

Positive enhancement of *Lactobacillus fermentum* HY01 on intestinal movements of mice having constipation

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Abstract *Lactobacilli* have been used to treat many gastrointestinal disorders. But the outcome of *Lactobacilli* are strain specific. The strain *Lactobacillus Fermentum*, HY01, (LF-HY01) has a good performance in the environment of gastrointestinal tract. In this study, the aim is to investigate the preventive effects of LF-HY01 against activated-carbon-induced constipation in mice. Mice are randomized into four groups. Normal group was fed a normal diet, model group also has the same with activated carbon treatment, and low and high concentration groups are treated with LF-HY01. We have determined many indexes such as body weight, water content in faeces, defecation conditions, the level of small intestinal villi damages and levels of various neurotransmitters in serum, including motilin (MTL), gastrin (GT), endothelin (ET), somatostatin (SST), acetylcholinesterase (AchE), substance P (SP), and vasoactive intestinal peptide (VIP). LF-HY01 has no

significant difference in each group, but it can significantly improve water content of faeces, defecation time of first black stool and activated carbon propelling rate in small intestine as compared of model group. Furthermore, LF-HY01 can effectively prevent small intestinal villi damages, which is less than that of model group. Moreover, LF-HY01 has the consistency to increase the levels of MTL, GT, ET, AchE, SP and VIP, and LF-HY01 can also have the ability to reduce the level of SST. These results suggest that *Lactobacillus Fermentum*, HY01, has a great impact in enhancing intestinal peristalsis ability and has the ability to prevent from activated-carbon-induced constipation in mice.

Keywords Activated carbon · Constipation · Intestinal movement · *Lactobacillus Fermentum* · Traditional fermented yak yoghurt

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Introduction

Constipation is one of the most common functional bowel disorders, which is characterized by the difficulty or infrequent timing of defecation, sensation of incomplete evacuation or accompanied by straining. Constipation has a high incidence, affecting about 14% of the adult populations on a global scale, mainly women, and it can significantly decrease the quality of life with a range of symptoms including anorexia, distension, abdominal pain and nausea [1–3]. However, the exact pathogenesis of constipation is unknown to many grounds, so it still needs more exploration. Previous researches have shown that constipation is probably related to multiple factors, for instance, the amount and morphological changes of the enteric nervous system (ENS) or interstitial cells of Cajal (ICC) [4], dysfunction of autonomic nervous system [5] and morphologic changes in the myenteric and submucosal plexus [6]. They also support that it is also related to decreased neurotransmitter levels (such as 5-HT, NO and VIP) [7–9] and increased neurotransmitter levels (such as SP) [10]. Recently, an increasing number of studies have indicated that the intestinal flora is closely related to many human diseases, such as inflammation, obesity and constipation [11]. Therefore, maintaining a perfect balance of intestinal flora plays an important role in the body's health.

It is well known that probiotics have many functions, which include regulating and improving the intestinal flora, reducing allergen and enhancing immunity and anti-inflammatory effects [12–15]. Besides, probiotics are conductive in the prevention and alleviation of constipation [16]. The possible explanation is that the production of organic acids (lactic acid and acetic acid) by probiotics and the decreased pH value effectively suppress pathogenic bacteria in intestinal tract and regulate intestinal flora balance, which help to improve the gastrointestinal digestion system and the contraction functions of smooth muscles [17, 18]. Furthermore, probiotics can increase the level of neurotransmitters (5-HT and dopamine) by playing an important role in regulating the intestinal immune system [19, 20]. At present, constipation is treated by drugs, such as cisapride, metoclopramide, and erythromycin [21]. But studies have found that drug treatment had side effects and many complications if taken for a long time, such as flatulence, abdominal pain, diarrhoea and nausea. By contrast, probiotics are safe and reliable method of treatment, which can be used for a longer period [22]. So screening *Lactobacillus* for the prevention of constipation is important, and this can lay a good foundation in further studies of clinical application.

