Yak yoghurt is a traditional fermentation dairy product local to the Qinghai-Tibet Plateau area. Rich in nutrition, it is an antioxidant, decreases cholesterol and adjusts the immune capacity of organs (1). It was found that a high lactic acid bacteria population in the yoghurt contributes to its healthcare function. There are many factors that determine the species of lactic acid bacteria population in yak yoghurt, such as the living habitats of herdsmen, the fermentation vessels, temperature, and fermentation time. There is a significant difference between lactic acid bacteria from yak yoghurt and lactic acid bacteria available for commercial purposes (2). We isolated and characterized a microorganism from yak yoghurt from Tibetan habitats and designated the lactic acid bacteria as *Lactobacillus fermentum* Zhao (LF-Zhao).

Constipation is defined medically as fewer than three stools per week and severe constipation as less than one stool per week. It occurs when the colon has absorbed too much water (3). According to the Rome III criteria, functional constipation can be diagnosed by bowel movements, straining, frequency of hard stools, and sensation of incomplete evacuation (4). Constipation can also be checked by stool status, defecating time, and serum levels under laboratory conditions (5). In the latest study, activated carbon was orally administered to mice. Activated carbon attaches to gastrointestinal (GI) mucosal surfaces and reduces the drainage of the GI tract, causing GI fluid reduction and slowing of GI movement, with weakness of the spleen and stomach, resulting in a constipation mouse model (5). Previous studies have used the constipation model induced by activated carbon to demonstrate the effects of drugs used for constipation treatment (6, 7). One study had demonstrated that a large dose of activated carbon results in digestive tract obstruction (8). Therefore, in the latest study, we examined the functional effects of LF-Zhao in the alimentary tract using an activated carbon-induced constipation mouse model. We examined GI transit distance, time for the first black stool status, gastrointestinal transit and stool time, in addition to motilin (MTL), gastrin (Gas), endothelin (ET), somatostatin (SS), acetylcholinesterase (AChE), substance P (SP) and vasoactive intestinal peptide (VIP) levels in serum were monitored to evaluate the preventive effects of LF-Zhao on constipation. Bisacodyl, a laxative drug, was used as a positive control.

### Summary

The aim of this study was to investigate the effects of *Lactobacillus fermentum* Zhao (LF-Zhao) on activated carbon-induced constipation in ICR mice. ICR mice were administered lactic acid bacteria by gavage for 9 d. Body weight, diet intake, drinking amount, stool status, gastrointestinal transit distance and stool time, in addition to motilin (MTL), gastrin (Gas), endothelin (ET), somatostatin (SS), acetylcholinesterase (AChE), substance P (SP) and vasoactive intestinal peptide (VIP) levels in serum were monitored to evaluate the preventive effects of LF-Zhao on constipation. Bisacodyl, a laxative drug, was used as a positive control. Times to the first black stool for normal (untreated), control (no lactic acid bacteria treatment but activated carbon treated), bisacodyl-treated and *L. delbrueckii* subsp. *bulgaricus* (LB), LF-Zhao (L) (low concentration of $1 \times 10^8$ CFU/mL)- and LF-Zhao (H) (high concentration of $1 \times 10^9$ CFU/mL)-treated mice induced by activated carbon were 90, 218, 117, 180, 169 and 156 min, respectively. Following the consumption of LB, LF-Zhao (L) and LF-Zhao (H) or the oral administration of bisacodyl, the gastrointestinal transit distances were reduced by 55.2%, 61.3%, 70.6% and 94.6%, respectively. The serum levels of MTL, Gas, ET, AChE, SP and VIP were significantly increased and the serum levels of SS were reduced in the mice treated with LF-Zhao compared with those in the control mice ($p < 0.05$). These results demonstrated that lactic acid bacteria demonstrate preventive effects on mouse constipation and that LF-Zhao alleviated constipation symptoms better than LB.

**Key Words** *Lactobacillus fermentum* Zhao, activated carbon, constipation, bisacodyl, gastrointestinal transit
stool, histological sections, and serum assays of motilin (MTL), gastrin (Gas), endothelin (ET), somatostatin (SS), acetylcholinesterase (AChE), substance P (SP) and vasoactive intestinal peptide (VIP) levels. Bisacodyl was used as a positive control. Bisacodyl is a laxative drug that stimulates intestinal peristalsis and acts directly on the colon to produce bowel movement. It is typically prescribed for the relief of constipation and for the management of neurogenic bowel dysfunction, as well as for bowel preparation prior to medical examinations (9–11).

