Lactobacillus plantarum YS-3 Prevents Activated Carbon-Induced Constipation in Mice

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ABSTRACT The aim of this study was to determine the effects of *Lactobacillus plantarum* YS-3 (LP-YS3) on activated carbon-induced constipation in Kunming mice. The results of the experiment show that the antigastric acid activity and bile salt tolerance of LP-YS3 were stronger than those of *Lactobacillus bulgaricus* (LB). LP-YS3 inhibited loss of body weight caused by constipation and further reductions in fecal weight, particle number, and water content in mice. Moreover, LP-YS3 elevated the gastrointestinal transit rate and reduced the time required for initial black stool defecation. LP-YS3 also elevated motilin (MTL), endothelin (ET), acetylcholinesterase (AChE), substance P (SP), and VIP serum levels and reduced somatostatin (SS) levels in constipated mice. Hematoxylin–eosin (H&E) staining revealed that high concentration of LP-YS3 reduced the incidence of injuries to small intestine villi and the intestinal wall compared to carbon-induced constipation groups. Reverse transcription-polymerase chain reaction and western blot experiments demonstrated that LP-YS3 upregulated c-Kit, stem cell factor, and glial cell line-derived neurotrophic factor mRNA and protein expression and downregulated transient receptor potential vanilloid 1 and nitric oxide synthase expression in small intestine tissue from constipated mice. In conclusion, high concentrations of LP-YS3 had stronger and more beneficial effects than LB. Based on these results, we conclude that LP-YS3 can effectively inhibit constipation.

KEYWORDS: • constipation • gene expression • *Lactobacillus bulgaricus* • *Lactobacillus plantarum* YS-3

INTRODUCTION

YAK SOUR MILK, a naturally fermented dairy product native to the Tibetan area of the Qinghai Tibet Plateau, is very rich in many types of nutrients with functionalities related to antioxidation, cholesterol reduction, and immune system regulation.¹ Yak yogurt is formed under special natural fermentation conditions that includes fermented raw milk, fermentation temperature, total time, fermentation vessel, and especially the fermentation microorganism, which produces a distinct flavor and quality of yak yogurt.² In this study, we isolated and identified lactic acid bacteria from yak sour milk in the Yushu Tibetan Autonomous Prefecture of Qinghai. We named one of these strains *Lactobacillus plantarum* YS-3 (LP-YS3) and performed further research.

Constipation is defined as a state in which the weekly defecation frequency is less than three, and severe con-

stipation is when the frequency further decreases to once per week. Constipation is a threat to human health and has remarkably complex symptoms affecting colon health.³ Constipation is usually not considered as a disease, as it can be prevented or cured by regulating diet and lifestyle habits.⁴ In this study, we analyzed food physiological effects on constipation by establishing an animal constipation model. For this, we applied gastric-activated carbon to mice to activate the surface of the mucous membrane of the gastrointestinal (GI) tract. Activated carbon reduces water content and gastric juices in the digestive tract, thereby slowing GI motility and causing constipation.⁵

Large doses of activated charcoal can be used to block defecation. Our team previously investigated the preventive effects of lactic acid bacteria on constipation and observed reductions of initial black defecation time. Moreover, the preventive effects of lactic acid bacteria against constipation were further confirmed based on various indices such as serum motilin (MTL), gastrin (Gas), endothelin (ET), somatostatin (SS), acetylcholinesterase (AChE), substance P (SP), and vasoactive intestinal peptide (VIP) levels, which are commonly used evaluation indices when assessing *Lactobacillus* effects on constipation.⁶ Further, molecular

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level experiments can detect related mRNA expression levels in small intestine tissue to confirm the physiological effects of lactic acid bacteria.

In this study, the effects of LP-YS3 on constipation were evaluated, and *Lactobacillus bulgaricus* (LB) was used for the control group. First, we performed *in vitro* experiments to compare the tolerances of LP-YS3 and LB in artificial gastric juice and bile salt, thereby providing a preliminary understanding of the physiological role of LP-YS3 in GI activity. We further studied the active effects of LP-YS3 on constipation through animal experiments. The results of this study provide a theoretical foundation for the further development of LP-YS3 as a cure for constipation.

MATERIALS AND METHODS

Experimental microorganism strains

The lactic acid bacterium used was LP-YS3, and this strain was preserved in the China Center for Type Culture Collection (CCTCC No: 2016749, Wuhan, China) with the preservation number M2016749. *L. bulgaricus* (LB, CCTCC AB 200048) was purchased from the CCTCC.

