



## Food Microbiology

# Screening and characterization of lactic acid bacterial strains that produce fermented milk and reduce cholesterol levels



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## ABSTRACT

**Objective:** To screen for and characterize lactic acid bacteria strains with the ability to produce fermented milk and reduce cholesterol levels.

**Methods:** The strains were isolated from traditional fermented milk in China. *In vitro* and *in vivo* evaluation of cholesterol-reduction were used to identify and verify strains of interest. Characteristics were analyzed using spectrophotometry and plate counting assays.

**Results:** The isolate HLX37 consistently produced fermented milk with strong cholesterol-reducing properties was identified as *Lactobacillus plantarum* (accession number: KR105940) and was thus selected for further study. The cholesterol reduction by strain HLX37 was 45.84%. The isolates were acid-tolerant at pH 2.5 and bile-tolerant at 0.5% (w/v) in simulated gastric juice (pH 2.5) for 2 h and in simulated intestinal fluid (pH 8.0) for 3 h. The auto-aggregation rate increased to 87.74% after 24 h, while the co-aggregation with *Escherichia coli* DH5 was 27.76%. Strain HLX37 was intrinsically resistant to antibiotics such as penicillin, tobramycin, kanamycin, streptomycin, vancomycin and amikacin. Compared with rats in the model hyperlipidemia group, the total cholesterol content in the serum and the liver as well as the atherogenic index of rats in the viable fermented milk group significantly decreased by 23.33%, 32.37% and 40.23%, respectively. Fewer fat vacuoles and other lesions in liver tissue were present in both the inactivated and viable fermented milk groups compared to the model group.

**Conclusion:** These studies indicate that strain HLX37 of *L. plantarum* demonstrates probiotic potential, potential for use as a candidate for commercial use for promoting health.

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## Introduction

Cholesterol is an indispensable nutrient in the human body,<sup>1</sup> as it serves as a component of the cell membrane and as a precursor for cholic acid and steroid hormone synthesis. However, excess cholesterol is a major contributor to hyperlipidemia and cardiovascular and cerebrovascular diseases.<sup>2,3</sup> According to the statistics from the National Health and Family Planning Commission of China (2015), cardiovascular and cerebrovascular diseases have become a leading cause of death worldwide, with as many as 15 million people dying from these diseases annually. Research shows that the probability of suffering from cardiovascular disease decreases by 2–3%<sup>4</sup> with every 1% decrease in the serum cholesterol level. Therefore, an effective method for reducing high cholesterol to a healthy level could protect against these diseases.

Studies have shown that some therapeutics, such as 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, effectively reduce serum cholesterol.<sup>5</sup> Along with these therapeutics, a person's diet should also be regulated to include foods effective at preventing and reducing cholesterol. Numerous experiments have demonstrated that probiotic lactic acid bacteria can also reduce cholesterol via adsorption, conversion, co-precipitation and bile salt hydrolase (BSH) enzymatic degradation.<sup>5,6</sup> However, the oral intake of probiotics is not palliative for most people.

Fermented milk obtained through the lactobacillus fermentation of fresh milk is a dairy product with a number of nutritional and health advantages. The fermentation process also involves the growth of lactic acid bacteria that convert the lactose and milk protein into lactic acid, galactose, amino acids and small peptides, which are easily digested and absorbed by the human body. Moreover, the nutritional value of fermented milk products generated by various lactic acid bacteria can also be different. In addition to satisfying nutritional requirements, some fermented milk products can improve the absorption and uptake of nutritive substances, such as calcium-containing substances. There are numerous lactic acid bacteria that can inhabit the internal wall of the intestinal tract and form microbial communities and a biological barrier. Inhibition of the growth of pathogenic bacteria in the intestinal tract<sup>7,8</sup> and the regulation of the balance of intestinal microflora in such a way may have effects similar to those of probiotics and metabolites.<sup>9,10</sup>

For these reasons, we set out to screen for lactic acid bacterial (LAB) strains capable of producing fermented milk and reducing cholesterol levels for use as starter cultures. Furthermore, this study investigated the ability of these strains in fermented milk to reduce cholesterol in high-fat rats, and evaluated these strains as candidate commercial probiotics to promote health.

## Materials and methods

### Samples and media

Traditional fermented milk samples were screened for lactic acid bacteria strains able to reduce cholesterol levels. Selected

strains were cultivated in fresh milk collected from a farmer in Mangdang Town, Yanping District, Nanping City, in the Fujian Province of China (26.510°N, 118.090°E). Traditional fermented milk was made as follows: the fresh milk from Chinese Holstein cows was incubated at 15 °C for 35 days to ferment; when the pH value reached approximately 4.4, fermented milk had been produced. Man, Rogosa and Sharpe (MRS) medium was used for culturing the LAB. A basal diet was obtained from the Center for Disease Control and Prevention (Fujian, China) and contained 10% moisture, 22% crude protein, 4% crude fat, 5% crude fiber, and 8% crude ash. A hypercholesterol forage consisted of 57% basal diet, 13% lard, 5% peanuts, 2% sesame oil, 20% sucrose, 3% cholesterol (Zhengzhou Tianjian Food Technology Co., Ltd, Hunan Province, China), and 0.1% sodium cholate (Zhengzhou Tianjian Food Technology Co., Ltd., Hunan Province, China).

### Animals

Sprague-Dawley (SD) male rats (weighing 150–170 g) were purchased from the Hayes Lake Laboratory Animal Co., Ltd. (Shanghai, China) [license number: SCXK (Shanghai 2007-0005)]. The rats were housed under standard controlled laboratory conditions (temperature of 24 ± 3 °C, humidity of 50 ± 10% and 12 h light/12 h dark cycle), with unrestricted access to food and water. All animals were handled in accordance with the animal care and use guidelines established by the Fujian University of Traditional Chinese Medicine (Fujian, China), which entailed minimizing both the number and suffering of the animals used for the experiments. All experimental procedures involving rats were approved by the Fujian Association for Laboratory Animal Science (FALAS).