*L. delbrueckii* subsp. *bulgaricus* (LB), which is used in yoghurt production, was used as a reference strain. By comparing the cholate tolerance and hydrophobic property of LF-Zhao and LB, we could elucidate the ability of LF-Zhao to pass through the stomach and gut and to adhere to the small intestine. The results from this study could warrant the development of certain lactic acid bacteria as a novel treatment for constipation.

**MATERIALS AND METHODS**

**Microorganism strains.** LF-Zhao was isolated from yak yoghurt from the Hongyuan county (Ngawa Prefecture of Sichuan Province, China) and preserved at the China Center for Type Culture Collection (CCTCC, Wuhan, Hubei, China) under CCTCC Accession Number M2013513. *L. delbrueckii* subsp. *bulgaricus* was purchased from the Institute of Microbiology of the Chinese Academy of Sciences, Beijing, China.

**Animals.** Seven-week-old female ICR mice (*n* = 120) were purchased from the Experimental Animal Center of Chongqing Medical University (Chongqing, China). The mice were maintained in a temperature- and humidity-controlled (temperature 25 ± 2°C, relative humidity 50 ± 5%) facility with a 12-h light/dark cycle and free access to a standard mouse chow diet and water.

**Tolerance of lactic acid bacteria to pH 3.0 artificial gastric juice.** The artificial gastric juice was made with 0.2% NaCl and 0.35% pepsin, adjusted to pH 3.0 and then vacuum-filtered. Five milliliters of bacteria culture was centrifuged at 3,000 rpm for 10 min. The bacteria pellet was collected and re-suspended in 5 mL of sterile saline. One milliliter of the suspension was mixed for 30 s and allowed to stand undisturbed to separate the aqueous phase and the organic solvent phase. The aqueous layer was measured for its OD value, and the tolerance of the bacteria to oxgall was determined by comparison to the control tube (12).

**Determination of bacterial tolerance of bile salt (oxgall) at different concentrations.** One hundred microliters of bacteria culture was inoculated into 5 mL MRS-thio (MRS plus 0.2% of sodium thiglycolate) broth which contained 0.0% (as control), 0.3%, 0.5%, and 1.0% oxgall. After incubation at 37°C for 24 h, each culture was measured for its OD value, and the tolerance of the bacteria to oxgall was determined by comparison to the control tube (12).

**Determination of the hydrophobic property of lactic acid bacteria.** Five milliliters of bacteria culture was centrifuged at 3,000 rpm for 10 min. The bacteria pellet was collected and re-suspended with 5 mL of PBS buffer (50 mM, pH 6.5). The process was repeated. Using PBS buffer only as the blank for absorption, the final bacteria suspension was adjusted by PBS buffer to 1.00 absorbance at 560 nm. Dimethylbenzene (0.8 mL) was added to 4 mL of the adjusted bacteria suspension. The suspension was mixed for 30 s and allowed to stand undisturbed to separate the aqueous phase and the organic solvent phase. The aqueous layer was measured for the absorbance at 560 nm (blank: PBS buffer) (13).

**Induction of constipation in mice.** To investigate the preventive effects of LF-Zhao against activated carbon-induced constipation, the animals were divided into 6 groups with 20 mice each. The normal and control groups were fed normal diets for 9 d. The LF-Zhao was suspended in water to make the lactic acid bacteria suspensions at concentrations of 1 \times 10^8 and 1 \times 10^9 CFU/mL. They were allowed to pass through the stomach and gut and to adhere to the small intestine. The results from this study could warrant the development of certain lactic acid bacteria as a novel treatment for constipation.
control, bisacodyl, LB, and LF-Zhao groups received an oral administration of 10% activated carbon while the mice in the normal group received an oral administration of 10% arabic gum. Thirty minutes later, mice were sacrificed by cervical dislocation under anesthesia with diethyl ether. Ten mice in each group were dissected and the small intestine from the pylorus to the caecum was carefully removed. The GI transit distance for each mouse was calculated as the percentage of the distance traveled by the activated carbon meal relative to the total length of the small intestine (GI transit (%) = distance traveled by the activated carbon/total length of the small intestine × 100). The remaining 10 mice of each group were used to measure the time for the first black stool following oral administration of 10% activated carbon.