Detection of lactic acid bacteria tolerant to pH 3 artificial gastric juice

NaCl and pepsin were dissolved in distilled water with pH adjusted to 3 using 1 mol/L of HCl to obtain a mass ratio of 0.2% NaCl and 0.35% pepsin, followed by vacuum filtration to remove bacteria. Activated LP-YS3 was cultured, and 5 mL was extracted by centrifugation at 1100 g (iCEN-24, Allsheng, Hangzhou, Zhejiang, China) for 10 min to collect bacteria. Saline was added to obtain a 5-mL bacterial suspension, which was mixed with artificial gastric juice at a volume ratio of 1–9. After shaking and culture for 3 h at 37°C, we carefully measured the number of viable LP-YS3 bacteria at 0 and 3 h to calculate the tolerance of lactic acid bacteria to artificial gastric juice (%) = viable count (CFU/mL) at 3 h/viable count (CFU/mL) at 0 h × 100%.⁷

Detection of bile salt tolerance of lactic acid bacteria

LP-YS3 (2% inoculum concentration) was inoculated into MRS-THIO culture medium containing 0.0%, 0.3%, 0.5%, and 1% ox bile salt and then cultured for 24 h at 37°C. Culture medium without LP-YS3 was used as the control group. We measured the absorbance value at 600 nm to obtain the bile salt tolerance of lactic acid bacteria (%) = Bile salt culture medium OD₆₀₀/blank medium OD₆₀₀ × 100%.⁷

Animal experiment

Seven-week-old female Kunming mice were purchased from the experimental animal center of Chongqing Medical University (SYXK [Chongqing] 2012-0001). Mice were fed in a normal air, temperature, and humidity environment (temperature [25°C ± 2°C], relative humidity [50% ± 5%]) under a 12 h light/12 h dark cycle and with free access to standard mouse feed and water. A total of 100 Kunming

mice weighing about 25 g each were divided into five groups: normal group, constipation control group, LB treatment group, low LP-YS3 concentration treatment group, and high LP-YS3 concentration treatment group. After 1 week of feeding, the normal and constipation control groups were allowed free access to normal diet and water for 2 weeks. The LB treatment group, low LP-YS3 concentration treatment (LP-YS3-L) group, and high LP-YS3 concentration (LP-YS3-H) group were fed 2 mL of LB (1.0 × 10⁹ CFU/kg), 2 mL of LP-YS3 (1.0 × 10⁸ CFU/kg), and 2 mL of LP-YS3 (1.0 × 10⁹ CFU/kg), respectively, for 2 weeks in addition to normal diet and drinking water. After 2 weeks, mice from all groups except the normal group were intragastrically infused with 2 mL of 10% activated carbon water (10% activated carbon mixed with 10% Arabia gum according to mass ratio to maintain the suspension) for 3 days. Meanwhile, lactic acid bacteria were fed to all mice.⁵ Weight, dietary intake, water consumption, fecal weight, and fecal humidity were measured at 9:00 a.m. daily during the experiment. The protocol of this experiment was approved by the Animal Ethics Committee of Chongqing Medical University.

Determination of small intestinal propulsive rate and excretion time of activated carbon

On day 17 of the experiment, after intragastric administration of activated carbon water, food was withheld from all mice, including the normal group, for 24 h, although drinking water was still available. After 24 h, 0.2 mL of glacial activated carbon water was administered to all mice. Then, after 30 min, half of the mice (10) from each group were killed by cervical dislocation and blood collected. The small intestine was removed to determine the recommended rate of activated carbon in the small intestine of mice. The recommended rate (%) = activated carbon propulsive distance/total length of small intestine × 100. The remaining 10 mice in each group were used to determine the time of initial black stool.

Determination of serum MTL, Gas, ET, SS, AChE, SP, and VIP levels in mice

The collected mouse blood samples were centrifuged at 1650 g (iCEN-24, Allsheng, Hangzhou, Zhejiang, China) for 15 min to obtain serum, which was used to determine the levels of motilin (MTL), gastrin, endothelin-1 (ET-1), substance P (SP), somatostatin (SS), VIP, and acetylcholine enzyme (AChE) according to the kit instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China).

Determination of small intestine tissue MPO, NO, GSH, and MDA levels in mice

Small intestine tissues (100 mg) were homogenized using phosphate-buffered saline on ice. Small intestine levels of myeloperoxidase (MPO), nitric oxide (NO), glutathione (GSH), and malondialdehyde (MDA) were determined using commercial kits (Cayman Chemical Co., Ann Arbor, MI, USA) according to the manufacturer's protocols.