### Screening for strains capable of fermenting milk and reducing cholesterol levels

Natural fermented milk samples (1 mL) were serially diluted, and 10<sup>-5</sup>, 10<sup>-6</sup> and 10<sup>-7</sup> dilutions in saline were plated on solid MRS agar plates. The plates were incubated at 42 °C for 2 days. Colonies with phenotypes and characteristics similar to those of *Lactobacillus* were selected and sub-inoculated into MRS medium three times. The isolated strains were inoculated into 10 mL of MRS medium and incubated anaerobically at 42 °C for 1 day. A 1.0-mL aliquot of the culture broth was sub-inoculated into a fermenter containing 100 mL of pasteurized milk (Changfu Dairy Group Co., Ltd. Fujian Province, China), which had been sterilized at 115 °C for 20 min and incubated at 42 °C for 12 h. Following fermentation, any strain that consistently resulted in solidified fermented milk with a pleasing smell and a pH value ≤ 4.4 was selected, labeled and stored in 20% glycerol at -20 °C until further analysis. To screen for strains that could reduce milk cholesterol levels, a 1-mL aliquot of the culture broth from the tested fermentation strains was transferred into a fermenter containing 100 mL of cholesterol (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) screening medium and incubated at 42 °C for 2 days. Any strain that produced turbidity in the culture broth was tested for cholesterol reduction ability. LAB cultures were grown in MRS medium supplemented with water-soluble cholesterol (approximately 200 mg/L) for

24 to 72 h and then centrifuged at  $9000 \times g$  for 15 min at  $4^\circ\text{C}$ . The cholesterol in the supernatant was quantified using the O-phthaldehyde method described by Rudel et al.,<sup>11</sup> and cholesterol was used for the standard curve. The control broth was uninoculated MRS broth containing approximately 200 mg/L of water-soluble cholesterol. The ability of each strain to reduce the amount of cholesterol was calculated with the following equation:

$$\% \text{ of cholesterol reducing rate} = \left[ \frac{C_0 - C}{C_0} \right] \times 100,$$

where  $C_0$  is the control broth and  $C$  is fermented broth. The strain that resulted in the highest yogurt fermentation and cholesterol reduction was selected for further study.

#### Identification of strains able to both ferment milk and reduce cholesterol

The colony morphology of the isolates incubated on MRS medium plates at  $42^\circ\text{C}$  for 2 days was evaluated. Gram staining was performed, and the bacteria were examined using a YS100 type microscope (Nikon, Japan). Cell morphology was determined using a JSM-6380/LV scanning electron microscope (Electron Optics Laboratory Co., Ltd., Japan). In addition, the 16S rRNA and RNA polymerase alpha subunit (*rpoA*) genes of the strains were partially sequenced using the primers F<sub>9-27</sub>/R<sub>1525-1542</sub> (5'-GAG TTT GAT CCT GGC TCA G-3'/5'-AGA AAG GAG GTG ATC CAG CC-3') and *rpoA*-21-F/*rpoA*-22-R (5'-ATGATYGARTTTGAAAAACC-3'/5'-ACYTTVATCATNT CWGVYTC-3'), as described by Naser et al.<sup>12</sup> respectively. The PCR reaction mixture comprised 2.5  $\mu\text{L}$  of  $10\times$  PCR buffer (Tiangen Biotech Co., Ltd. Beijing, China), 2.5  $\mu\text{L}$  each of 2 mM dNTPs (dATP, dCTP, dGTP, and dTTP) (Tiangen Biotech Co., Ltd. Beijing, China), 1  $\mu\text{L}$  of 50 mM  $\text{MgCl}_2$  (Tiangen Biotech Co., Ltd. Beijing, China), 2  $\mu\text{L}$  of each primer (5 pmol/L), 0.5  $\mu\text{L}$  of BIOTaq DNA polymerase (2.5 U/L), 1  $\mu\text{L}$  of template, and distilled  $\text{ddH}_2\text{O}$  to a final volume of 25  $\mu\text{L}$ . The PCR program on an Eppendorf AG included 35 amplification cycles of 1 min at  $94^\circ\text{C}$  for denaturation, 1 min at  $55^\circ\text{C}$  (16S rRNA) or  $50^\circ\text{C}$  (*rpoA*) for annealing, and 1 min at  $72^\circ\text{C}$  for extension, followed by a final extension cycle at  $72^\circ\text{C}$  for 10 min. In addition, the 16S rDNA and *rpoA* partial sequences obtained were analyzed using BLAST on the NCBI server (<http://www.ncbi.nlm.nih.gov/>). Phylogenetic analyses of the 16SrRNA and *rpoA* gene sequences were performed using the ClustalW program with the MEGA4 software version 14.0.0.162.<sup>13</sup>

#### Acid tolerance and bile tolerance test

The acid tolerance of the strains was evaluated as described by Archer et al.<sup>14</sup> with slight modifications. Briefly, MRS prepared at pH values of 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0, inoculated with a 5% overnight bacterial suspension, and grown anaerobically at  $37^\circ\text{C}$  for 24 h. Aliquots were taken at intervals of 0, 2 and 4 h, serially diluted, and plated onto MRS agar plates. After incubating anaerobically at  $37^\circ\text{C}$  for 48 h, the number of viable colonies was counted. Similarly, MRS broth supplemented with 0.1%, 0.2%, 0.3%, 0.4% and 0.5% bile salts (w/v) (Huankai Microbial Sci Co., Ltd. China) was freshly prepared and inoculated with a 5% overnight bacterial suspension. Aliquots were

taken at intervals of 0, 2 and 4 h, and the number of viable colonies on MRS agar plates was counted. Samples taken at 0 h were used as a control.

