J. Microbiol. Biotechnol. (2014), 24(2), 225–235
http://dx.doi.org/10.4014/jmb.1308.08075

jmb

Potential Probiotic Characterization of *Lactobacillus plantarum* Strains Isolated from Inner Mongolia "Hurood" Cheese

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Received: August 26, 2013
Revised: November 8, 2013
Accepted: November 12, 2013

First published online
November 13, 2013

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pISSN 1017-7825, eISSN 1738-8872

Total 121 lactic acid bacteria were isolated from homemade Inner Mongolia extra hard Hurood cheese. Seven of these strains, identified as *Lactobacillus plantarum*, were studied for probiotic characteristics. All seven strains survived at pH 3.0 for 3 h, or in the presence of oxgall at 0.3% or 0.6% for 4 h, but their viabilities were affected to different extents at pH 2.0 for 3 h. Strains C37 and C51 showed better adherence to Caco-2 cells, and higher hydrophobicity. The seven *L. plantarum* strains were different in *in vitro* free radical scavenging activities and cholesterol-reducing ability. *In vivo* evaluation of the influence of *L. plantarum* C37 on the intestinal flora in a mouse model showed strain C37 could increase the viable counts of lactobacilli in feces of mice and decrease the viable counts of enterococci. When *L. plantarum* C37 was used to prepare probiotic Hurood cheese, it was able to maintain high viable counts (>7.8 log CFU/g) during the whole storage period, but the composition of the cheese was not changed. These results indicate that *L. plantarum* C37 could be considered as a promising probiotic strain.

Keywords: Hurood, cheese, *Lactobacillus plantarum*, probiotic characterization

Introduction

"Probiotic" is defined by the United Nations and World Health Organization Expert Panel as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2002). It exercises its effect mainly by regulating the gastrointestinal microbiota of humans and animals [47].

Lactobacillus plantarum is a heterofermentative and versatile species that exists in a variety of environmental niches, including various plant, meat, and dairy products [15]. *L. plantarum* strains were reported to possess different probiotic properties, such as reducing incidence of diarrhea, pain and constipation associated with irritable bowel syndrome, mitigating inflammation and flatulence, displacing enteropathogens from Caco-2 cells, and lowering the cholesterol in blood [12, 43, 61]. Many *L. plantarum* strains showed the ability to survive gastric transit and colonize the intestinal tract of humans and other mammals [15, 18, 37]. Some *L. plantarum* strains showed better probiotic properties than *L. casei* strains isolated from the same cheese [20]. Ingestion with the bacterial powder preparation of *L. plantarum* or cheese containing *L. plantarum* (10.0 log CFU per day for 3 weeks) was found to be safe [52]. Recently, *L. plantarum* strains have been isolated in a wide variety of traditional cheese products, such as different Italian and Iranian cheese varieties [9], Greek Melichloro cheese [42], Polish golka cheese [51], Serbian Zlatar cheese [56], Turkish Karin Kaymak cheese [55], Tibetan Qula cheese [17], Indian camel cheese [40], Brazilian ovine cheese [38], and West African soft cheese [4].

Previously in our laboratory, we characterized two potential probiotic *L. plantarum* strains, K25 and S4-1, from Tibet kefir and Chinese sauerkraut, respectively [60, 61]. In this study, similar experimental methods were employed to isolate lactic acid bacteria (LAB) from Hurood cheese, which is a home-made extra-hard type of cheese (moisture content about 25%) consumed in the traditional nomadic

district Inner Mongolia of China for hundreds of years. Among 121 isolates of LAB from Hurood cheese, seven of them identified to be *L. plantarum* were investigated for probiotic characteristics, including tolerance to acid and bile salt, ability to adhere to Caco-2 cells, surface hydrophobicity, and *in vitro* cholesterol-lowering and antioxidant activities. One strain with promising *in vitro* probiotic characteristics was further examined for *in vivo* regulatory effect on the intestinal flora in a mouse model. To test the potential of the selected strain for possible application, Hurood cheese containing the strain was prepared and the strains survivability in cheese and influence on the cheese basic composition were studied.

Materials and Methods

Microorganisms and Cell Line

L. rhamnosus GG (LGG) was isolated from a commercial fermented milk product (Yili, China). Human colon adenocarcinoma cell line (Caco-2) was provided by the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences.

Isolation and Identification of LAB

LAB were isolated from Hurood cheese obtained from farms in Inner Mongalia of China. Hurood cheese (5 g) was mixed with 45 ml of sterile saline solution (0.85% (w/v)) and homogenized. The mixture was serially diluted and plated on de Man Rogosa Sharpe (MRS) agar (Difco, USA) plates. After anaerobic incubation at 37°C for 48 h, all isolates were cultivated in MRS broth (Difco) at 37°C for 16 h and stored at −80°C in MRS broth supplemented with 20% (v/v) glycerol. The isolates were subjected to microscopy observation and gram staining and catalase reactions. The gram-positive, catalase-negative, non-motile, and randomly isolated bacterial rods were selected and identified to the species level by API 50 CHL tests (bio-Mérieux, France) and 16S rDNA sequence analysis. All strains were subcultured twice prior to the following experiments.

Acid and Bile Tolerance

Acid and bile tolerance was performed as described by Anderson *et al*. [6] with modifications. The overnight cultures of lactobacilli cells were harvested by centrifugation at 4,000 ×*g* for 10 min at 4°C. After washing twice with sterile saline solution (0.85% NaCl (w/v)), cell pellets were either resuspended in MRS broth (pH 6.6) or MRS adjusted to pH 2.0 or pH 3.0 with HCl, and then the mixtures were incubated at 37°C for 3 h. For the bile tolerance assays, *Lactobacillus* cultures were inoculated into MRS broth containing 0.1%, 0.3%, and 0.5% (w/v) oxgall (Sigma, USA) that were subsequently incubated at 37°C for 3 h. MRS broth without oxgall was used as the control. The viable counts of lactobacilli were determined by plate counting using MRS agar. The plates were incubated at 37°C for 48 h under anaerobic conditions.