Reverse transcription-polymerase chain reaction assay

Small intestine tissue from mice were crushed, and general RNAs of colon tissue were extracted by RNazol (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The total RNA concentration was diluted to 1 $\mu\text{g}/\mu\text{L}$. Then, 2 μL of diluted general RNA extraction solution was added to 1 μL each of oligodT18, RNase, dNTP, and MLV enzymes (Thermo Fisher Scientific, Inc.) and 10 μL of 5 $\times$ buffer to obtain cDNA at 37°C for 120 min, 99°C for 4 min, and 4°C for 3 min. Reverse transcription-polymerase chain reaction (RT-PCR) was performed to amplify c-Kit, stem cell factor (SCF), transient receptor potential cation channel subfamily V member 1 (TRPV1), glial cell-derived neurotrophic factor (GDNF), and NO synthase (NOS) (Thermo Fisher Scientific, Inc.) mRNA expression (Table 1), and the housekeeping gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as the reference. Finally, electrophoresis on 1% agar gel was performed to measure amplified PCR products.⁸ ImageJ 1.44 software was used to conduct semi-quantitative analysis of the results.

Western blot assay

Small intestine tissue (0.5 g) was placed into 1 mL of cell lysate, ground with a homogenizer, and then centrifuged at 4°C and 440 g (iCEN-24, Allsheng, Hangzhou, Zhejiang, China). The upper layer was then removed and stored at -80°C. The protein standard curve was drawn according to the instructions of the BCA Protein Concentration Assay Kit (Thermo Fisher Scientific, Inc.), and the protein concentration in the sample was measured from the standard curves. The amount of protein was mixed with 2 $\times$, and 12% SDS-PAGE (Thermo Fisher Scientific, Inc.) gel electrophoresis (140 V, 2 h) was carried out. Proteins were transferred onto a nitrocellulose membrane (4°C, 100 mA, 1 h) and blocked (5% skim milk at 25°C shaker 1 h). After incubation with TBST three times for 10 min each, the membrane was kept overnight at 4°C. The membrane was then incubated at 25°C

for 2 h with secondary antibody and then washed three times with TBST for 10 min. Finally, the gray value of each group was compared.⁹

Data analysis

The results of three parallel repeated experiments were averaged. SAS9.1 software was used to analyze significant differences in the data of each group at $P < .05$ level by using one-way ANOVA.

RESULTS

Acid and bile salt resistance of LP-YS3

In vitro experiments showed that LP-YS3 had better survivability in artificial gastric juice (pH 3) and stronger growth in bile salt than LB (Table 2). Specifically, survivability of LP-YS3 in artificial gastric juice (pH 3) was 72.38%, which was three times greater than that of LB (23.87%). Growth of LP-YS3 in 1.0% bile salt (11.02%) was also higher than that of LB (1.13%). On the whole, the acid and bile salt resistance abilities of LP-YS3 were better than those of commercial lactic acid bacteria LB, LP-YS3 had the better potential for use as a probiotic than LB.

Body weights of LP-YS3 treated mice

Body weights of mice significantly changed ($P < .05$) between days 1 and 14 (Fig. 1). Activated carbon treatment induced body weight reduction in mice. Control mice showed the lowest body weight, whereas LB and LP-YS3 treatments prevented loss of body weight. Body weight of mice treated with high concentrations of LP-YS3 (LP-YS3-H) were similar to that of normal mice. The constipation induced by activated carbon resulted in the loss of weight in mice, and LP-YS3 could alleviate the loss of weight, and the effect was better than that of LB.

Stool status of LP-YS3 treated mice

Stool weight, number of stool particles, and water content of stool were significantly different among the different groups of mice from days 1 to 14 (Table 3). Following induction of constipation by activated carbon, control mice showed the lowest stool weight, stool particle counts, and water content of stool. On the other hand, LP-YS3-H elevated all three stool parameters in comparison to control

TABLE 1. SEQUENCES OF REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION PRIMERS USED IN THIS STUDY

Gene name	Sequence
c-Kit	Forward: 5'-AGA CCG AAC GCA ACT-3' Reverse: 5'-GGT GCC ATC CAC TTC A-3'
SCF	Forward: 5'-AAA CTG GTG GCG AAT-3' Reverse: 5'-CAC GGG TAG CAA GAA-3'
TRPV1	Forward: 5'-AGC GAG TTC AAA GAC-3' Reverse: 5'-TTC TCC ACC AAG AGG-3'
GDNF	Forward: 5'-TTT TAT TCA AGC CAC CAT-3' Reverse: 5'-AGC CCA AAC CCA AGT-3'
NOS	Forward: 5'-CCA CAT CTG GCA GGA-3' Reverse: 5'-AGG CAC AGA ACT GAG-3'
GAPDH	Forward: 5'-CGG AGT CAA CGG ATT-3' Reverse: 5'-AGC CTT CTC CAT GGT-3'