#### Simulated gastric juice and intestinal fluid tolerance tests

Gastric juice was simulated using a solution of 125 mM NaCl, 7 mM KCl, 45 mM  $\text{NaHCO}_3$  and 3 g/L pepsin adjusted to pH values of 2.5 and 3.0 and prepared according to the method described by Archer et al.<sup>14</sup> Bacterial cultures were grown for 24 h, centrifuged for  $5000 \times g$  for 15 min at  $4^\circ\text{C}$  and added to the same volume of normal saline (0.9%) to replace the broth, while 10% was inoculated into the gastric fluid and incubated at  $37^\circ\text{C}$  in an incubator (MS-2102, LNB, Shanghai, China) with shaking at 180 rpm. Samples were taken at 0, 1, 2, 3 and 4 h of incubation, and the number of bacterial colonies on MRS agar plates was counted. Intestinal fluid consisting of 0.1% (w/v) pancreatin and 0.15% (w/v) ox-bile was adjusted to pH 8 or 7.5. In a manner similar to the gastric juice assay, the bacterial culture was used to inoculate intestinal fluid and then incubated. Viable cell counts were obtained by plating samples taken at 0, 3 and 6 h of incubation. Samples taken at 0 h were used as controls. The percent survival was calculated using the following formula:

$$\text{Bacterial survival (\%)} = \frac{\text{CFU}_{\text{assay}}}{\text{CFU}_{\text{control}}} \times 100,$$

where CFU assay is the cell count after incubation in simulated gastric juice or intestinal fluid, and CFU control is the cell count after incubation in normal MRS at 0 h (control).

#### Aggregation activity

Cultures of HLX37 and *Escherichia coli* DH5 $\alpha$  were grown in MRS and Luria-Bertani medium (LB, Canspec, Inc., China) overnight, respectively, and then centrifuged at  $3000 \times g$  for 10 min at  $4^\circ\text{C}$ . The cells were washed twice, resuspended in phosphate-buffered solution (PBS), and adjusted to an initial  $\text{OD}_{600}$  of  $0.4 \pm 0.1$  ( $A_0$ ). The cell suspensions were then incubated at room temperature at  $25^\circ\text{C}$  for 24 h to allow phase separation, and the final  $\text{OD}_{600}$  value ( $A_{24}$ ) of the aqueous phase was measured. The percentage of auto-aggregation was expressed as follows:  $A_{\text{auto}}\% = (A_0 - A_{24})/A_0 \times 100$ .<sup>15</sup> Similarly, equal volumes of LAB and *E. coli* DH5 $\alpha$  were mixed, adjusted to an initial  $\text{OD}_{600}$  of  $0.6 \pm 0.1$  ( $A_0$ ) in PBS and incubated at room temperature for 24 h without agitation. The  $\text{OD}_{600}$  value ( $A_{24}$ ) of the aqueous phase was measured. The co-aggregation percentage was expressed as follows:  $A_{\text{co}}\% = (A_0 - A_{24})/A_0 \times 100$ .

#### Antibiotic resistance

The antibiotic resistance patterns of strain HLX37 were determined by a disk diffusion method using the Kirby-Bauer technique.<sup>16</sup> MRS agar plates with a thickness of  $4 \pm 1$  mm were evenly spread with 100  $\mu\text{L}$  of strain HLX37 ( $8.0 \text{ lg cfu/mL}$ ) using a sterile swab. Antibiotic discs (Hangzhou Microbial Reagent Co., Ltd., Guangdong Province, China) were placed on the plates. The plates were then incubated for 36 h at  $37^\circ\text{C}$ . The diameter of the inhibition zone was measured with a

Vernier caliper, and the antibiotic resistance was determined according to CLSI.<sup>17</sup>

### Cholesterol reduction ability in a rat hyperlipidemia model

A fermented milk sample with HLX37 was prepared by fermenting fresh milk (whole milk, Changfu Dairy Group Co., Ltd., Fujian Province, China) with *Lactobacillus plantarum* HLX37 at 42 °C for 12 h until a viable count ( $8.30 \pm 0.01$  lg cfu/mL) was achieved and the acidity was  $82.32 \pm 0.99$  °T (as measured by titration with a GB5409-85, China). Fermented milk was produced every 7 days and stored at 4 °C, so the storage period of the fermented milk was 7 days. On the 7th day, the viable count was  $8.39 \pm 0.04$  lg cfu/mL ( $F = 13.73$ ,  $p = 0.021 > 0.05$ ), the viable count did not change significantly during storage. A total of 40 rats were randomly assigned to the following four groups ( $n = 10$ ): (1) an inactivated fermented milk group (Inactivated) in which the rats were fed a fermented milk sample that was inactivated in a 100 °C water bath for 30 min and a hypercholesterol chow, (2) a viable fermented milk group (Viable) in which the rats were fed a fermented milk sample and hypercholesterol chow, (3) a hyperlipidemia model group (Model) in which the rats were fed a saline solution and a hypercholesterol chow, and (4) a normal control group (Normal) in which the rats were fed saline and only a basal diet provided by the Center for Disease Control and Prevention, Fujian, China. The fermented milk (1 mL/100 (d g) of rat body weight) or saline was fed to the rats for 62 d by gavage. The rats were allowed free access to the experimental diet and water; food consumption and body weight were monitored. The blood and liver were collected after 12 h of fasting under anesthesia by injected sodium pentobarbital (40 mg/kg body weight of rat, Shanghai Chemical Reagents Procurement Supply Station, China). Blood collected from the postcava serum was isolated by centrifuging the blood samples at  $3000 \times g$  for 10 min. A portion of the liver was immediately fixed in 10% formalin buffer for freezing and liquid histological sectioning, and the remaining tissues were flash frozen in liquid nitrogen and stored at  $-80$  °C until use. The total cholesterol (TC) level in the serum and liver were determined using a total cholesterol assay kit (Jiancheng, Nanjing, China). High-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) in the liver were measured using high-density and low-density lipoprotein cholesterol assay kits, respectively (Nanjing Jiancheng

Bioengineering Institute, China). The atherogenic index (AI) was expressed as follows:  $AI = (TC - HDL - C) / HDL - C$ .<sup>18</sup>

### Statistical analysis

All experimental samples were tested in triplicate, and the resulting values are expressed as the mean  $\pm$  standard deviation (sd). The statistical analysis was performed using SPSS 17.0 software. A one-way ANOVA was performed across multiple groups to test significance, and  $p < 0.05$  was considered significant.