Microbial Adhesion to Solvents

The bacterial adhesion to hydrocarbons assay was carried out according to Zoueki *et al*. [62] with slight modification. Strains were grown in MRS broth at 37°C for 18 h. Cells were harvested by centrifugation at 4,000 ×*g* for 10 min at 4°C, and the pellets were washed twice with PBS (pH 7.4). The cell density was adjusted for each sample to approximately OD_{600} = 0.4 (A_0). The cell suspension (3 ml) was mixed with 1 ml of xylene, chloroform, and ethylene acetate, respectively. The mixture was vigorously vortexed for 1 min and allowed to stand for 30 min to separate into two phases. The absorbance of the aqueous phase was determined at 600 nm (A_1). Cell surface hydrophobicity was expressed as follows:

$$H\% = (A_0 - A_1)/A_0 \times 100.$$

According to the hydrophobic characteristics of the bacterial surface, strains are usually classified into three categories: low (0%−35%), medium (36%−70%), and high (71%−100%).

Caco-2 Cell Culture and *In Vitro* Adhesion Assay

The Caco-2 cell line was routinely cultured in Dulbecco's minimal essential medium (Gibco, USA) supplemented with 10% (v/v) heat-inactivated fetal calf serum and antibiotic antimycotic solution (Sigma) to give a final concentration of 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B, at 37°C in a 5% CO_2/RH 90% atmosphere.

The adherence of *Lactobacillus* strains to the Caco-2 cell lines was evaluated using the method described by Dhanani *et al*. [16]. The results of the adhesion assay were expressed as adhesion percentage (%); namely, the ratio between adherent bacteria and added bacteria per well. The experiments were repeated twice in duplicates in two successive passages.

Assay of *In Vitro* Antioxidant Activity

Preparation of intracellular cell-free extract. The cultures were centrifuged at 7,500 ×*g* for 10 min (4°C) and washed three times with PBS (pH 7.2). Cells were resuspended in PBS solution and adjusted to 10^9 and 10^{10} CFU/ml. Bacterial cells were disintegrated twice by using a French press at 1,500 psi pressure. Cell debris was removed by centrifugation at 15,000 ×*g* for 10 min (4°C), and the resulting supernatant was the intracellular cell-free extract.

Determination of hydroxyl radical scavenging activity. The hydroxyl radical scavenging assay was conducted according to the method described by Wang *et al*. [57]. Briefly, the reaction mixture containing 1.0 ml of brilliant green (0.435 mM), 2.0 ml of $FeSO_4$ (0.5 mM), 1.5 ml of H_2O_2 (3.0% (w/v)), and 1.0 ml of intracellular cell-free extract in different concentrations was incubated at room temperature for 20 min, and the absorbance was measured at 624 nm. The absorbance change of the reaction mixture indicated the scavenging ability of the *L. plantarum* strains for hydroxyl radicals. Hydroxyl radical scavenging activity is expressed as Scavenging activity (%) = $[(A_s - A_0)/(A - A_0)] \times 100$, where A_S is the absorbance in the presence of the sample, A_0 is the

absorbance of the control in the absence of the sample, and A is the absorbance without the sample and hydroxyl radicals.

Determination of DPPH radical scavenging activity. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity of lactobacilli was determined according to the method described by Kao and Chen [21] with some modifications. Briefly, 1.0 ml of cell-free extracts prepared with 10^9 and 10^{10} CFU/ml cell suspension was added to 2.0 ml of ethanolic DPPH radical solution (0.05 mM). The mixture was mixed vigorously and incubated at room temperature in the dark for 30 min. The controls included only deionized water and DPPH solution. The blanks contained only ethanol and the cells. The absorbance of the resulting solution was measured in triplicate at 517 nm after centrifugation at 8,000 ×*g* for 10 min. The scavenging ability was defined as

$$\text{Scavenging activity (\%)} = [1 - (A_{sample} - A_{blank})/A_{control}] \times 100.$$

Determination of superoxide anion radical scavenging activity. The superoxide anion radical scavenging capacity of lactobacilli was determined according to the method described by Wang *et al*. [57] with some modifications. Briefly, 1.0 ml of cell-free extracts prepared with 10^9 and 10^{10} CFU/ml culture suspension (or deionized water as a control) were mixed with 3.0 ml of 1.0 M Tris-HCl buffer (pH 8.2) containing 0.1 mM EDTA and incubated at 25°C for 25 min, and then 40 µl of 45 mM pyrogallol (25°C) was added. The mixture was mixed and incubated at 25°C for 10 min. The absorbance of the resulting solution was measured in triplicate at 325 nm. The scavenging ability was defined as

$$\text{Scavenging activity (\%)} = [1 - (A_{11} - A_{10})/(A_{01} - A_{00})] \times 100$$

where A_{11} and A_{10} are the absorbance of sample with and without addition of pyrogallol, respectively; and A_{01} and A_{00} are the absorbance of deionized water with and without addition of pyrogallol, respectively.