Traditional fermented yak yoghurt is a type of very special traditional food in the China's Qinghai-Tibet

Plateau area. The primary microorganisms contributing to traditional fermented yak yoghurt are lactic acid bacteria (LAB) and yeast [23]. Due to the altitude and climate of Qinghai-Tibet Plateau area, the living habits of herdsman, yak milk varieties and fermentation temperature [24–26], LAB from traditional fermented yak yoghurt has more superiority properties (such as endurance ability, fermentation property and functional features). *Lactobacillus Plantarum*, Lp3, is isolated from traditional fermented Tibetan yak milk, which has lower cholesterol and survival rate in simulated gastric and intestinal juices. Moreover, *Lactobacillus Plantarum*, Lp3, can significantly lower cholesterol and triglyceride levels in serum and liver. It also has the ability to decrease lipid deposition in the cytoplasm of rat's liver tissue [27]. Exopolysaccharides (LW1 and LW2) produced by *Lactobacillus Casei*, SB27, isolated from the yak milk of the Gansu Tibetan area of China, show a significant inhibition in the proliferation of HT-29 colorectal cancer cells and enhance the expression of Bad, Bax, Caspase-3 and Caspase-8 genes. On the other hand, LW1 and LW2 can induce HT-29 cells apoptosis, which we observed by TEM images [27]. *Lactobacillus Fermentum*, HY01 (LF-HY01), is isolated from traditionally fermented yak yoghurt, which have a good endurance ability in simulated gastric juice and bile salt. The mice model of activated-carbon-induced constipation is often used for evaluating the therapeutic effectiveness of drugs in many studies. In this study, we tested the hypothesis that LF-HY01 can regulate intestinal movements of mice which is having activated-carbon-induced constipation by evaluating faecal characteristics, small intestine propelling rate, damage to the small intestinal villi and neurotransmitter levels in serum.

Methods

Stain

Lactobacillus fermentum HY01 (LF-HY01) is isolated and identified from traditional fermented yak yoghurt (Ngawa Tibetan and Qiang Autonomous Prefecture, Sichuan, China) and deposited at the China Center for Type Culture Collection (CCTCC, Wuhan, Hubei, China), bearing CCTCC Accession Number M2015792.

Animals

Forty female Kunming mice (20 ± 2 g) were purchased from the Experimental Animal Center of Chongqing Medical University (Chongqing, China). Animal permit number is SYXK (Yu) 2012–0001. They are exposed in the conditions (room temperature 25 ± 2 °C, relative humidity

$50 \pm 5\%$, 12 h light/12 h dark cycle), and they had free access to mice chow and drinking water.

Survival rate in simulated gastric juice

Simulated gastric juice (100 ml) composed of pepsin (0.35 g) and NaCl (0.2 g). The pH value of the solution is adjusted to 3.0 pH with 1 mol/L HCl using pH meter (pHS-3C, Shanghai Lei Ci Instrument Factory, Shanghai, China) and sterilized by filtering through 0.45- μm filter (Millipore, Massachusetts, American). Ten millilitres of inoculums is centrifuged at 4 °C, 4000 r/min for 10 min followed by washing twice with 10 ml sterilizing saline (0.9%) and resuspended in the same buffer. One millilitre of the suspension is mixed with 9 ml simulated gastric juice and incubated in shaking incubator (37 °C, 150 r/min) for 3 h. And its colony-forming units (CFU) are separately counted at 0 h and 3 h. The survival rate is calculated using the following equation: $\text{SR} (\%) = (N_3/N_0) \times 100$, where SR is the survival rate of *Lactobacillus* and N_0 is the number of viable cells (CFU/ml) at 0 h and N_3 is the number of viable cells (CFU/ml) at 3 h [28].

Bile salt tolerance

Hundred microlitres of inoculums is inoculated into 5 ml MRS-THIO (MRS broth plus 0.2% of sodium thioglycolate) with or without 0.3% (w/v) Ox gall. Bacterial cell in the culture broth is measured by reading the optical density (OD) at 600 nm after 24 h incubation at 37 °C. The percentage of bile tolerance is calculated using the following equation: $\text{BT} (\%) = (A/A_0) \times 100$, where BT is the bile tolerance of *Lactobacillus* and A is the OD of bacterial cell with 0.3% (w/v) Ox gall and N_0 is the OD of bacterial cell without 0.3% (w/v) Ox gall [28].