## Results

### Screening and isolation of bacterial strains able to ferment milk and reduce cholesterol

Strains capable of fermenting milk and reducing cholesterol were screened and isolated from traditional fermented milk samples collected from a farmer. A preliminary screening yielded 22 isolates that had a phenotype and characteristics similar to *Lactobacillus* spp. on solid MRS plates. These isolates were transferred to pasteurized milk. Of these 22 isolates, 8 isolates fermented milk and were selected and designated as HLX31–HLX38. Of these 8 isolates, 2 strains (HLX33 and HLX37) produced turbidity in a cholesterol screening medium, so they were further tested for their ability to reduce cholesterol. The cholesterol content and the residual cholesterol in the culture broth during growth are shown in Table 1. Strains HLX33 and HLX37A constantly reduced cholesterol in the broth throughout the fermentation process. After 72 h of incubation, strain HLX37 showed the highest cholesterol reduction, 45.84%, in a culture broth containing 123.1  $\mu$ g/mg of cholesterol; the  $F$  value between strains = 21.016, and  $p = 0.000 < 0.01$ , which indicates that the ability of strain HLX37 to lower cholesterol was significantly better than strain HLX33. thus, HLX37 was selected for further study.

### Characterization and identification of strain HLX37

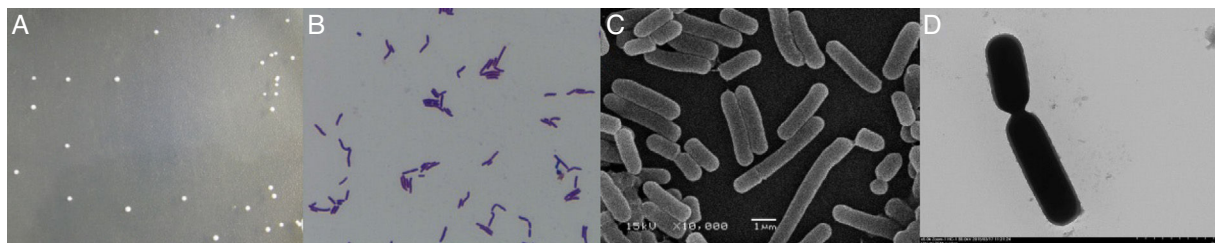
The morphology of strain HLX37 is shown in Fig. 1. Colonies on MRS plates were white, round, opaque, Gram-positive, and rod-shaped with a cell size of  $(1.8\text{--}3.5) \times (0.7\text{--}0.9)$   $\mu$ m. LAB

**Table 1 – Ability of lactic acid bacterial strains to lower cholesterol. The data are expressed as  $\bar{x} \pm sd$  ( $n = 3$  for each sample). Note: The LAB strains were grown in a cholesterol medium (MRS medium+ approximately 200  $\mu$ g/mL cholesterol). The  $F$  value between strains = 21.016, and  $p = 0.000 < 0.01$ , which indicates that the ability of strain HLX37 to lower cholesterol was significantly better than strain HLX33.**

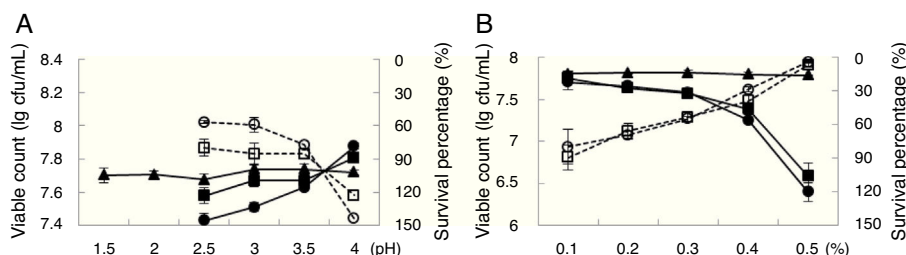
Incubation time (h)	Incubation temperature (°C)	HLX33		HLX37	
		Cholesterol content ( $\mu$ g/mL)	Cholesterol reduction (%)	Cholesterol content ( $\mu$ g/mL)	Cholesterol reduction (%)
0	42	227.30 $\pm$ 5.80		227.30 $\pm$ 5.80	
24	42	172.80 $\pm$ 9.84	23.88 $\pm$ 4.33	143.69 $\pm$ 1.80	36.78 $\pm$ 0.79
48	42	168.59 $\pm$ 6.74	25.83 $\pm$ 2.97	143.12 $\pm$ 9.39	37.04 $\pm$ 4.13
72	42	158.88 $\pm$ 0.77	30.10 $\pm$ 0.34	123.10 $\pm$ 23.05	45.84 $\pm$ 10.14

LAB, lactic acid bacteria; MRS, Man, Rogosa and Sharpe.





**Fig. 1 – Colony characteristics and morphology of *L. plantarum* strain HLX37. Note: (A) Colony characteristics inoculated in MRS medium; (B) Gram staining; (C) Scanning Electron Microscopy (SEM); (D) Transmission Electron Microscope (TEM).**



**Fig. 2 – The viable count (lg cfu/mL) and survival (%) of *Lactobacillus plantarum* HLX37 incubated in (A) acidic and (B) bile-containing medium with time. The viable counts were measured after  $\blacktriangle$ , 0 h;  $\blacksquare$ , 2 h; and  $\bullet$ , 4 h. The survival was measured after  $\square$ , 2 h and  $\circ$ , 4 h.**

strains were identified by BLAST sequences of the 16S rRNA gene and were found to have >99% homology with *Lactobacillus plantarum* and *Lactobacillus pentosus*. *L. plantarum* and *L. pentosus* were differentiated by a *rpoA* partial sequence (861 bp) that was also analyzed by BLAST and were found to have 99% homology with *L. plantarum* but only 97% homology with *L. pentosus*. This result was further confirmed by mapping the strain phylogeny; thus, strain HLX37 was identified as *L. plantarum*. The 16S rDNA sequence (1425 bp) for this strain was deposited in the GenBank database under the accession number KR105940. Additionally, HLX37 was added to the China General Microbiological Culture Collection Center (CGMCC) collection under the number 10632.