SCF, stem cell factor; TRPV1, transient receptor potential cation channel subfamily V member 1; GDNF, glial cell-derived neurotrophic factor; NOS, nitric oxide synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

TABLE 2. ACID AND BILE SALT RESISTANCE ACTIVITIES OF *LACTOBACILLUS PLANTARUM* YS3 AND *LACTOBACILLUS BULGARICUS*

Strain	Survival in pH 3.0 artificial gastric juice (%)	Growth in bile salt (%)		
		0.3%	0.5%	1.0%
LP-YS3	72.38 $\pm$ 7.62	19.33 $\pm$ 1.28	16.82 $\pm$ 2.68	11.02 $\pm$ 1.89
LB	23.87 $\pm$ 4.25	2.28 $\pm$ 0.58	1.52 $\pm$ 0.44	1.13 $\pm$ 0.21

Values presented are the mean $\pm$ standard deviation.

LP-YS3, *Lactobacillus plantarum* YS3; LB, *Lactobacillus bulgaricus*.

FIG. 1. Effects of *Lactobacillus bulgaricus* and *Lactobacillus plantarum* YS3 on body weight changes in activated carbon-induced constipated mice during the experiment ($N=10/\text{group}$). ^{a-e}Mean values with different letters over the bars are significantly different ($P<.05$) according to Duncan's multiple-range test. LB: *L. bulgaricus* (1.0×10^9 CFUs/kg bw); LP-YS3-L: *L. plantarum* YS3 low dose (1.0×10^8 CFU/kg bw); LP-YS3-H: *L. plantarum* YS3 high dose (1.0×10^9 CFU/kg bw). BW, body weight; CFU, colony-forming unit.

mice. Moreover, LP-YS3-H provided better results than the same concentration of LB and low concentration of LP-YS3 (LP-YS3-L) but slightly worse results compared to normal mice. Constipation led to abnormality of feces, and LP-YS3 could normalize the excrement of the constipated mice, and was more effective than in the LB treated mice.

Initial black stool defecation time of LP-YS3 treated mice

On the last day of the animal experiment, all mice were treated with activated carbon. Initial black stool defecation times of mice in the normal, control, LB, LP-YS3-L, and LP-YS3-H groups were 83 ± 9 min, 195 ± 28 min, 163 ± 22 min, 147 ± 26 min, and 112 ± 19 min, respectively (Fig. 2). Initial black stool defecation time was reduced in mice treated with LP-YS3 compared with control mice, and LP-YS3-H showed the best results overall. Constipation led to difficulty in defecation and increased the time of the mice to discharge the initial black stool excretion. LP-YS3 could

reduce the time of the constipated mice to discharge the initial black stool excretion, and the effect was better than that of LB.

GI transit capability of LP-YS3 treated mice

After activated carbon treatment for 30 min, control constipated mice showed the longest GI transit time. Mice in the LB and LP-YS3 groups showed faster GI transit times (Table 4). Regarding overall GI and small intestinal transit times, normal mice showed the fastest transit time based on activated carbon propulsive rate. Transit times of the LP-YS3-H, LP-YS3-L, and LB groups declined, indicating faster propulsive rates, compared with that of control mice. Activated carbon induced constipation could slow down the actions of activated carbon in the small intestine, and the residence time of activated carbon in the small intestine is long. LP-YS3 could accelerate the GI of activated carbon in the small intestine, and the effect was better than LB.

Serum levels of LP-YS3 treated mice

As shown in Table 5, serum levels of MTL, Gas, ET, AChE, SP, and VIP in control mice were the lowest. On the contrary, serum levels of these parameters in normal mice were the highest. LB and LP-YS3 group mice showed increased levels of MTL, Gas, ET, AChE, SP, and VIP in addition to reduced SS levels compared with control mice. Meanwhile, LP-YS3-H treated mice showed remarkable increases in MTL, Gas, ET, AChE, SP, and VIP levels almost up to those of normal mice. Mice in the LP-YS3-H group also showed a weak SS level, which was slightly stronger than that of normal mice.

Small intestine tissue levels of LP-YS3 treated mice

As shown in Table 6, normal mice showed the lowest MPO, NO, and MDA intestine tissue levels and the highest GSH level. On the contrary, control mice had the highest MPO, NO, and MDA levels and lowest GSH level. LB and LP-YS3 group mice showed increased GSH levels and reduced MPO, NO, and MDA levels compared with control mice, and LP-YS3-H treatment showed the strongest effects.