Animal experiments

To investigate the preventive effects of *Lactobacillus Fermentum*, HY01, in constipation, 40 female Kunming mice are randomly divided into normal group (N), model group (M), low concentration group (L) and high concentration group (H) with 10 mice in each group. Normal group is fed with a normal diet from day 1 to day 10. Model group is fed with a normal diet from the day 1 to day 10 and also treated with 0.2 ml 10% pre-cooling activated carbon (100 ml water contains 10 g activated carbon) by gavaging once a day from the day 1 to day 10. Low concentration group (10^8 CFU/kg BW) and high concentration group (10^9 CFU/kg BW) are treated with *Lactobacillus Fermentum*, HY01, by gavaging once a day, having fed normal diet from the day 1 to day 10, and treated with 0.2 ml 10% pre-cooling activated carbon by

gavaging once a day from the day 8 to day 10. Body weight and water content of faeces of experimental animal are determined daily. On the 11th day, all mice are gavaged 0.2 ml 10% pre-cooling activated carbon. Half of the mice in each group are used for observing the defecation time of first black stool. After the last time of gavage of activated carbon about half an hour later, the rest of mice in each group are put to death to measure activated carbon propelling rates in small intestine. The activated carbon propelling rates is calculated using the following equation: the activated carbon propelling rates (%) = distance travelled by the activated carbon/total length of the small intestine $\times 100\%$.

Histological examination

Small intestinal villi damage is evaluated by hematoxylin–eosin staining. Small intestinal villi are fixed in 10% formalin and embedded in paraffin. Four-micrometer slices are cut from paraffin blocks to study the histological changes using microscope (Olympus BX43, Olympus Co., Japan).

Serum cytokine levels

Serum supernatant, which are isolated from blood sampled using enucleating eyeball (4 °C, 3000 r/min for 10 min), were tested for MTL, GT, ET, SST, AchE, SP, and VIP concentrations using specific ELISAs (Cloud-Clone Corp, Houston, Texas, USA), according to the instructions of the manufacturer.

Statistical analysis

Statistical analysis is performed using the SPSS version 17.0 (SPSS Inc, Chicago, Illinois, USA). All data are reported as mean \pm SD. Differences between groups were analysed using one way analysis of variance (ANOVA) with Duncan's multiple range test. P value below 0.05 is considered statistically significant.

Result

Morphological characteristics

As shown in Fig. 1, LF-HY01 colony shows white, circular, opaque, moist and smooth in surface, convex in centre as well as neat in edge after LF-HY01 was cultured in MRS agar plate at 37 °C for 18 h (Fig. 1A). Moreover, LF-HY01 is a gram-positive bacterium after gram staining, and cell morphology showed short and straight rod and both ends of dull circle (Fig. 1B).

In vitro characteristics of strain

To assess the tolerances of LF-HY01 in gastrointestinal tract environment, we examined the survival rates of LF-HY01 in simulated gastric juice and the growth efficiency of LF-HY01 in bile salt. As shown in Table 1, LF-HY01 is well tolerated in simulated gastric juice. The increase of bile salt concentration resulted in the decrease in growth efficiency, but it still possessed well growth efficiency in 0.3% bile salt. These results indicate that LF-HY01 has a good performance against gastrointestinal tract environment.

Body weight changes

To assess the effects of LF-HY01 to body weight of mice, body weight of each mouse are measured every day. During the experiment, the body weight changes of each group are shown in Fig. 2. The body weight in each group has no significant differences with activated carbon and LF-HY01 as compared with the normal group, and the growth trend of body weight with activated carbon and LF-HY01 are quite near to the normal group. These results indicate that LF-HY01 has no significant effect on body weight of mice with constipation.

Water content of faeces changes

Water content of faeces is an important indicator to measure the severity of constipation. Generally speaking, water content of faeces (stool stem node) is low in patients with constipation. As shown in Fig. 3, compared to normal group, water content of faeces has dropped remarkably after treating with activated carbon. However, water content of faeces has improved significantly after treating with LF-HY01 and the best way to improve the water content of faeces is by applying high concentration of LF-HY01. These results indicate that LF-HY01 has the effects on inhibiting the stool stem node of mice with constipation.