Assay of *In Vitro* Cholesterol Assimilation

Lactobacillus cells were inoculated into sterile MRS broth containing 100 µg/ml cholesterol (Sigma) and 0.3% (w/v) oxgall (Sigma) at 37°C for 24 h. Untreated cells were used as a control. After the incubation period, cells were centrifuged (6,000 ×*g*, 4°C, 10 min) and the remaining cholesterol concentration in the broth was determined using a colorimetric method as described by Kumar *et al*. [24]. The observations were compared with a standard curve prepared by using suitable concentrations of cholesterol read at 550 nm. The percent reduction was determined in the spent broth by comparing values with uninoculated control. All experiments were replicated three times.

Regulatory Effect of *L. plantarum* on the Intestinal Flora in a Mouse Model

In order to evaluate the regulatory effect of potential probiotic *L. plantarum* strain on the intestinal flora in mice, 5-week-old Kunming male mice were used. Mice were housed and fed in accordance with the Provisions and General Recommendations of Chinese Experimental Animal Administration Legislation. Mice were divided randomly into two groups of 15 mice each. The mice were fed by oral gavage. Mice of group 1 received 0.4 ml of PBS (Control group). Group 2 mice were fed with 0.4 ml of PBS containing *L. plantarum* strain at a cell concentration of 1×10^9 CFU/ml. After 14 days of feeding, oral gavage was stopped and the mice were then fed with basal diet for another 7 days. At 0, 7, 14, and 21 days, three mice of each group were killed by cervical dislocation. The fresh stool samples in the rectum were collected, serially diluted, and plated on LBS, MRS+NNLP, VRBDA, BEA, and TSC agar to estimate the number of viable lactobacilli, bifidobacteria, enterobacter, enterococci, and *Lostridium perfringens*, respectively [46]. The plates were incubated at 37°C for 24 or 48 h. The results are reported as log10 CFU per gram of stool sample.

"Hurood" Cheese Manufacturing with *L. plantarum*

The viable cell preparation of potential probiotic *L. plantarum* strain in 12% reconstituted skim milk supplemented with 5% glucose was lyophilized into powder and store at −80°C for use. The powder contained 11.0 log CFU/g of viable *L. plantarum*. The acid whey was prepared by fermenting the cheese whey with 3% inoculum of starter cultures (provided by Inner Mongolia Agricultural University, China) containing lactococci of 9.1 log CFU/ml.

Two batches of cheese were manufactured with 10 L of milk each. The control batch was made according to the traditional method. Raw milk (3.30% fat, 3.00% protein) was pasteurized. Then the acid whey was added to lower the pH of milk to 5.0. When the milk began to coagulate, the temperature was increased to 65°C and the milk stirred for 30 sec. Then the curds were collected and milled. The milled curd particles were pressed in a round cheese mold. The final product was attained by drying the pressed curd at 40°C to a final moisture content of 25% and stored at 4°C. For the batch of Hurood cheese containing *L. plantarum*, lyophilized powder of *L. plantarum* (1% (w/w)) was added in the milling step. The two batches of cheeses were produced in the same day with the same batch of pasteurized milk, and duplicate replications were made in the following weeks.

The gross composition of the cheeses was analyzed after 1 month of storage for moisture by the oven-drying method [1], fat by the Röse-Gottlieb method [2], and total protein by the Kjeldahl method [3]. The pH of a slurry, prepared by macerating 10 g of grated cheese with 12 ml of cold deionized water, was measured by using a calibrated pH meter (Mettler Toledo, Switzerland). To assess the viability of strains during the storage, samples of cheese at 0, 1, 2, and 4 weeks were studied. The sample (11 g) was diluted in 99 ml of sterile 2% (w/v) trisodium citrate at 40°C. The sample was homogenized in an Ultra-Turrax (IKA Labortechnik, Germany) for 2 min at high speed in a beaker to obtain slurry for the first dilution, and subsequent serial dilutions were performed in 0.9% (w/v) saline solution. *L. plantarum* was enumerated on selective media under anaerobic incubation at 37°C for 72 h [11].

Table 1. Effects of acid and bile on the survival of *Lactobacillus* strains (log CFU/ml).

Strains	Initial count	pH 3.0 (3 h)	pH 2.0 (3 h)	0.3% oxgall (4 h)	0.6% oxgall (4 h)
LGG	8.15 ± 0.36^{a}	$8.12 \pm 0.31^{\alpha\beta\gamma}$	$7.42 \pm 0.19^{\eta}$	$8.13 \pm 0.23^{\gamma\delta}$	$8.11 \pm 0.16^{\gamma}$
C35	$8.56 \pm 0.15^{\beta}$	$8.00 \pm 0.12^{\alpha\beta}$	$0.00 \pm 0.00^{\alpha}$	$8.60 \pm 0.28^{\varepsilon}$	$8.45 \pm 0.20^{\delta}$
C37	$8.54 \pm 0.16^{\beta}$	$8.57 \pm 0.18^{\delta}$	$6.88 \pm 0.14^{\zeta}$	$8.48 \pm 0.45^{\delta\varepsilon}$	$8.43 \pm 0.09^{\delta}$
C42	$8.52 \pm 0.17^{\beta}$	$8.22 \pm 0.14^{\beta\gamma}$	$5.34 \pm 0.10^{\delta}$	$8.50 \pm 0.21^{\delta\varepsilon}$	$8.48 \pm 0.15^{\delta}$
C51	$8.03 \pm 0.13^{\alpha}$	$7.86 \pm 0.12^{\alpha}$	$4.22 \pm 0.09^{\beta}$	$7.23 \pm 0.14^{\beta}$	$7.04 \pm 0.13^{\beta}$
C55	$7.96 \pm 0.11^{\alpha}$	$7.97 \pm 0.08^{\alpha\beta}$	$6.26 \pm 0.07^{\varepsilon}$	$7.30 \pm 0.02^{\beta}$	$7.13 \pm 0.06^{\beta}$
C60	$8.27 \pm 0.08^{\alpha\beta}$	$8.40 \pm 0.11^{\gamma\delta}$	$4.60 \pm 0.08^{\gamma}$	$5.93 \pm 0.09^{\alpha}$	$5.20 \pm 0.19^{\alpha}$
C62	$8.02 \pm 0.15^{\alpha}$	$8.64 \pm 0.17^{\delta}$	$6.93 \pm 0.23^{\zeta}$	$7.97 \pm 0.22^{\gamma}$	$8.16 \pm 0.07^{\gamma}$

$^{\alpha-\eta}$Means in the same column followed by different superscripts are significantly different ($P < 0.05$).