TABLE 3. STOOL STATUS OF LACTIC ACID BACTERIA TREATED MICE DURING THE EXPERIMENT

Groups	Normal	Control	LB	LP-YS3-L	LP-YS3-H
1–14 days (lactic acid bacteria administration period, but not induction of constipation)					
Stool weight (g)	0.92 ± 0.05^a	0.92 ± 0.05^a	0.91 ± 0.04^a	0.92 ± 0.05^a	0.92 ± 0.05^a
Particle counts of stool	39 ± 3^a	39 ± 4^a	40 ± 4^a	40 ± 3^a	40 ± 3^a
Water content of stool (%)	48 ± 4^a	48 ± 3^a	49 ± 4^a	49 ± 3^a	50 ± 4^a
15–17 days (lactic acid bacteria administration period, induction of constipation)					
Stool weight (g)	0.96 ± 0.04^a	0.43 ± 0.05^d	$0.62 \pm 0.05^{c,d}$	0.64 ± 0.05^c	0.76 ± 0.05^b
Particle counts of stool	40 ± 5^a	22 ± 2^d	27 ± 3^c	$29 \pm 4^{b,c}$	33 ± 4^b
Water content of stool (%)	49 ± 5^a	17 ± 4^d	32 ± 4^c	$34 \pm 4^{b,c}$	41 ± 4^b

Values presented are the mean $\pm$ standard deviation ($N=10/\text{group}$).

^{a-d}Mean values with different letters in the same row are significantly different ($P<.05$) according to Duncan's multiple-range test. LB: *L. bulgaricus* (1.0×10^9 CFUs/kg bw); LP-YS3-L: *L. plantarum* YS3 low dose (1.0×10^8 CFU/kg bw); LP-YS3-H: *L. plantarum* YS3 high dose (1.0×10^9 CFU/kg bw).

BW, body weight; CFU, colony-forming unit.

FIG. 2. Initial black stool defecation time in *L. bulgaricus* and *L. plantarum* YS3 treated activated carbon-induced constipated mice. ^{a-e}Mean values with different letters over the bars are significantly different ($P < .05$) according to Duncan's multiple-range test. LB: *L. bulgaricus* (1.0×10^9 CFUs/kg bw); LP-YS3-L: *L. plantarum* YS3 low dose (1.0×10^8 CFU/kg bw); LP-YS3-H: *L. plantarum* YS3 high dose (1.0×10^9 CFU/kg bw).

Pathological observations in small intestine

Mice in the normal group showed homogenous small intestinal wall thickness and complete small intestinal villi. On the other hand, small intestinal villi in control mice were all injured and impaired, and thickness of the small intestinal wall was heterogeneous. The LB group mice showed a reversal of villi injury and impairment and reduced heterogeneity of the small intestinal wall. The protective effects of LP-YS3 in the small intestine were greater than those of LB. These effects were further enhanced by LP-YS3-H and were slightly stronger than that of LP-YS3-L (Fig. 3).

c-Kit and *SCF* mRNA and protein expression levels in small intestine tissue

As shown in Figure 4, small intestinal tissue from normal mice showed the highest *c-Kit* (9.49 ± 0.42 and 1.91 ± 0.12 -folds of control) and *SCF* (9.37 ± 0.45 and 2.42 ± 0.19 -folds

of control) mRNA and protein expression levels, whereas control mice showed the weakest expression. LB and LP-YS3 treatments enhanced *c-Kit* and *SCF* expression in comparison to untreated control mice. Lastly, LP-YS3-H significantly enhanced *c-Kit* (7.24 ± 0.33 and 1.69 ± 0.11 -folds greater than control) and *SCF* (7.01 ± 0.53 and 2.03 ± 0.17 -folds greater than control) expression levels almost up to those of the normal group.

TRPV1, *GDNF*, and *NOS* mRNA and protein expression levels in small intestine tissue

As shown in Figure 5, normal mice showed the strongest *GDNF* (5.77 ± 0.51 and 2.32 ± 0.32 -folds higher than control) mRNA and protein expression along with the weakest *TRPV1* (0.16 ± 0.03 and 0.57 ± 0.05 -folds of control) and *NOS* (0.24 ± 0.04 and 0.57 ± 0.05 -folds of control) expression in the small intestine. LB, LP-YS3-L, and LP-YS3-H treatments elevated *GDNF* expression and reduced *TRPV1* and *NOS* expression in small intestinal tissue compared with control mice. Lastly, LP-YS3-H treatment elevated *TRPV1* (0.38 ± 0.03 and 0.66 ± 0.05 -folds of control), *GDNF* (4.21 ± 0.31 and 1.93 ± 0.22 -folds of control), and *NOS* (0.33 ± 0.05 and 0.62 ± 0.05 -folds of control) expression levels almost up to those of normal mice.