Defecation time of first black stool

To assess the effects of LF-HY01 to the defecation situation of mice with constipation, we measured the defecation time of first black stool in each group after the last time to gavage activated carbon about half an hour later. As shown in Fig. 4, the defecation time of first black stool in model group is significantly longer than normal group. The defecation time of first black stool in LF-HY01 group is significantly longer than normal group, but the defecation time of first black stool in low or high concentration group took shorter time than that of model group. These results indicate that LF-HY01 are helpful for defecation of mice with constipation, and it is the best way to shorten the defecation time of first black stool by applying high concentration of LF-HY01.

Activated carbon propelling rates in small intestine

Constipation is closely related to the ability of intestinal peristalsis, and activated carbon propelling rates in small intestine have been normally used in the evaluation of intestinal peristalsis ability. As shown in Fig. 5, compared to normal group, the activated carbon propelling rates in small intestine are declined significantly in model group. After treating with LF-HY01, the activated carbon propelling rates in small intestine are higher than model group. Among treating with low and high concentration of LF-HY01, high concentration of LF-HY01 played a better role in promoting activated carbon propelling rates in small intestine. These results clearly indicate that LF-HY01 can effectively improve intestinal peristalsis ability.

Histological analyses

The intestinal peristalsis ability depends on the integrity of the intestinal villi, as shown in Fig. 6, and pathological sections of small intestine in normal group are distributed evenly and orderly and have no damage. On the contrary,

Fig. 1 Morphological characteristics of LF-HY01

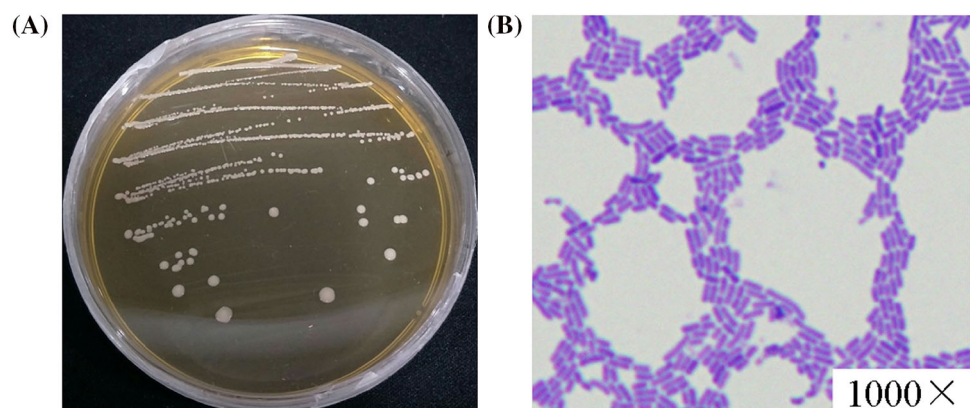


Table 1 Effect of simulated gastric juice and bile salt on viability of LF-HY01

Train	Survival in pH 3.0 simulated gastric juice (%)	Growth efficiency in bile salt (%)			
		0.05	0.1	0.2	0.3
LF-HY01	104.60 ± 6.50	98.00 ± 0.97	61.30 ± 0.43	29.10 ± 0.77	21.10 ± 0.24