Statistical Analysis

Data analysis was carried out with the Statistical Package for Social Science (SPSS, Windows ver. 12.0; SPSS Inc., USA). One-way analysis of variance was used to study differences between means at the 0.05 probability level. The multiple comparisons among treatment means were performed by the Duncan method. All data are presented as the mean ± standard deviation of means.

Results and Discussion

Identification of Lactobacilli

Among 121 LAB isolated from Hurood cheese, the API 50 CHL results showed that the fermentation profiles of C35, C37, C42, C51, C55, C60, and C62 were 36.1%, 85.2%, 96.2%, 99.9%, 26.3%, 82.6%, and 97.7% identical to *L. plantarum* species, respectively. 16S rDNA analysis also showed these strains are *L. plantarum* species.

Tolerance to Acid and Bile

The gastrointestinal tract was considered to be the major location to affect the viability of LAB cells. In this study, the viable counts of *Lactobacillus* strains were determined after 3 h incubation in MRS broth (pH 2.0, 3.0). As shown in Table 1, most strains tested were sensitive to acid, especially in the environment of pH 2.0, where no survival (C35) or drastic decrease in viability (C42, C51, C60) was found after 3 h incubation; other strains were from 2.0% to 22.9%. All tested strains were able to survive the MRS broth at pH 3.0, and strains C60 and C62 could even reproduce with slightly increased bacterial counts after 3 h incubation at pH 3.0.

The bile in the intestine was reported to be an important factor affecting the LAB viability [35]. Although the composition of human bile juice was shown to be not exactly the same as that of the 0.3% oxgall solution, most studies use oxgall as one substitute for human bile because of their similarity [34]. In this study, *L. plantarum* strains were cultured in MRS broth with 0.3% and 0.6% oxgall to evaluate their bile tolerance. After 4 h cultivation, effects of bile salt on the growth of LAB were observed (Table 1). Strains LGG, C35, C37, C42, and C62 were highly tolerant to oxgall, and other strains were sensitive to oxgall.

Hydrophobicity

In this study, the cell surface hydrophobicity of the 7 *L*

Table 2. Surface hydrophobicity of *Lactobacillus* strains.

	Xylene (%)	Chloroform (%)	Ethyl acetate (%)
LGG	$22.35 \pm 3.40^{\beta}$	$30.10 \pm 0.96^{\delta}$	$16.82 \pm 1.54^{\delta}$
C35	0^{α}	$10.34 \pm 2.53^{\alpha}$	$13.02 \pm 3.71^{\gamma\delta}$
C37	$86.70 \pm 1.70^{\delta}$	$93.91 \pm 2.85^{\zeta}$	$54.25 \pm 3.16^{\zeta}$
C42	$45.80 \pm 1.94^{\gamma}$	$26.14 \pm 0.54^{\gamma}$	$10.02 \pm 2.30^{\beta\gamma}$
C51	$96.03 \pm 2.14^{\varepsilon}$	$98.93 \pm 0.79^{\eta}$	$38.29 \pm 2.86^{\varepsilon}$
C55	$23.89 \pm 0.86^{\beta}$	$73.49 \pm 1.17^{\varepsilon}$	$13.11 \pm 3.92^{\gamma\delta}$
C60	$88.11 \pm 1.96^{\delta}$	$92.90 \pm 2.25^{\zeta}$	$5.54 \pm 1.47^{\beta}$
C62	0^{α}	$20.27 \pm 1.46^{\beta}$	$0 \pm 0.00^{\alpha}$

$^{\alpha-\eta}$Means in the same column followed by different superscripts are significantly different ($P < 0.05$).

plantarum strains in comparison with LGG was characterized by using the microbial partition to different solvents such as xylene, chloroform and ethyl acetate, and the results were presented in Table 2. The ability of partition to xylene was assayed to estimate cell surface hydrophobicity or hydrophilicity. The results showed that strains C37, C51, and C60 were highly hydrophobic, whereas strains C35 and C62 were fully hydrophilic. The electron donor (basic) and acceptor (acidic) characteristics of the bacterial cell surfaces were assessed by measuring the bacterial partition to chloroform (a monopolar and acidic solvent as an electron acceptor) and ethyl acetate (a monopolar and basic solvent as an electron donor). The seven *L. plantarum* strains and LGG showed various hydrophobicities for chloroform. The highest value of 98.93% hydrophobicity was found for the strain C51, followed by strains C37 (93.91%) and C60 (92.90%), indicating that they were strong electron donors. Except for strains C37 (54.25%) and C51 (38.29%), other strains showed low hydrophobicity for ethyl acetate, indicating that these strains were weak electron acceptors.