DISCUSSION

Lactobacillus play a probiotic role and show physiological function in the strong bile acid environment of the stomach and intestine.¹⁰ Here, we studied the potential probiotic effects of lactic acid bacteria by establishing an *in vitro* virtual model, which is a common method to detect the antigastric acid activity and bile salt tolerance of lactic acid bacteria.¹¹ We observed that LP-YS3 had stronger antigastric activity and bile salt tolerance than LB, which has good physiological activity.

Body weight change is an important indicator of constipation in mice, and our study as well as many other

FIG. 3. Pathological observation of small intestine tissue in *L. bulgaricus* and *L. plantarum* YS3 treated activated carbon-induced constipated mice. LB: *L. bulgaricus* (1.0×10^9 CFUs/kg bw); LP-YS3-L: *L. plantarum* YS3 low dose (1.0×10^8 CFU/kg bw); LP-YS3-H: *L. plantarum* YS3 high dose (1.0×10^9 CFU/kg bw).

TABLE 4. GASTROINTESTINAL TRANSIT CAPABILITY AND FIRST BLACK STOOL DEFECATION TIME IN *LACTOBACILLUS BULGARICUS* AND *LACTOBACILLUS PLANTARUM* YS3 TREATED ACTIVATED CARBON-INDUCED CONSTIPATED MICE

Groups	Length of small intestine (cm)	Length of GI transit (cm)	Activated carbon propulsive rate (%)
Normal	41.2 ± 4.0 ^a	39.2 ± 4.1 ^a	95.1 ± 1.1 ^a
Control	40.3 ± 3.9 ^a	6.3 ± 1.2 ^e	15.6 ± 2.2 ^e
LB	40.8 ± 4.3 ^a	23.8 ± 2.2 ^d	58.3 ± 2.4 ^d
LP-YS3-L	40.8 ± 4.5 ^a	28.3 ± 1.9 ^c	69.4 ± 2.5 ^c
LP-YS3-H	40.9 ± 4.1 ^a	31.2 ± 2.5 ^b	76.3 ± 2.2 ^b

Values presented are the mean ± standard deviation (N = 10/group).

^{a-e}Mean values with different letters in the same column are significantly different ($P < .05$) according to Duncan's multiple-range test. LB: *L. bulgaricus* (1.0×10^9 CFU/kg bw); LP-YS3-L: *L. plantarum* YS3 low dose (1.0×10^8 CFU/kg bw); LP-YS3-H: *L. plantarum* YS3 high dose (1.0×10^9 CFU/kg bw).

GI, gastrointestinal.

studies have shown that mice with active carbon-induced constipation weigh less than health mice.¹² For instance, hydrochloric acid amine-induced constipation in mice can lead to body weight loss in mice.¹³ Our study results corroborate literature reports that animal constipation can lead to weight loss, as LP-YS3 showed the greatest inhibitory effects against weight loss caused by constipation.

Stool quality, fecal grains, and fecal water content are important indicators of fecal status and reflect an aggravated degree of constipation when low.¹² Many studies have shown that lactic acid bacteria can improve fecal particle number and water content in constipated mice.^{13,14} In this study, the lactic acid bacterium LP-YS3 relieved constipation by improving defecation quality, defecation times, and water content in mice feces.

Constipation can reduce bowel motility and increase fecal transit time, leading to the accumulation of harmful bacteria that feed on fecal matter and thereby threatening intestinal health.¹⁵ Propelling length and propulsive rate of the small intestine can be used to evaluate intestinal activity and constipation in activated carbon-induced constipated mice.⁶

In this study, LP-YS3 reduced the amount of activated carbon in the small intestine of mice, and propulsive rate and length were higher than those of LB mice. The effects of LP-YS3-H were even more obvious.

In terms of establishing an animal model of constipation, mice were chosen since they are less tolerant than rats. According to Chinese medicine, obstruction of air caused by cold temperatures can induce constipation in mice in a manner similar to activated carbon water.¹⁶ Constipation causes retardation of intestinal movement and longer fecal retention times, thereby enlarging initial black stool discharge.⁶ A shorter initial black stool excretion time indicates normal intestinal movements. In this study, LP-YS3 could reduce the initial black stool excretion time, and the effect stronger than LB.

MTL is known to stimulate pepsin production and promote intestinal motility.¹⁷ Gas plays an important role in the GI tract, as it can promote GI secretion, motility, pyloric relaxation, and relief of constipation.⁶ ET plays an important role in vascular tone stability and maintenance of the basic cardiovascular system.¹⁸ SS is used to stimulate bowel movements.¹⁹ AChE promotes muscle contraction and mucus secretion, resulting in muscle relaxation and fecal excretion.²⁰ SP also promotes intestinal peristalsis,²¹ maintaining normal levels of VIP in the intestinal wall as an important means of stabilizing gut function.²² The collective effects of these factors ease constipation. The results of this study show that LP-YS3 normalizes serum levels of these factors and relieves constipation.