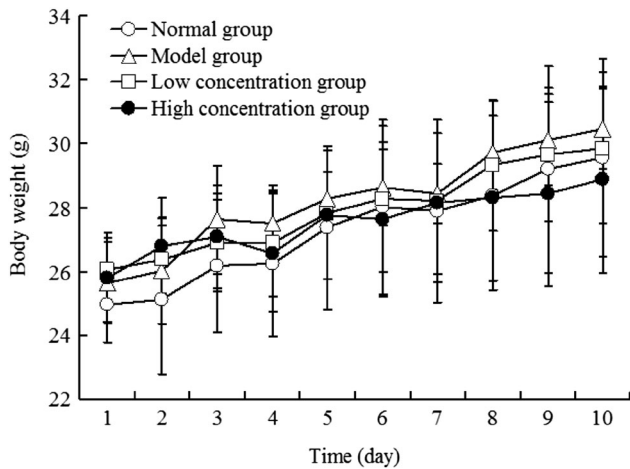


Fig. 2 Effects of LF-HY01 to body weight

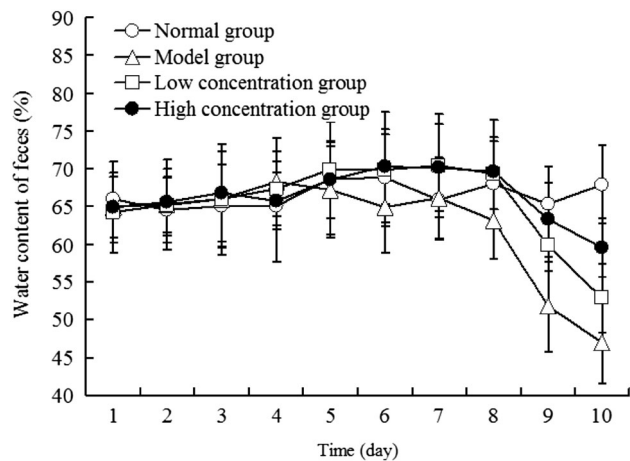


Fig. 3 Effects of LF-HY01 on water content of faeces

the small intestinal villus in model group (treat with activated carton) shows an obvious damage, including breaking, losing and disorganizing. After treating with low and high concentration of LF-HY01, the level of small intestinal villus damage is recovered as compared with model group. However, the small intestinal villus damage in low concentration group still shows breaking, losing and disorganizing. But in high concentration group, the small intestinal villus only had atrophy and mild damage. These results indicate that LF-HY01 has the function to protect intestinal function and promote intestinal peristalsis.

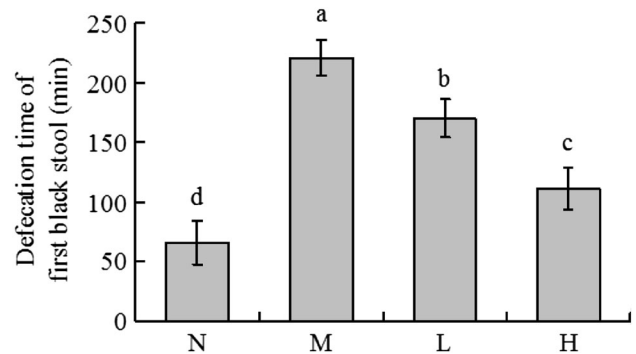


Fig. 4 Effects of LF-HY01 on defecation time

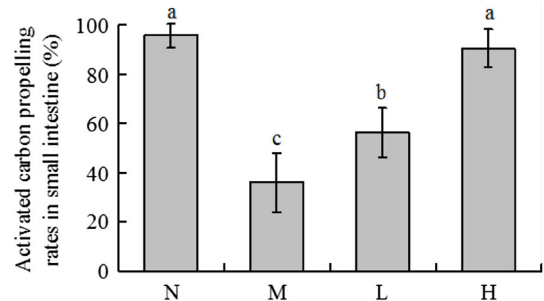
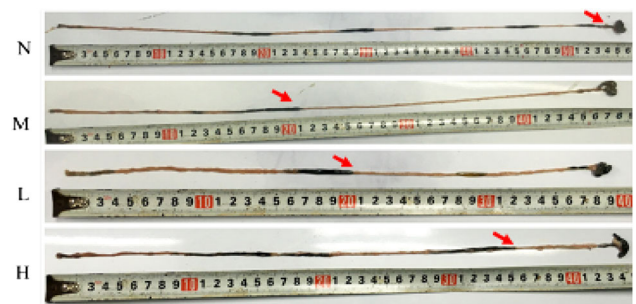
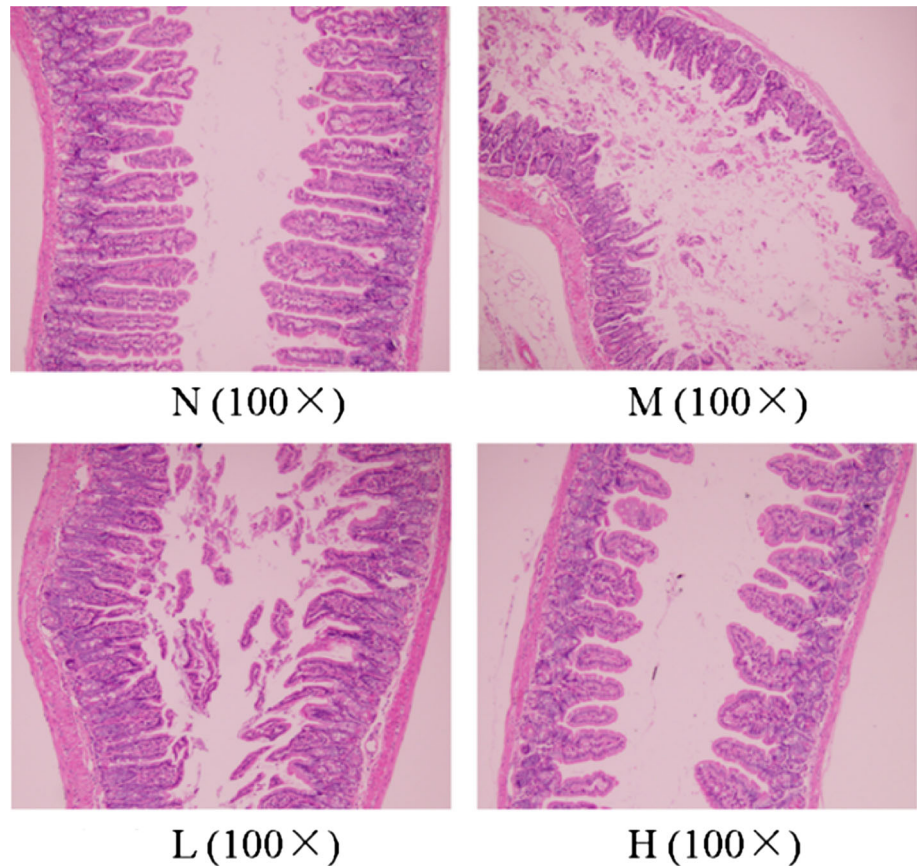