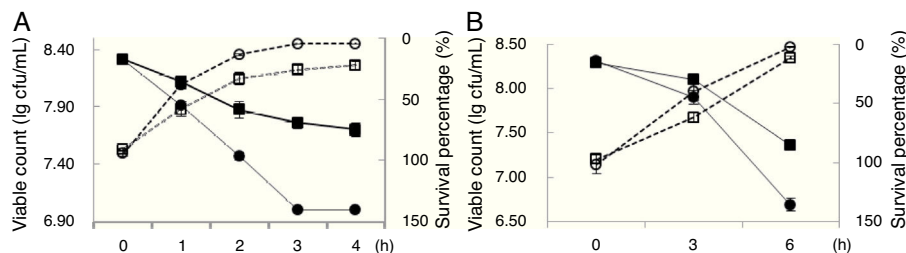
#### Acid and bile tolerance

The effect of acidic conditions on the viability of *L. plantarum* HLX37 is shown in Fig. 2. Strain HLX37 survived well under acidic conditions ( $\text{pH} \geq 2.5$ ), and the viable cell count reached  $7.43 \pm 0.05$  lg cfu/mL. The survival rate was  $56.84 \pm 0.57\%$  at  $\text{pH} = 2.5$  after 4 h of incubation at  $37^\circ\text{C}$ . Survival reached

122.86% and 143.81% after 2 and 4 h incubation periods at a pH of 4.0. The bile tolerance of the strain was considered an important characteristic because it enables the strain to survive and remain functional following gastrointestinal transit.<sup>15</sup> Strain HLX37 was grown in the presence of 0.1%, 0.2%, 0.3%, 0.4% and 0.5% bile salts and was determined to have a high bile tolerance. The rate of survival in 0.3% bile salts after 2 h was  $53.24 \pm 4.09\%$  and that after 4 h was  $54.68 \pm 2.03\%$ , with a viable count of  $7.57 \pm 0.03$  lg cfu/mL. This strain could also tolerate up to 0.5% bile salts, and the viable count range was  $6.40 \pm 1.10$  to  $6.5 \pm 0.16$  lg cfu/mL after 2–4 h of incubation. This result suggests that HLX37 can tolerate a low pH and a high concentration of bile salts.

#### Simulated gastric and intestinal fluid tolerance tests

The effect of simulated gastric fluids and intestinal fluids on the viability of strain HLX37 is shown in Fig. 3. Strain HLX37 could survive at pH 3.0 for 2 h with a survival rate of 33.41%. Strain HLX37 also had a survival rate greater than 13.66% at a pH 2.5 after 2 h in gastric fluid with a viable count



**Fig. 3 – Viable count (lg cfu/mL) and survival (%) of *Lactobacillus plantarum* HLX37 after exposure to simulated gastric juice (A) and intestinal fluid (B). The viable count was measured in the presence of  $\bullet$ , pH 2.5 in A or pH 8.0 in B; and  $\blacksquare$ , pH 3.0 in A or pH 7.5 in B. The survival was measured in the presence of  $\circ$ , pH 2.5 in A or pH 8.0 in B; and  $\square$ , pH 3.0 in A or pH 7.5 in B.**

**Table 2 – Adhesive ability of *L. plantarum* HLX37 (n = 3, x ± sd).**

Tests	A <sub>0</sub>	A <sub>24</sub>	Aggregation percentage (%)
Auto-aggregation	0.5131 ± 0.0045	0.0629 ± 0.0369	87.74
Co-aggregation with <i>Escherichia</i> DH5α	0.5133 ± 0.0082	0.3708 ± 0.0355	27.76

greater than  $7.47 \pm 0.03$  lgcfu/mL (Fig. 3A) and greater than  $40.05 \pm 7.09\%$  at pH 8.0 after 3 h with a viable count greater than  $7.90 \pm 0.07$  lgcfu/mL in intestinal fluid (Fig. 3B). This result suggests strain HLX37 could tolerate simulated digestive juice.

### Aggregation activity

The results of assays to measure aggregation are shown in Table 2. Auto-aggregation increased to 87.74% after 24 h at room temperature (25 °C), while the co-aggregation percentage was 27.76% for *Escherichia* DH5α after 24 h in a mixed suspension.

### Antibiotic resistance

The susceptibility patterns of *L. plantarum* HLX37 are shown in Table 3. No inhibition zone was observed in the presence of tobramycin. Furthermore strain HLX37 was resistant to kanamycin, streptomycin and vancomycin according to the

CLSI with *Staphylococcus aureus* ATCC25923 as the indicator bacterium. The diameters of the inhibition zone of penicillin and amikacin were less than 2.8 cm and 1.4 cm, respectively, indicating resistance. The antimicrobial susceptibility type was Intermediary (I) with gentamicin. No resistance to the other antibiotics tested was evident, and the clear zones ranged from 19.43 to 44.67 cm.