There were several mechanisms about the adhesion of microorganisms to intestinal epithelial cells. Cell hydrophobicity was one of the factors that might contribute to adhesion of bacterial cells to host tissues. This property could indicate an advantage and importance for bacterial maintenance in the human gastrointestinal tract [49]. Previous studies showed that hydrophobic strains did not always adhere best to hydrophobic substrate, probably due to bacterial adaption of their surface hydrophobicity in response to environmental changes like pH, ionic strength, or surface structure [44, 45]. For example, *L. acidophilus* BFE 719, characterized by an extremely low hydrophobicity of 2%, was also able to bind to HT29 MTX cells, showing 40% adhesion as compared with 25% for *L. rhamnosus* GG [49]. However, in this study, strains with a high cell surface hydrophobicity generally adhered to Caco-2 cells at a high level.

Adhesion Ability to Caco-2 Cells

In this study, Caco-2 cells were used to compare the ability of the *Lactobacillus* cells to adhere to epithelial cell lines. Fig. 1 shows different adhesion capacities of the eight strains to Caco-2 cells. Strains C37 and C51 possessed higher adhesion capacity (6.8% and 6.2%, respectively) compared with the well-known probiotic *L. rhamnosus* GG (4.3%). Strain C60 adhered moderately (3.0–4.0%), whereas other strains adhered poorly (< 2%). Therefore, the adhesion capacity was not dependent on the bacterial species but rather was strain specific.

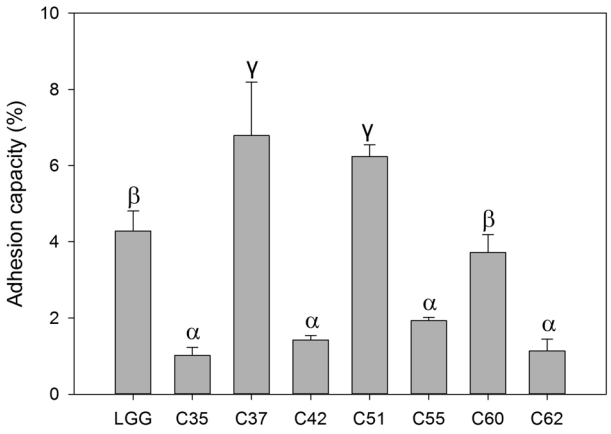

Fig. 1. Adhesion of *L. plantarum* strains to a Caco-2 cell monolayer after 90 min of incubation at 37°C in a 5% CO_2/RH 90% atmosphere.
Different letters (α, β, and γ) represent significant difference ($P < 0.05$).

The ability to adhere can give information about the possibility of probiotics to colonize and may modulate the host immune system. Adherence to intestinal epithelium was considered as an important criterion for selection of probiotics [14, 27], and *in vitro* models with intestinal cell lines, such as Caco-2 and HT-29 cell lines, were widely adapted for this assessment [25]. Several researchers have reported that high cell surface hydrophobicity may favor the colonization of mucosal surfaces and play a role in the adhesion of bacteria to epithelial cells [23, 49]. In this study, strains C37 and C51 with a high cell surface hydrophobicity adhered to Caco-2 cells at a high level. Strains C35 and C62 with an extremely low hydrophobicity were only able to bind to Caco-2 cells with 10% adhesion. It may be shown that cell surface hydrophobicity was related to epithelial cells adhesion properties. However, strains with a high cell surface hydrophobicity did not adhere at a high level to HT-29 cells. Strain *L. pentosus*, ST712BZ characterized by a relatively low hydrophobicity (38%), adhered to HT-29 cells at 63% [54]. Thus, hydrophobicity may assist in adhesion, but bacterial adhesion to the gastrointestinal tract is a complex mechanism that involves extracellular and cell surface receptors.

***In Vitro* Antioxidant Activity**

As shown in Fig. 2, all strains exhibited certain free radical scavenging activities against all three radicals tested, which increased with the viable counts in a dose-dependent manner. Lactobacilli had various hydroxyl radical scavenging activities and presented a certain dose-

