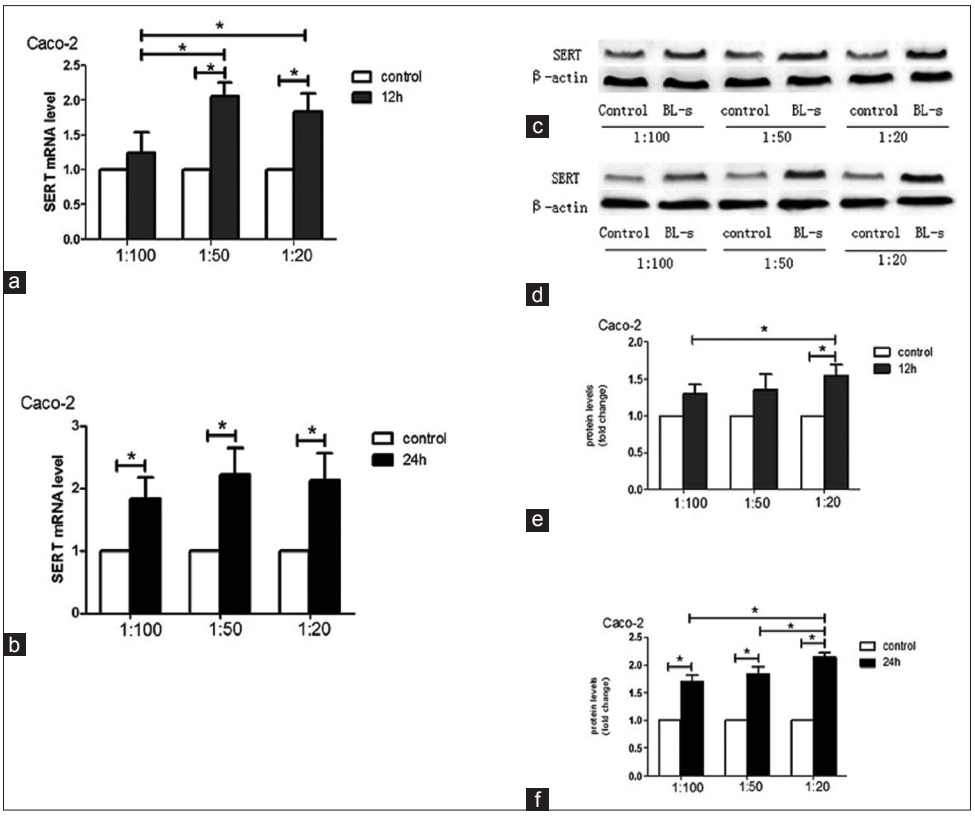


**Figure 4:** SERT in Caco-2 cells treated with B. longum supernatant. The levels of mRNA of 12h (a), 24h (b); the protein of 12h (c and e), 24h (d and f). *: *P*<0.05, a significant difference

In the present study, the expression levels of SERT mRNA and protein were significantly upregulated in HT-29 cells treated with different concentrations of *L. acidophilus* supernatant at 12 h and 24 h, and was found to be time-dependent. The expression levels of SERT mRNA and protein stimulated by each concentration of *L. acidophilus* supernatant for 24 h was higher than that for 12 h. However, the *L. acidophilus* supernatant diluted to 1:100 upregulated the SERT mRNA and protein levels in Caco-2 cells when treated for 12 h and 24 h without statistical significance. Similar to the *L. acidophilus* supernatant, the *B. longum* supernatant diluted to 1:50 and 1:20 increased the expression levels of SERT mRNA and protein in HT-29 cells when treated for 12 and 24h. The *B. longum* supernatant diluted to 1:100 significantly increased the expression levels of SERT mRNA in HT-29 cells when treated for 24 h; however, the expression levels of the protein in HT-29 cells were increased after treatment for 24 h without statistical significance. This phenomenon might be attributed to the fact that in addition to *B. longum* supernatant, other unknown factors might effect the SERT protein expression. Moreover, the expression levels of SERT mRNA and protein were significantly upregulated in Caco-2 cells treated with different concentrations of *B. longum* supernatant at 12 h and 24 h similar to that in HT-29 cells.

The supernatant of *L. acidophilus* and *B. longum* could up-regulate the expression of SERT mRNA in HT-29 and Caco-2 cells, which is time- and concentration-dependent and the current data were not sufficient in order to demonstrate the statistical differences between the two probiotics. The level of expression of SERT protein in the stimulated groups was not identical to that of the mRNA expression, which may be attributed to various unknown factors regulating the process of translation from mRNA to protein; however, the trend of mRNA and protein expression was similar.

Considering that enteric 5-HT is responsible for the secretion, motility, and perception of the bowel.[4,21] High 5-HT is commonly associated with depressed SERT mRNA in patients with IBS as compared to healthy controls.[22,23] The difference in the expression of SERT between IBS patients and healthy controls might suggest that SERT plays an essential role in IBS pathogenesis,[24-29] and could serve as a novel therapeutic target for IBS. SERT could be regulated by several factors, including gene polymorphisms,[30,31] microRNAs,[32] immunity, and inflammation,[33] and growth factors.[34-36] Our study showed

that *L. acidophilus* supernatant and *B. longum* supernatant could increase the expression of SERT in the intestinal epithelial cells. Based on the results of the current study, we aspire to explore similar macromolecular proteins between different probiotics strains in order to identify the factors that stimulate the upregulation of SERT expression.

## CONCLUSION

In summary, we demonstrate that the supernatants of L. acidophilus and B. longum upregulate the expression SERT in HT-29 and Caco-2 cells. Since the levels of 5-HT are decreased, this may indicate a potential mechanism for the usage of probiotics.

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### Conflicts of interest

There are no conflicts of interest.

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