Interstitial cells of Cajal (ICC) are slow wave pacemaker cells in the gut and play an important role in enteric nerve signaling. Indeed, ICC affect GI function.²³ Research has shown that ICC from patients with intestinal constipation have lower density than normal ICC. This causes responses that decrease the availability of neurotransmitters, resulting in spontaneous rhythmic slow wave loss and leading to irregular colonic motility and intestinal functional effects.^{23–25} c-Kit is a specific marker of ICC and a key factor in the proliferation of ICC.²⁶ SCF concentration is important for ICC production, as ICC do not grow in the absence of SCF. Animal experiments have shown that the ICC content of colon tissue from constipated mice is very low, and the

TABLE 5. SERUM LEVELS OF MTL, GAS, ET, SS, AChE, SP, AND VIP IN *LACTOBACILLUS BULGARICUS* AND *LACTOBACILLUS PLANTARUM* YS3 TREATED ACTIVATED CARBON-INDUCED CONSTIPATED MICE

Levels (pg/mL)	Normal	Control	LB	LP-YS3-L	LP-YS3-H
MTL	217.8 ± 17.8 ^a	87.0 ± 7.9 ^e	132.3 ± 12.1 ^d	152.7 ± 13.2 ^c	187.2 ± 9.3 ^b
Gas	93.2 ± 5.1 ^a	30.8 ± 3.3 ^e	49.8 ± 2.9 ^d	60.3 ± 2.5 ^c	71.3 ± 2.8 ^b
ET	19.3 ± 1.3 ^a	4.1 ± 0.6 ^e	7.0 ± 0.4 ^d	8.9 ± 0.7 ^c	13.5 ± 0.8 ^b
SS	26.3 ± 2.8 ^c	71.3 ± 3.8 ^a	55.9 ± 2.9 ^b	40.8 ± 2.2 ^c	33.5 ± 1.1 ^d
AChE	39.2 ± 1.5 ^a	8.3 ± 0.5 ^e	14.5 ± 0.6 ^d	24.2 ± 0.7 ^c	31.2 ± 1.4 ^b
SP	73.2 ± 2.6 ^a	28.3 ± 2.2 ^e	50.3 ± 2.5 ^d	59.3 ± 1.5 ^c	66.3 ± 2.7 ^b
VIP	65.3 ± 2.1 ^a	24.6 ± 2.3 ^e	40.3 ± 2.6 ^d	48.3 ± 2.1 ^c	58.3 ± 1.9 ^b

Values presented are the mean ± standard deviation (N = 10/group).

^{a-e}Mean values with different letters in the same row are significantly different ($P < .05$) according to Duncan's multiple-range test. LB: *L. bulgaricus* (1.0×10^9 CFU/kg bw); LP-YS3-L: *L. plantarum* YS3 low dose (1.0×10^8 CFU/kg bw); LP-YS3-H: *L. plantarum* YS3 high dose (1.0×10^9 CFU/kg bw).

MTL, motilin; gastrin, ET-1, endothelin; SS, somatostatin; AChE, acetylcholinesterase; SP, substance P; VIP, vasoactive intestinal peptide.

TABLE 6. SMALL INTESTINE TISSUE LEVELS OF MPO, NO, GSH, AND MDA IN *LACTOBACILLUS BULGARICUS* AND *LACTOBACILLUS PLANTARUM* YS3 TREATED ACTIVATED CARBON-INDUCED CONSTIPATED MICE

Group	MPO (mU/mg)	NO (μ mol/gprot)	MDA (nmol/mg)	GSH (μ mol/mg)
Normal	6.12 $\pm$ 0.21 ^d	0.32 $\pm$ 0.08 ^d	0.38 $\pm$ 0.05 ^d	8.31 $\pm$ 0.62 ^a
Control	17.22 $\pm$ 0.56 ^a	1.92 $\pm$ 0.22 ^a	1.06 $\pm$ 0.13 ^a	4.58 $\pm$ 0.31 ^d
LB	12.05 $\pm$ 0.42 ^b	1.15 $\pm$ 0.12 ^b	0.78 $\pm$ 0.08 ^b	6.68 $\pm$ 0.22 ^c
LP-YS3-L	11.02 $\pm$ 0.32 ^b	0.82 $\pm$ 0.10 ^b	0.66 $\pm$ 0.08 ^b	7.03 $\pm$ 0.15 ^c
LP-YS3-H	8.52 $\pm$ 0.32 ^c	0.61 $\pm$ 0.05 ^c	0.48 $\pm$ 0.06 ^c	7.58 $\pm$ 0.14 ^b

Values presented are the mean $\pm$ standard deviation ($N=10$ /group).