### Cholesterol reduction ability of the bacterial strains in rats

#### Effects of HLX37 fermented milk in rats

Food consumption was significantly greater in the normal group than in the other groups, but no significant differences were noted for the inactivated, viable and model groups. Meanwhile, the value of body final weight was significantly greater in the normal group than in the inactivated and model groups. No significant differences were found between the viable group and the other groups (Table 4). The TC levels in the rat's serum and liver are shown in Fig. 4A. Compared to the hyperlipidemia model group, the TC levels in the serum and liver of the rats in the viable group were significantly less by 23.33% and 32.37%, respectively, and a significant decrease of 27.46% in the serum was observed for the inactivated group. The order of the TC content values in the rats for each treatment were Model > Inactivated > Viable > Normal. Rats in the inactivated group showed a significant decrease in serum LDL-C of 15.19% despite their high-fat diet (Fig. 4B), but this was not observed in viable group. No significant differences were found in the HDL-C in the inactivated, viable and model groups. Additionally, the viable group showed a significant decrease in AI (40.23%; Fig. 4C).

#### Microscope morphological analysis

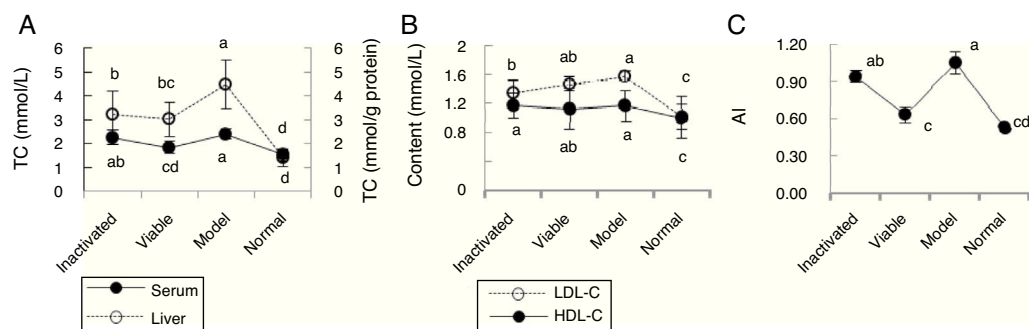
Morphological differences were noticed in the treated rat liver tissue biopsies compared to the healthy tissues from normal rats (Fig. 5). In the model group, numerous fat vacuoles, hepatic lobules containing debris, loose cytoplasm and a folded membrane that was pushed to one side were found in the liver biopsies, confirming that the model group was unhealthy compared to the normal group. Only a few fat vacuoles were noted in the rats in the inactivated and viable bacterial fermented milk groups, which were notably less than in the hyperlipidemia model group. Furthermore, in the inactivated and viable bacterial fermented milk groups, the hepatic lobules were clear, the cells were neatly arranged, and the liver sinusoid was normal with no significant lesions. The occurrence of fatty liver lesions was significantly reduced in the high-fat rats, and liver adipose degeneration was ameliorated to some degree in the inactivated and viable groups, indicating a beneficial effect on the liver.

**Table 3 – Antibiotic susceptibility of *L. plantarum* HLX37 using the Kirby-Bauer method (n = 3, x ± sd.) Note: (S), (I) and (R) denote sensitive, intermediary and resistant strains according to the breakpoints established by CLSI (2007).**

Antibiotic disks	Paper content µg/piece	Diameter of zone of inhibition (cm)	Antimicrobial susceptibility type
<i>Beta-amide/penicillin</i>			
Penicillin (P)	10	23.00 ± 2.83	R
<i>Cephalosporins</i>			
Cephalexin (CA)	30	23.17 ± 5.103	S
Ceftriaxone (CTR)	30	44.67 ± 0.473	S
<i>Aminoglycosides</i>			
Amikacin (AK)	30	13.33 ± 0.24	R
Gentamicin (GM)	10	12.33 ± 2.17	I
Tobramycin	10	–	R
Kanamycin (K)	30	–	R
Streptomycin (S)	10	–	R
<i>Tetracyclines</i>			
Tetracycline (TE)	30	19.43 ± 0.50	S
<i>Beta-lactam/Beta-inhibitor</i>			
Amoxicillin (AMX)	20	33.17 ± 1.18	S
<i>Phenicol</i>			
Chloroamphenicol (C)	30	21.33 ± 4.11	S
<i>Glycopeptides</i>			
Vancomycin (VA)	30	–	R
<i>Macrolides</i>			
Erythromycin (E)	15	32.50 ± 0.41	S

**Table 4 – Food consumption and final body weight of rats for 62 d ( $\bar{x} \pm \text{sd}$ ,  $n = 10$ ). Note: Values with the same letter are not significantly different ( $p > 0.05$ ), while different superscript letters indicate significance at  $p < 0.05$ .**

Diet treatment	Inactivated group	Viable group	Model group	Normal group
Consumption of diet [g/(one rat-d)]	24.94 $\pm$ 2.26 <sup>b</sup>	25.34 $\pm$ 1.91 <sup>b</sup>	25.64 $\pm$ 2.02 <sup>b</sup>	36.74 $\pm$ 1.36 <sup>a</sup>
Body final weight (g/one rat)	345.61 $\pm$ 54.03 <sup>b</sup>	376.41 $\pm$ 38.51 <sup>ab</sup>	361.27 $\pm$ 41.25 <sup>b</sup>	431.73 $\pm$ 42.83 <sup>a</sup>