Fig. 5 Effects of LF-HY01 on activated carbon propelling rates in small intestine

MTL, GT, ET, SST, AchE, SP and VIP levels in serum

Intestinal peristalsis is regulated by related neurotransmitter. So evaluating related neurotransmitter levels can explain the ability of intestinal peristalsis. As shown in Fig. 7(A, B, C, E, F, G) compared to normal group, MTL, GT, ET, AchE, SP and VIP Levels are significantly reduced in model group. After treating with LF-HY01,

Fig. 6 Histopathology observation of small intestine



MTL, GT, ET, AchE, SP and VIP Levels are higher than model group. But the change of SST levels is opposite (Fig. 7D). Among treating with low and high concentration of LF-HY01, high concentration of LF-HY01 played a better role in positive regulation of neurotransmitter levels. These results indicate that LF-HY01 can effectively improve intestinal peristalsis ability.

Discussion

The main finding in this study is *Lactobacillus Fermentum*, HY01 (LF-HY01) which can effectively enhance intestinal movement of mice with activated-carbon-induced constipation.

Constipation is a typical malfunction of bowel with a high incidence in a crowd, so it exerts not only a big health burden to patient, but also big economic burden to patient and society [29]. Probiotics treatment is a new and scientific idea of therapy to constipation, and has gained worldwide attention [30]. Therefore, screening lactic acid bacteria and assessing its functional characteristics played a key role in prevention and treatment of diseases. Traditional fermented yak yoghurt, as a type of traditionally fermented food, has always been loved by the people in the

Qinghai-Tibet Plateau Area, China. Because of the influence of milk source, fermentation process, as well as the climate, and altitude of the Qinghai-Tibet Plateau, China, it became preferred source of lactic acid bacteria with good fermented and functional characteristics [16, 31].

Probiotics are recognized as living microorganisms which usually confer health benefits to the host when administered in adequate, and its health-promoting mechanism involves regulation of intestinal flora, enhancement of intestinal barrier function, inhibition the growth and colonization of pathogens in the intestines, and modulation of the immune system [32]. *Lactobacillus* is the main probiotics, but not all the *Lactobacillus* are probiotic in nature. Generally speaking, probiotics are determined by a series of critical assessment standards, including withstand transit through the gastrointestinal tract, the capacity to inhibit intestinal pathogens, the ability to colonize the intestinal tract, health benefits and so on. Among these assessing standards, it is the general selection criteria to survive through gastrointestinal tract. Therefore, resisting gastric juice and bile salts in the gastrointestinal tract are the fundamental requirement [33, 34]. LF-HY01 is isolated from traditional fermented yak yoghurt. Based on gastrointestinal tolerance evaluation in vitro, we defined that LF-HY01 is not affected at pH 3.0-simulated gastric juice,