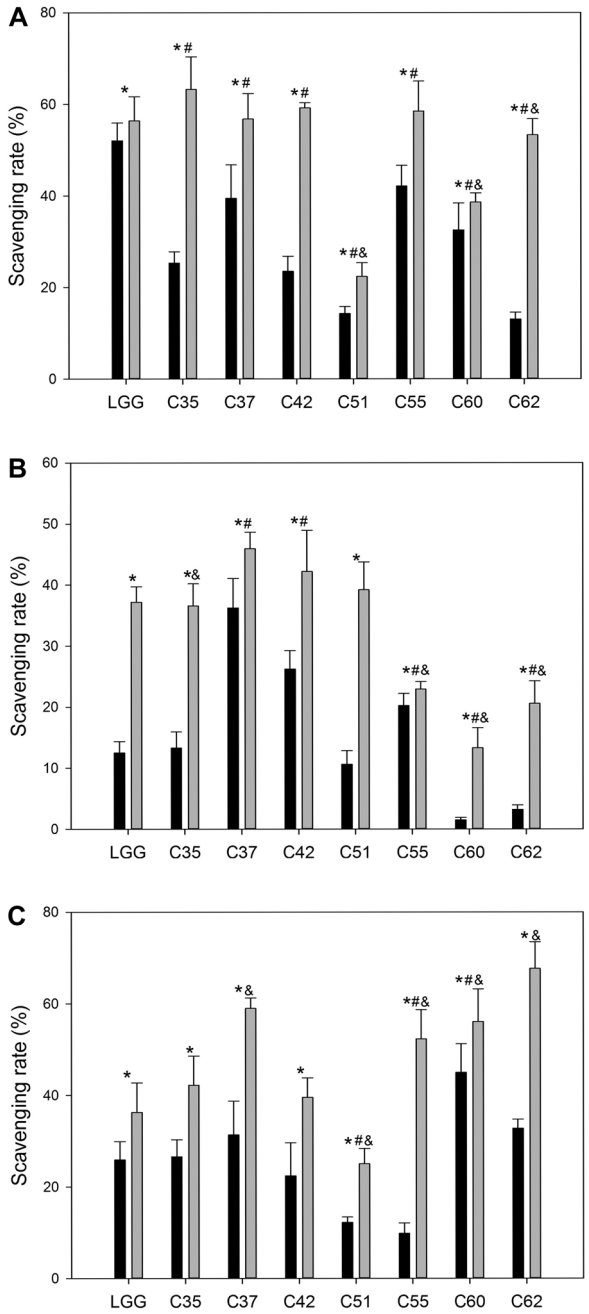

Fig. 2. Scavenging activities of *L. plantarum* strains against hydroxyl radicals (**A**), DPPH radicals (**B**), and superoxide anion (**C**), at cell concentration 10^9 CFU/ml (■) and 10^{10} CFU/ml (■). *Significantly different between cell concentration 10^9 CFU/ml and 10^{10} CFU/ml of each strain; #Significantly different from control (LGG) at cell concentration 10^9 CFU/ml; &Significantly different from control (LGG) at cell concentration 10^{10} CFU/ml ($P < 0.05$).

response relationship (Fig. 2A). Strain C35 (at 10^{10} CFU/ml) was the most active among the strains, for which hydroxyl radical scavenging activity was 63.2% in cell-free extract. The scavenging capacity of strains C37, C42, C55, and C62 was more than 50.0%, and other strains showed relatively low activity in cell-free extract from 10^{10} CFU/ml of cell suspension. The results presented in Fig. 2B show that all the cell-free extracts had DPPH radical scavenging activity. When cell-free extracts from 10^{10} CFU/ml of cell suspension were added, the DPPH radicals scavenging effects of *L. plantarum* C37, C42, and C51 were higher than those of LGG (37.0%), with the highest value for strain C37 (46.0%); whereas other strains were lower than that of LGG, with the lowest for strain C60 (13.3%). The scavenging activities of eight strains against the superoxide anion radicals are shown in Fig. 2C. All strains exerted inhibitory effects on the generation of the superoxide radical, and the highest value was found for strain C62 at 10^{10} CFU/ml (67.7%), followed by strain C37 (59.0%) and strain C60 (56.1%).

Owing to peroxidant, oxidative stress accumulation was reported to play an important pathological role in causing human diseases, such as cancer, emphysema, cirrhosis, atherosclerosis, and arthritis [5, 30]. In the past decade, increased attention was paid to using *Lactobacillus* and *Bifidobacterium* spp. as natural antioxidants for their potential roles in promoting human health by decreasing the risk of accumulation of reactive oxygen species during ingestion of food [28, 33]. Because there were evidences showing that the antioxidant activity of cell-free extract was stronger than that of intact cells [13], the cell-free extracts of eight *Lactobacillus* strains were prepared to test *in vitro* antioxidant capacity, including hydroxyl, DPPH, and superoxide anion radical scavenging capacity. Our results confirm these findings, as our data show that the cell-free extracts of *L. plantarum* strains C35 and C37 (Fig. 2) had significant ability to scavenge hydroxyl, DPPH, and superoxide anion radicals. The radical scavenging activity of LAB might be due to intracellular antioxidant enzymes, including superoxide dismutase, glutathione peroxidase, and catalase, being released after cell lysis. Cells lysates of some LAB may also produce non-enzymatic antioxidant components such as glutathione and thioredoxin. In addition, the antioxidant activities of intact cells of LAB could also be attributed to their production of cell-surface compounds, such as extracellular polysaccharides produced by *Lactococcus lactis* subsp. *lactis* 12 [41] and *Bifidobacterium animalis* RH [58], and lipoteichoic acid from the cell surface of bifidobacteria [59]. Previous work from our laboratory reported that the cell surface proteins or polysaccharides of *L. plantarum* C88 were involved in the antioxidant activity of this strain, since removing these cell surface compounds resulted in

significant decrease of the DPPH free radical scavenging capacity of the strain [32]. Thus, LAB possess enzymatic and non-enzymatic antioxidant defense and repair systems that have evolved to protect them against oxidative stress.

In Vitro Cholesterol-Lowering Activity

Cholesterol-removal activities of *L. plantarum* strains in this study are shown in Fig. 3. All eight *Lactobacillus* strains were capable of removing cholesterol from the growth medium after 24 h of incubation, but the removal ratio varied considerably among the strains. *L. plantarum* C55 was most active in removing cholesterol (68.4%). *L. plantarum* strains C35 and C42 removed cholesterol by 35.5%–48.0% and other strains removed cholesterol by 58.8%–68.1%.

Hypercholesterolemia is considered to be a risk factor of cardiovascular disease, the leading cause of morbidity and mortality in many countries [22]. Recent studies indicated that administration of *L. plantarum* strains exhibited cholesterol-reducing effects in both *in vitro* studies and *in vivo* models [61]. Using *in vitro* experiments, Lee *et al.* [29] reported that *L. plantarum* NR74 was able to take up cholesterol from media by bile salt hydrolytic (BSH) activity. Our previous study indicated that mice fed with probiotic cheese contaning *L. plantarum* K25 showed a decrease in serum total cholesterol levels [61]. A similar observation was reported by Jeun *et al*. [19] that *L. plantarum* KCTC3928 might have hypocholesterolemic effects in mice primarily due to induction of fecal bile acid secretion followed by increased degradation of hepatic cholesterol into bile acids. Results from the present study further confirmed that *L. plantarum* strains isolated from Inner Mongolia Hurood cheese exhibited a high ability of cholesterol assimilation.