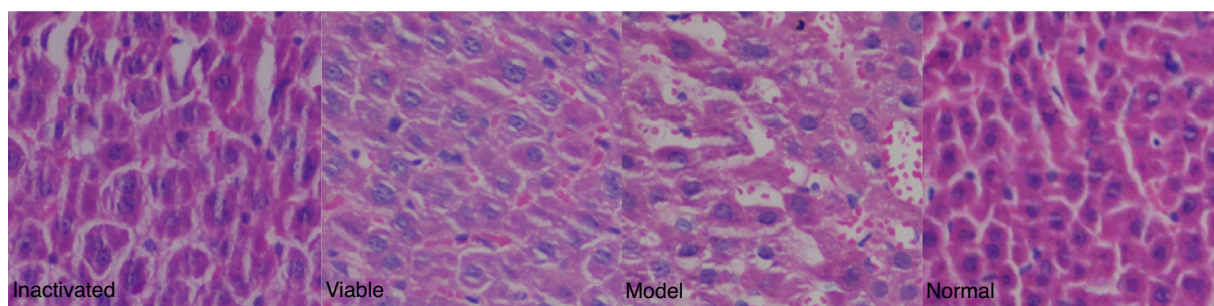
**Fig. 4 – Effects of *L. plantarum* HLX37 fermented milk in rat. (A) Total cholesterol (TC) levels in serum (mmol/L) and liver (mmol/g protein), (B) LDL-C and HDL-C levels in serum following treatment, (C) the atherogenic index (AI) = (TC-HDL-C)/HDL-C. Note: (1) Inactivated: the fermented milk sample was inactivated in a 100 °C water bath for 30 min and the rats were fed the hypercholesterol chow; (2) Viable: the fermented milk sample and the rats were fed the hypercholesterol chow; (3) Model: saline and the rats were fed the hypercholesterol chow; (4) Normal: saline and the rats were fed a basal diet. Values with the same letters were not significantly different  $p > 0.05$ , while different superscript letters indicate significance at  $p < 0.05$ .**

## Discussion

Traditional fermented foods such as cabbage, cucumber and milk, which provide products with defined consistency and have additional health-promoting (functional) properties, have usually been used as a source for screening various LAB.<sup>19,20</sup> Screening for strains with the ability to ferment milk and reduce cholesterol levels identified a LAB strain designated as HLX37, which was subsequently cultured. This strain could ferment milk and reduce the cholesterol level in MRS medium by 45.84%; consequently, it became the subject for further study. A morphological analysis and 16S rDNA sequencing identified the isolate HLX37 as *Lactobacillus* spp. To further identify HLX37 to the species level, we used *rpoA* gene sequences that offer a reliable identification system for nearly all species of the genus *Lactobacillus*,<sup>12</sup> and strain HLX37

was identified as *L. plantarum* (Accession no. KR105940). We found that *L. plantarum* HLX37 could lower cholesterol and was suitable for thermophilic fermentation to produce fermented milk. This strain was listed in the Strain List Applicable to Food specified in File No. [2010]65 by the health office of the China Ministry of Health, which supervises edible microorganism.

An FAO/WHO working group confirmed that some bacterial species such as *L. fermentum* can be designated as probiotics when delivered in food and food supplements at a minimum level of 9 lg cfu viable cells per serving.<sup>21</sup> This indication means that consuming enough fermented milk (i.e., more than 10 mL fermented milk that contains 8 lg cfu/mL) per serving not only tastes excellent and provides a high nutritional value but also is a good delivery system for probiotics. Humans can consume sufficient fermented milk to exert a beneficial probiotic effect to regulate the intestinal microecology. However, before these probiotics can reach the distal part of the intestinal tract



**Fig. 5 – Rat liver morphology as indicated by staining with hematoxylin and eosin (20  $\times$  10 magnification). Note: (1) Inactivated: the fermented milk sample was inactivated in a 100 °C water bath for 30 min and the rats were fed the hypercholesterol chow; (2) Viable: the fermented milk sample and the rats were fed the hypercholesterol chow; (3) Model: saline and the rats were fed the hypercholesterol chow; (4) Normal: saline and the rats were fed a basal diet.**

and exert a probiotic effect, the LAB must pass through the highly acidic (approximately pH 3) gastric fluid in the stomach and the weakly basic (pH of 7.8–8.4) intestinal juice that contains 0.3–2% (w/v) bile-salts in the upper part of the intestinal tract, including a residence time of 1–2 h.<sup>22–24</sup> Therefore, it was necessary to determine if the strain could function as desired after overcoming the stress from acidic gastric juice in addition to stress from weakly basic and bile-salt-containing intestinal juice. Strain HLX37 was acid tolerant, as indicated by testing in simulated gastric juice (pH 2.5) for 2 h. Specifically, in simulated gastric juice (pH = 3 for 2 h) or in acid (pH 3.0, for 2 h), the survival rates were 33.41% or 85.45%, respectively, while human gastric juice showed survival rates of 15 and 45%.<sup>20</sup> Similar results were obtained by other researchers such as Zielińska et al.,<sup>20</sup> who noted that the survival rate of bacteria of the genus *Lactobacillus* in a low pH ranged from 30 to 100%. Meanwhile, HLX37 was also able to tolerate a simulated intestinal fluid (pH 8.0) for 3 h and bile-salts 0.3% (w/v) for 2 h with a survival rate ranging from 53.27 to 95.83%. This result was also confirmed by Mathara et al.,<sup>19</sup> who found that *L. plantarum* strains isolated from fermented milk products were resistant to 0.1–0.5% bile salts. This result suggests that our isolates can tolerate low pH as well as a suitable bile concentration.

Some lactic acid bacteria can produce cell surface proteins, which often not only degrade substrates and ingest products<sup>24</sup> but also aid in binding the bacteria to the epithelium of the human gastrointestinal tract. This binding phenomenon provides the required conditions for the immunoregulation<sup>25</sup> necessary to remove pathogenic bacteria<sup>26</sup> from the host. Therefore, the adhesive capacity of a bacterial strain in the intestinal mucosa is an important index for evaluating the *in vivo* niches of a probiotic. In this study, the auto-aggregation rate of strain HLX37 and the coagulation rate of *Escherichia* DH5 $\alpha$  were 87.74% and 27.76%, respectively. These results demonstrated that the HLX37 strain has a strong adhesive capacity conducive to strong adherence in the intestinal tract. Research has shown that a number of proteins occur on the surface of *L. plantarum*. Several single proteins could mediate the *in vitro* epithelial adhesive capacity of the bacteria. Importantly, a mannose-specific adhesive factor participates in the binding of strains to the intestinal tract.<sup>27</sup> Further studies are required to determine whether specific proteins and/or a mannose-specific adhesive factor is present on the surface of strain HLX37.