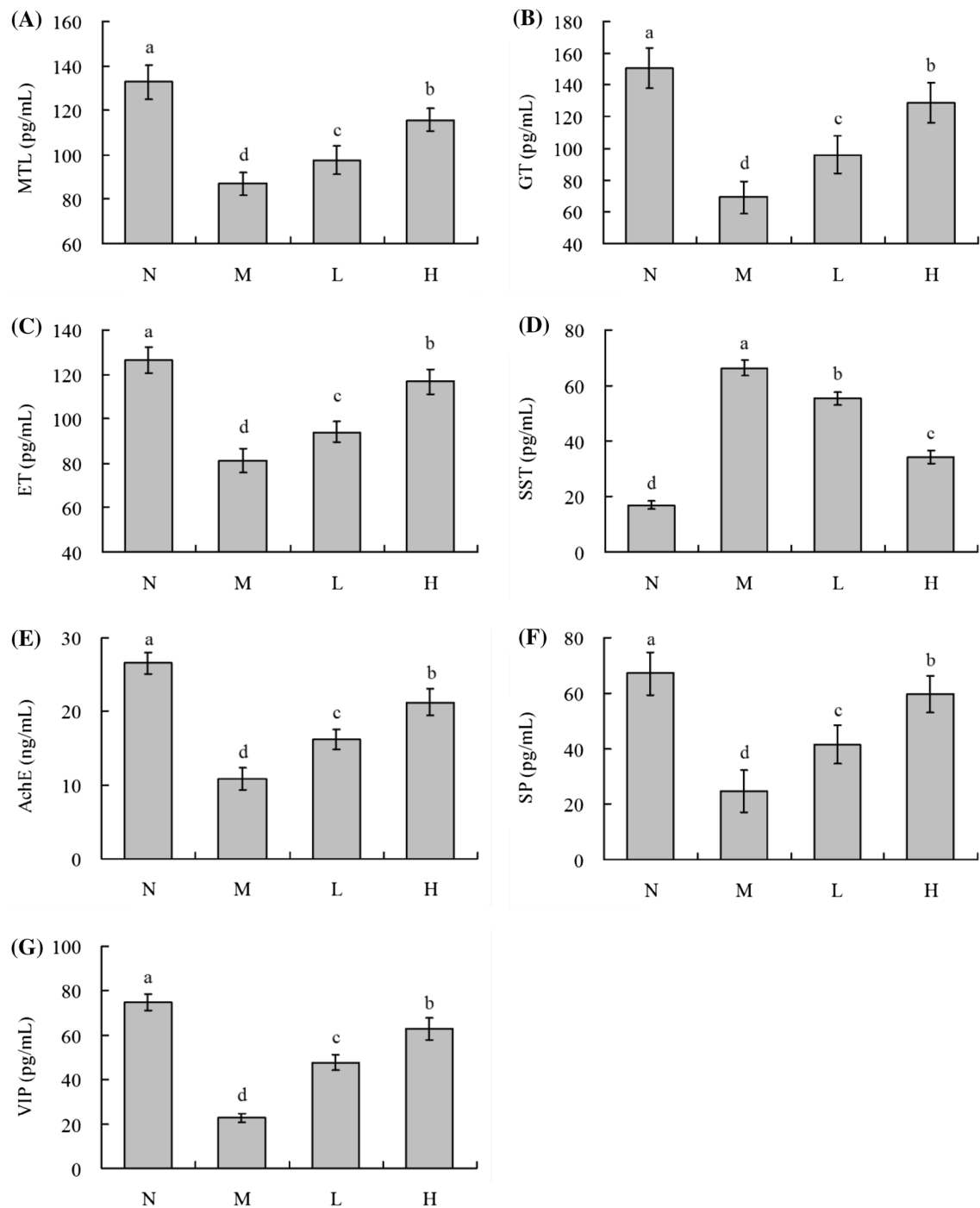


Fig. 7 Effects of LF-HY01 on the levels of serum

and growth efficiency is also well enough in bile salts. This result shows that LF-HY01 can be used for the further study due to its functional effects showed in vivo experiments.