### Table 1. Resistance to biological barriers and the hydrophobicity of *L. fermentum* Zhao (LF-Zhao).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Survival in pH 3.0 artificial gastric juice (%)</th>
<th>Hydrophobic property (%)</th>
<th>Growth in bile salt (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.3%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.5%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.0%</td>
</tr>
<tr>
<td>LF-Zhao</td>
<td>76.68 ± 3.72*</td>
<td>60.11 ± 3.13*</td>
<td>20.65 ± 1.77*</td>
</tr>
<tr>
<td></td>
<td>63.72 ± 3.62*</td>
<td>57.62 ± 3.52*</td>
<td>17.56 ± 1.67*</td>
</tr>
<tr>
<td>LB</td>
<td>27.81 ± 3.41</td>
<td>25.56 ± 2.71</td>
<td>2.61 ± 0.34</td>
</tr>
<tr>
<td></td>
<td>24.78 ± 3.34</td>
<td>23.56 ± 2.61</td>
<td>1.57 ± 0.37</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.31 ± 0.22</td>
</tr>
</tbody>
</table>

*The LF-Zhao group was significantly different from the LB group (p<0.05).*

### RESULTS

**Biological barriers and the hydrophobicity of lactic acid bacteria**

Gastrointestinal survival of lactic acid bacteria was evaluated using artificial gastric juice, bile salt, and hydrophobic property tests. LF-Zhao showed much higher abilities than LB (Table 1). LF-Zhao had strong survival in pH 3.0 artificial gastric juice; this activity was about 2.8 times higher than that of LB. The hydrophobic property of LF-Zhao (60.11%) was also much higher than that of LB (25.56%). In different concentrations of bile salt, in particular, the growths of LF-Zhao were 10–12 times higher than those of LB.

**Body weight during the experiment**

The normal mice had a normal diet and their body weight increased during the experiment. The body weights of the control mice with activated carbon-induced constipation were significantly decreased after 7 d. As shown in Fig. 1, following the initiation of acti-
The stool time was the shortest (90±8 min) in the normal group and the longest (218±18 min) in the control group; the stool time in the bisacodyl group was 117±6 min, longer than that of the normal group. The times for the first black stool of the LB, LF-Zhao (L) and LF-Zhao (H) groups of mice were 180±13, 169±15 and 156±11 min, respectively. According to the stool time, LF-Zhao had more alleviating effects than LB.

**GI transit**

The constipation-alleviating effects of the treatments were determined by GI transit in mice following the administration of activated carbon (0.2 ml/mouse, 10% activated carbon). In the bisacodyl-treated group, the mean GI transit was higher than that of the control group (Table 3). The GI transits of the LB, LF-Zhao (L) and LF-Zhao (H) groups were also higher than in the control group, but lower than in the bisacodyl-treated group. GI transit of the LF-Zhao (H) group was highest among LB and LF-Zhao groups.

**Motilin (MTL), gastrin (Gas), endothelin (ET), somatostatin (SS), acetylcholinesterase (AChE), substance P (SP) and vasoactive intestinal peptide (VIP) levels in serum**

The MTL, Gas, ET, AChE, SP and VIP levels of normal mice were highest for all the groups, and the SS level was the lowest (Table 4). The control mice showed the opposite results, i.e., the control mice had the highest
level of SS, and the lowest levels of the others. The levels of bisacodyl-treated mice showed a similarity to the normal mice. The MTL, Gas, ET, AChE, SP and VIP levels of LF-Zhao-treated mice were higher than for LB-treated mice, but lower than for normal and bisacodyl-treated mice.

**DISCUSSION**

Probiotics including lactic acid bacteria are usually in food or taken in by oral administration. They have to survive the strong acid conditions in the stomach and upper intestinal tract, and reach the destination (usually the large intestine) to colonize for physiological efficacy (14). Therefore, in order to study whether lactic acid bacteria can pass through the stomach and intestine to colonize in intestinal tract, we built an external virtual model of the intestinal tract, and screened lactic acid bacteria capable of growing in that model. Their potential probiotic functions can be identified by measuring their acid-resistance, cholate tolerance and hydrophobic property (11). LF-Zhao showed better acid-resistance, cholate tolerance and hydrophobic property than LB, and these high qualities could result in functional effects of LF-Zhao for use in humans.