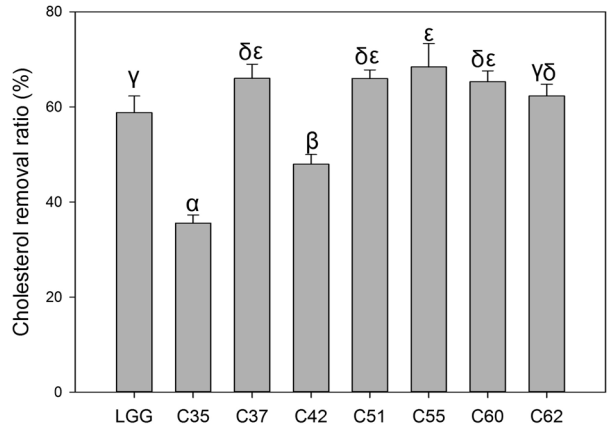

Fig. 3. The assimilation of cholesterol by *L. plantarum* strains. Different letters (α to ε) represent significant difference ($P < 0.05$).

However, the mechanism of *in vitro* cholesterol removal is unknown. Previously, several possible mechanisms for cholesterol removal or reducing the concentration of cholesterol in media by lactobacilli were proposed: assimilation of cholesterol during growth, binding of cholesterol to cellular surface, disruption of cholesterol micelle, deconjugation of bile salt, and BSH activity [36]. Further studies should be conducted to determine the mechanisms of *in vitro* cholesterol assimilation by *L. plantarum* strains.

Regulatory Effect of *L. plantarum* C37 on the Intestinal Flora of Mice

On the basis of the *in vitro* characterization of the *L. plantarum* strains as described above, strain C37 that showed promising probiotic properties was subjected to *in vivo* studies using a mouse model, focusing on its regulatory effect on the intestinal flora of mice. It has been reported that regulating the intestinal flora of the host is the fundamental function of probiotics [53]. The probiotic function exercised mainly through changing the composition of intestinal flora, which has the function of synthesizing and enhancing the bioavailability of nutrients, stimulating the immune system, reducing symptoms of diarrhea, and preventing infections [31]. Using the experimental model of this study, administration of *L. plantarum* C37 in mice was found to increase the number of lactobacilli in the fecal sample (Fig. 4A). Similarly, Angelakis *et al*. [7, 8] reported that the inoculation of *L. ingluviei* in chicks, ducks, and mice significantly increased the weight gain and liver weight of animals, and the DNA copy numbers of *Lactobacillus* spp. and *Firmicutes* in their feces. Moreover, we found that oral ingestion of *L. plantarum* C37 by mice reduced the number of enterococci (Fig. 4B), but there was no significant impact on the number of bifidobacteria, *Enterobacter,* and *Clostridium perfringens* in feces (Figs. 4C, 4D, and 4E). Previous studies also showed the cell numbers of fecal bacteria were not significantly altered by the clinical trials with *L. salivarius* Ls-33 conducted in obese adolescents [26]. However, Montesi *et al*. [39] reported that rats fed with diets supplemented with either prebiotics or probiotics induced a significant reduction of clostridia and *Bacteroides* spp. Thereby, for future probiotics, the most important requirements should include a demonstrated clinical benefit supported by understanding of the effect on the target bacterial population [48].

Viability of *L. plantarum* C37 in Hurood Cheese and Its Influence on Cheese Composition