Some human diseases usually accompanied with body weight gain or loss [35, 36]. The studies carried on the patients with constipation, previous experimental studies reported contradictory results. *Lactobacillus Fermentum*,

Suo alleviated body weight loss of activated-carbon-induced constipation mice [16], but *Lactobacillus Paracasei* subsp, *Paracasei* LC-01, has no effect on body weight gain or loss of compound diphenoxylate-induced constipation mice [37]. These contradictory results may be due to the differences of probiotic treatment, different experimental models, and duration of probiotic treatment. Finally, these differences may lead to different clinical situations [38]. In

the present study, the body weight in each group has no significant differences with LF-HY01 and activated carbon treatment.

The characteristics of faeces and defecation situation are considered to be the direct reflection of constipation [39]. Decreasing water content of faeces can lead to stool stem node, and increasing the difficulty of defecation. Defecation time of first black stool can be directly explained by the difference between defecation situation of LF-HY01 and activated carbon treated to constipated mice, and shorten the defecation time of first black stool has a direct effect of relieving constipation. Activated carbon propelling rates in small intestine are also an evaluating index to react defecation situation. A higher value of activated carbon propelling rates indicates a greater ability of intestinal peristalsis [40]. This result reflects that the body is more easily to defecate. In present study, we found LF-HY01 can significantly improve water content of faeces, defecation time of first black stool and activated carbon propelling rates in small intestine. In addition, we found a decrease of the small intestinal villi damage with LF-HY01 in this study. The small intestinal villi are associated with intestinal peristalsis. The larger damage of small intestinal villi indicates the less ability of intestinal peristalsis.

More importantly, we also found LF-HY01 treatment can positively regulate some neurotransmitter levels which are related to intestinal peristaltic function. MTL is an important index for reflecting the gastrointestinal tract movement and function, and it can promote gastrointestinal peristalsis, stimulate secretion of hydrochloric acid by parietal cells, and stimulate the secretion of pancreatic juices and bile [41]. GT not only can regulate the secretion of digestive system but also improve gastrointestinal peristalsis. Besides, it can also stimulate the production of pepsin and slow gastric emptying function [42]. ET plays demonstrate, keeping the stability of the vascular tension. It is closely related to constipation caused by diseases such as intestinal obstruction [40]. SST not only can reduce the release of gastrointestinal hormones but also reduce smooth muscle contractions. Moreover, it can also slow gastric emptying function [43]. Ach (Acetylcholine) can adjust bowel movement function. It plays an enlightening role for gastrointestinal peristalsis in combination with its receptors. Generally speaking, there is a positive correlation between the increase AchE levels and Ach levels [44]. SP can strongly stimulate the gastrointestinal smooth muscle contractions, so increasing SP levels can quickened to gastrointestinal contraction and movements [45]. VIP is a kind of neurotransmitters with the function of relaxing gastrointestinal tract, and it is also an important component of regulating intestinal peristalsis [46]. In present study, we found that LF-HY01 treatment can increase MTL, GT, ET, AchE, SP and VIP levels, and decrease SST level. All these

results indicate LF-HY01 can positively regulate intestinal movement of constipated mice treated with activated carbon.

This study has deficiency. We wanted to evaluate the effects of LF-HY01 to constipation. Based on the results of evaluating indexes in this experiment, we have known that LF-HY01 plays an important role in the protection from constipation. However, we are still not sure about the prevention mechanism of LF-HY01 from constipation. Previous studies suggest that bowel diseases are closely related to intestinal flora, and effects of bowel diseases could be reduced if we regulate intestinal flora. Therefore, to investigate intestinal flora changes was a key way to study the prevention mechanism of LF-HY01. In addition, it was also essential to assess abnormal effects of LF-HY01 by a relatively longer-term of experiment after gavaging LF-HY01 to mice with constipation.

In conclusion, our results clearly demonstrate that *Lactobacillus Fermentum*, HY01 could increase water content of faeces, defecation time of first black stool, activated carbon propelling rates in small intestine and MTL, GT, ET, AchE, SP and VIP levels, and decrease small intestinal villi damage and SST level, and it can also significantly improve intestinal movement function in constipated mice treated with activated carbon. However, probiotics are not only limited to the food industry, they are also applied in the fields of medicine and pharmacy. So we can also strengthen further research of LF-HY01 and discuss its application values in other areas [47].

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