The surviving lactic acid bacteria passing through the stomach would get in touch with the cholate in the small intestine. Cholate tolerance of lactic acid bacteria can be used as a main measure to estimate constipation. Therefore defecation is one of the criteria for constipation. This study evaluated stool status on three levels. First was stool weight (g), which can be used as a main measure to estimate constipation. If the stool weight was high, that meant the mice had good stool quality. Second was particle count of stool (pieces). More stool pieces would reflect good gastrointestinal movement. Third was water content of stool, which is also reflective of mice constipation. The higher the water content, the better the stool quality. The results of the stool status of mice treated with lactic acid bacteria demonstrated that LF-Zhao has better stool status after induced constipation, indicating that LF-Zhao can relieve constipation.

Anorexia is an important symptom in constipation (18). Measuring dietary and water intake in mice may
determine the level of constipation and the inhibitory effects of different substances on constipation. The definition of constipation includes infrequent bowel movements and difficulty passing stool (19, 20). Constipation most commonly occurs when the stool moves too slowly (slow transit) as it passes through the digestive tract. A low level of water intake and certain drug administration lead to slow transit. When stools move slowly, too much water is absorbed from the stool and it becomes hard and dry (21). Stool status, dietary intake, water consumption, stool time and GI transit are important standards when investigating constipation. The characteristics of LF-Zhao were the increase of the GI transit compared to the control, reduction in constipation, and the increase of the functional effect.

The serum levels of MTL, Gas, ET, AChE, SP and VIP in patients with constipation are lower than those in healthy individuals while the SS levels are higher (22–24). The main function of MTL is to increase the migrating myoelectric complex component of GI motility and stimulate the production of pepsin. It is one of the intestinal hormones responsible for the proper filling and emptying of the GI system in response to the intake of food, as well as hunger stimuli and responses (25). Gas is a polypeptide hormone secreted by certain cells of the pyloric glands, which strongly stimulates the secretion of gastric acid and pepsin, and weakly stimulates the secretion of pancreatic enzymes and gallbladder contraction (22). Gas produces effects throughout the GI tract, including promoting GI secretion, increasing GI movement and promoting pyloric sphincter relaxation. ET plays an important role in the stability of vascular tension and maintains the basic cardiovascular system. Constipation not only causes disease, including intestinal obstruction and other serious diseases, but it also induces or aggravates cardio cerebrovascular diseases in the elderly (26). SS is homologous with cortistatin and suppresses the release of gastrointestinal hormones, such as Gas and VIP. SS also could decrease the rate of gastric emptying and reduce smooth muscle contractions, all of which could lead to constipation (27). Stools are formed from the non-digestible components of food after water is either absorbed or secreted in the large intestine. Mucous is also produced in the large intestine to provide viscosity. Thin segments of muscle line the intestinal tract and contract and relax in concert to propel the stool forward. Muscle contraction and mucous secretion are regulated by acetylcholine (28). Patients with slow-transit constipation have abnormal neurotransmitter levels in the muscular layer of their intestinal walls. These abnormalities include a deficiency of a peptide known as SP, which is thought to contribute to peristalsis (29). Disturbances in the normal neural content of VIP in the bowel wall in idiopathic constipation and diverticulosis may initiate or contribute to the functional changes observed in these disorders (30).

The aim of this study was to investigate whether LF-Zhao has a preventative effect against activated carbon-induced constipation in mice. The results demonstrated that LF-Zhao has better qualities of acid-resistance, cholate tolerance and hydrophobic property than LB, and that the time taken for the first black stool for mice treated with LF-Zhao was slightly longer than that for the mice treated with bisacodyl. Furthermore, the GI transit was longer than that in the control mice and was similar to that in the bisacodyl group. The various serum levels of MTL, Gas, ET, AChE, SP and VIP in the LF-Zhao-treated mice were higher than that those in the control mice and the LB-treated mice, and the SS levels demonstrated the opposite. These results finally suggest that LF-Zhao has a significant preventive effect on activated carbon-induced constipation in mice.

**Acknowledgments**

X. Zhao, Y. Qian and H. Suo contributed equally to this work.

This research project was supported by the National Science and Technology Support Program (No. 2012BAD31B06–5), Natural Science Foundation of Chongqing Science & Technology Commission (No. cstc2013jcyJ ASD006), Fundamental Research Funds for the Central Universities (No. XDJK2013BD010) and Scientific and Technological Research Program of Chongqing Municipal Education Commission (No. KJ131503), China.

**Conflict of interest**

The authors declare no conflict of interest.

**REFERENCES**


the gastrointestinal tract. *Proc Natl Acad Sci USA** **100**: 8567–8570.