^{a-c}Mean values with different letters in the same column are significantly different ($P < .05$) according to Duncan's multiple-range test. LB: *L. bulgaricus* (1.0×10^9 CFU/kg bw); LP-YS3-L: *L. plantarum* YS3 low dose (1.0×10^8 CFU/kg bw); LP-YS3-H: *L. plantarum* YS3 high dose (1.0×10^9 CFU/kg bw). MPO, myeloperoxidase; NO, nitric oxide; MDA, malondialdehyde; GSH, glutathione.

expression level of c-Kit in colon tissue is also reduced.²⁷ Another study has shown that lactic acid bacteria can effectively increase c-Kit content in the intestinal tract of constipated mice and the number of ICC, thereby promoting intestinal peristalsis and relief of constipation.²⁸ In this

study, constipation reduced the expression of c-Kit and SCF in the small intestine of mice while LP-YS3 treatment effectively upregulated expression of c-Kit and SCF, thereby increasing the number of ICC in the intestinal tracts of constipated mice and inhibiting constipation.

FIG. 4. Small intestine tissue mRNA and protein expression levels of c-Kit and SCF in *L. bulgaricus* and *L. plantarum* YS3 treated activated carbon-induced constipated mice. LB: *L. bulgaricus* (1.0×10^9 CFUs/kg bw); LP-YS3-L: *L. plantarum* YS3 low dose (1.0×10^8 CFU/kg bw); LP-YS3-H: *L. plantarum* YS3 high dose (1.0×10^9 CFU/kg bw). SCF, stem cell factor.

FIG. 5. Small intestine tissue mRNA and protein expression levels of TRPV1, GDNF, and NOS in the LB and LP-YS3 treated activated carbon-induced constipation mice. LB: *L. bulgaricus* (1.0×10^9 CFUs/kg bw); LP-YS3-L: *L. plantarum* YS3 low dose (1.0×10^8 CFU/kg bw); LP-YS3-H: *L. plantarum* YS3 high dose (1.0×10^9 CFU/kg bw). TRPV1, transient receptor potential cation channel subfamily V member 1; GDNF, glial cell-derived neurotrophic factor; NOS, nitric oxide synthase.

Constipation is more common in elderly populations. Bacteria in the human gut produce a large number of toxins such as indole and indole acetic acid, which cause chronic poisoning upon absorption into the body and aging.²⁹ Constipation patients cannot rule out the presence of toxic substances, as the body accumulates large amounts of toxic substances as time passes. Toxic substances accumulated in the body can overwhelm the detoxifying ability of the liver, causing toxic substances to circulate in the blood into the brain, thereby accelerating aging. MPO, NO, GSH, and MDA all are aging-related factors.^{30,31} In this study, LP-YS3 significantly regulated the development of aging-related factors and helped alleviate aging and constipation.

TRPV1 has been shown to be closely related with defecation. Activation of TRPV1 triggers release of neurotransmitters, leading to movement disorders in the small intestine.³² Increased expression of TRPV1 is a significant symptom of intestinal injury, and GI disturbances result in higher TRPV1 expression in patients with constipation. There is a known relationship between constipation and the enteric nervous system. NO is a major inhibitory neurotransmitter in the enteric nervous system that induces smooth muscle relaxation and reduces GI motility. An increase in the number of positive fibers of NOS leads to elevation of NO content, thus affecting intestinal function and causing constipation.^{33,34} NOS also plays an important role in regulating GI motility.³⁵ Upregulation of NO causes serious colonic motility disorders, and controlling NOS could reduce the content of NO as a feasible means to control constipation.³⁶ Expression of these genes in the intestinal tract and thus constipation can be relieved by regulating the expression of TRPV1, GDNF, and NOS. Collectively, these findings demonstrate one of the mechanisms by which lactic acid bacteria (LY-PS3) can suppress constipation.

The Tibetan yak yogurt lactic acid bacterium LP-YS3 has excellent antigastric acid activity and bile salt tolerance, and effectively inhibits body weight loss in mice caused by constipation and changes in fecal weight, particle number, and water content. LP-YS3 also improves the transit rate of activated carbon in the small intestine and reduces initial black defecation time. Analysis of mouse serum found that LP-YS3 improved levels of MTL, ET, SS, AChE, SP, VIP, and SS in constipated mice. RT-PCR and Western blot experiments further showed that LP-YS3 upregulated mRNA and protein expression levels of c-Kit, SCF, and GDNF and downregulated expression of TRPV1 and NOS in constipated mice. The results show that LP-YS3 can effectively relieve constipation better than the commonly used *L. bulgaricus*.

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No competing financial interests exist.

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