Antibiotic resistance studies indicated that lactobacilli are usually resistant to classes of antibiotics such as aminoglycosides, beta-lactams, cephalosporins, quinolones, and glycopeptides.<sup>28–30</sup> Kattla et al.<sup>31</sup> confirmed that *Lactobacillus* is sensitive to tetracycline and chloramphenicol. *L. salivarius* strain MTC 1026 was found to be sensitive to penicillin, streptomycin and vancomycin.<sup>32</sup> In this study, strain HLX37 was also resistant to antibiotics, including almost all aminoglycosides as well as penicillin and vancomycin tested in this study, which was also reported in previous studies. Previous studies also indicated that antibiotic resistance, such as that observed for *L. plantarum* HLX37, was considered to be intrinsic or natural non-transmissible resistance.<sup>20,33–35</sup>

The significantly lower food consumption and final body weight showed that rats fed with hypercholesterol chow that

contains higher energy and is more difficult to digest could cause the rats to reduce their intake significantly. This result was accompanied by a slower weight gain, except in the viable group, which showed that feeding with strain *L. plantarum* HLX37 could increase the absorption and utilization of food in rats. In addition to TC, the LDL-C and HDL-C content and the AIs are important indicators that measure the predisposition for cardiovascular and cerebrovascular diseases. HDL-C is mainly synthesized in the liver. It moves systemic cholesterol to the liver for processing, which reduces the probability of cardiovascular disease, so it is known as good cholesterol. However, the function of LDL-C opposes that of HDL-C, and LDL-C levels are indirectly correlated with good health.<sup>36</sup> The concept of AI was proposed by Bhan et al.<sup>29</sup> in a study that had a large sample, and it was positively correlated with atherosclerosis, which suggested that atherosclerosis occurs in individuals, and increases the risk of individual cardiovascular and cerebrovascular disease. The effect of HLX37 fermented milk on cholesterol in rats was investigated in this study. We found that viable fermented milk rat groups showed a lower cholesterol level in the serum and liver following the intake of a high-fat diet as well as a lower AI compared with the model group, which indicated that *L. plantarum* strain HLX37 could lower cholesterol level *in vivo*. This result has also been confirmed by El-Gawad et al.<sup>37</sup> and Fazeli et al.<sup>38</sup> No significant difference in the HDL-C level between the viable group and model group was observed at the end of the 62 d experimental period. Endo et al.<sup>39</sup> and El-Gawad et al.<sup>37</sup> reported that the addition of a probiotic to the diet had no effect on the HDL-C level. Our data agreed with this finding. In addition, we also found that dead bacteria had no effect on the HDL-C level. HDL-C consists of cholesterol, lipoprotein, phospholipid and a small amount of fatty acid.<sup>29</sup> The HDL-C level in the normal group was lower than in the other experimental group, possibly because the experimental groups received a hypercholesterol diet that contained lard, peanut, sesame oil, sucrose and cholesterol, which could lead to an increase in the HDL-C level compared with the basal diet. Studies by Liong et al.,<sup>40</sup> Kimoto et al.<sup>41</sup> and Jiao et al.<sup>2</sup> noted that dead bacteria may be able to lower the cholesterol level. In this study, we also found that rats fed inactivated fermented milk showed an obviously lower cholesterol level in the liver and the presence of LDL-C in the serum. This strain may reduce the cholesterol in the media through various means, such as co-precipitation.

Meanwhile, a microscopic pathomorphism analysis noted fewer fat vacuoles and lesions in the liver in inactivated and viable groups compared to the untreated model control group. Both inactivated and viable fermented milk improved lipid metabolism with significant decreases in blood fat and inhibition of high cholesterol in rats and increased resistance to arteriosclerosis and repair of hepatic steatosis in the rats. Thus, it is possible that this strain can be used to create a fermented milk product that efficiently reduces cholesterol.

Nguyen et al.<sup>42</sup> found that *L. plantarum* PH04 had a high bile salt hydrolase enzymatic activity, which reduces the total cholesterol in a hyperlipidemia rat model, but Tok et al.<sup>43</sup> found that *L. delbrueckii* sp. had no BSH activity. The capacity to reduce cholesterol in the culture medium was directly correlated with the bacterial exopolysaccharide. In this study, viable fermented milk and inactivated fermented milk could



reduce the TC in the liver and the LDL-C level. These characteristics of strain HLX37 are combined with a very high coagulation rate. Further studies to investigate whether the metabolic substances in fresh milk fermented by this strain can reduce cholesterol and to elucidate the mechanism of action underlying this reduction are necessary.

In conclusion, in this study, we isolated the LAB strain *L. plantarum* HLX37, which could ferment milk and reduce cholesterol, from traditional fermented milk. An *in vitro* study confirmed that strain HLX37 could tolerate acid and bile salts and survive under simulated gastrointestinal conditions. Strain HLX37 was intrinsically resistant to the usual antibiotics tested compared to other *L. plantarum* for which previous reports exist, but more information is required to establish whether strain HLX37 contain any transferable antibiotic resistance genes. Strain HLX37 was also found to have strong adhesive properties, but more studies about the adhesive factor are required. Specifically, strain *L. plantarum* HLX37 can lower cholesterol, as confirmed by an *in vivo* study that showed a significant reduction of the TC level and AI in rats. Additionally, fewer fat vacuoles and liver lesions were found in rats fed viable fermented milk containing strain HLX37. *L. plantarum* strain HLX37 shows a commercial probiotic potential for use in promoting health.

### Compliance with ethics requirements

All of animals in this study were handled in accordance with the animal care and use guidelines established by the Fujian University of Traditional Chinese Medicine (Fujian, China), which entails minimizing both the number and suffering of the animals used for the experiments. All experimental procedures involving rats were approved by the Fujian Association for Laboratory Animal Science (FALAS).

### Conflicts of interest

None declared.

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### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.bjm.2017.02.011](https://doi.org/10.1016/j.bjm.2017.02.011).

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