Cheese has been considered to be a suitable carrier

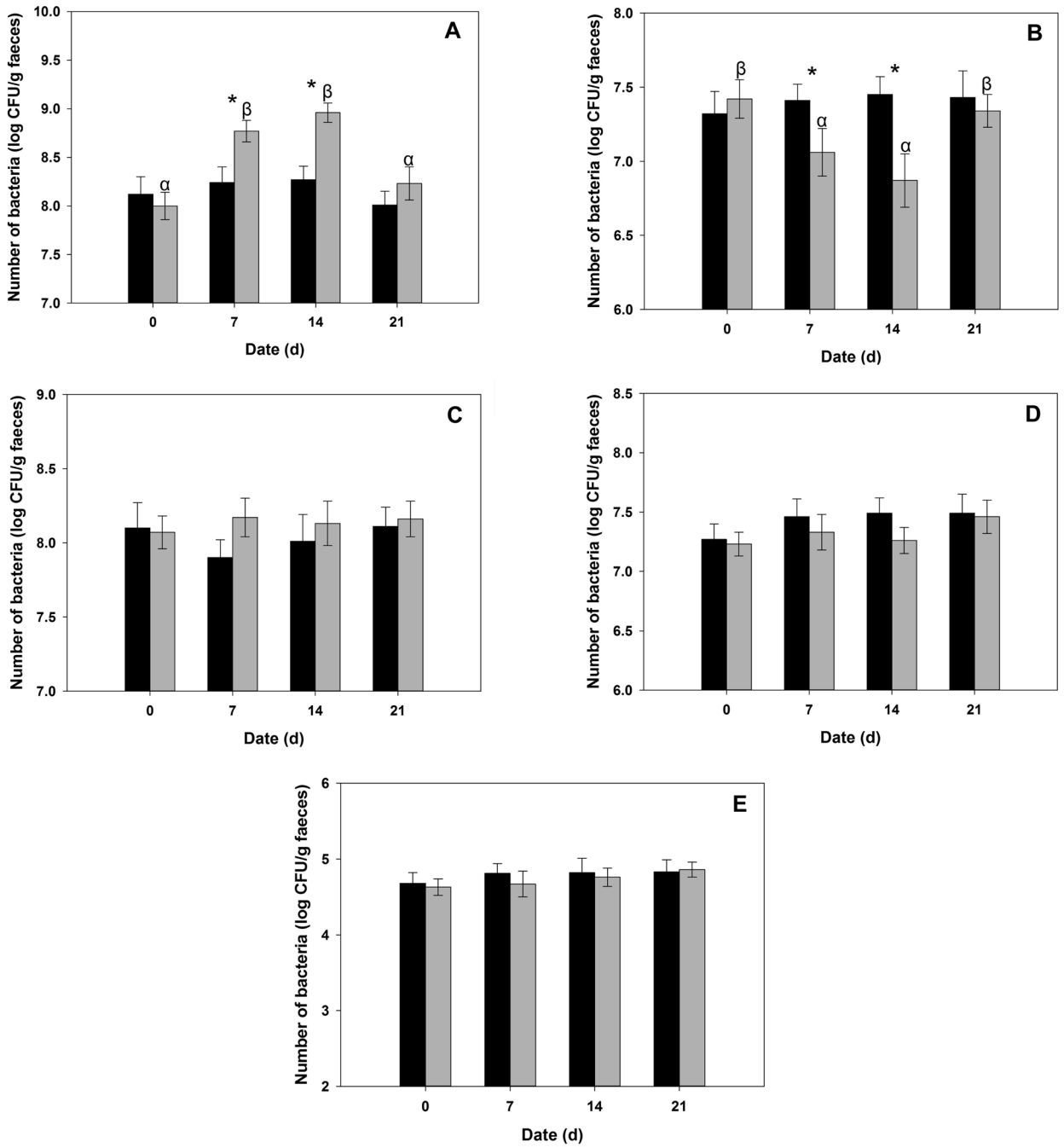

Fig. 4. Regulatory effect of *L. plantarum* C37 on the intestinal flora of mice including lactobacilli (**A**), enterococci (**B**), bifidobacteria (**C**), *Enterobacter* (**D**), and *Clostridium perfringens* (**E**): control group (■), *L. plantarum* C37 group (■).
*Significantly different from control at the same time point; Different letters (α and β) represent significant difference ($P < 0.05$).

for probiotics [10]. In this study, the probiotic adjunct *L. plantarum* C37 was inoculated into milk to produce probiotic Hurood cheeses. The compositions of Hurood cheeses with and without the addition of *L. plantarum* C37 are shown in Table 3. The use of probiotic culture of *L. plantarum* C37 did not influence the chemical compositions and pH of the cheese ($P < 0.05$). Addition of *L. plantarum* C37 into Hurood cheese had no adverse effect on the quality of cheese, which is an important technological feature to prevent deviations from the standard cheese-

Table 3. Composition of Hurood cheese (100 g) with and without *L. plantarum* C37.

Composition	Control cheese (g)	Probiotic cheese[a] (g)
Protein	28.67 ± 0.10	28.98 ± 0.24
Fat	36.73 ± 0.27	36.79 ± 0.25
Moisture	28.10 ± 0.13	28.23 ± 0.08
pH	5.05 ± 0.02	4.98 ± 0.04

No significant difference within the same row ($P < 0.05$).

[a]Probiotic cheese was produced with *L. plantarum* C37.

Table 4. Survival of lactococci and *L. plantarum* C37 during 1 month storage of probiotic Hurood cheese (log CFU/g).

Period of storage (weeks)	Control cheese		Probiotic cheese[a]	
	Lactococci	Lactobacilli	Lactococci	Lactobacilli
0	$4.86 \pm 0.06^{\alpha}$	0	$4.92 \pm 0.03^{\alpha}$	$8.95 \pm 0.07^{\alpha}$
1	$4.58 \pm 0.02^{\beta}$	0	$4.47 \pm 0.05^{\beta}$	$9.45 \pm 0.01^{\beta}$
2	$4.15 \pm 0.06^{\gamma}$	0	$4.28 \pm 0.07^{\gamma}$	$8.12 \pm 0.04^{\gamma}$
4	$4.05 \pm 0.03^{\delta}$	0	$4.22 \pm 0.06^{\gamma}$	$7.87 \pm 0.01^{\delta}$

[α–δ]Means in the same column followed by different superscripts are significantly different ($P < 0.05$).

[a]Probiotic cheese was produced with lactococci starter and *L. plantarum* C37.

making techniques. During the whole ripening period, *L. plantarum* C37 was found to maintain high viable counts (>7.8 log CFU/g) (Table 4) that met the least viable count (6.0 log CFU/g) requirement for probiotic foods [50]. In addition, unlike *L. plantarum* C37, the viability of starter lactococci in both the control and probiotic cheeses was very low during the whole storage process in spite of the initial high viable counts in the acid whey. The main reason of this might be the unfavorable high temperature used during the cooking step in cheese manufacturing.

In conclusion, seven *L. plantarum* strains isolated from Inner Mongolia Hurood cheese were evaluated for their probiotic potential by using both *in vitro* and *in vivo* methods. These strains were found to vary in probiotic characteristics, and none of them possessed all the desired properties. Among the seven selected strains evaluated, *L. plantarum* C37 was found to possess the best probiotic properties, including acid and bile salt tolerance, surface hydrophobicity properties, Caco-2 cells adhesion ability, and remarkable antioxidant activity and hypocholesterolemic properties. In addition, *L. plantarum* C37 was appropriate for use as adjunct cultures in Hurood cheeses because it increased the viable counts of probiotics in cheese, but had no adverse effect on cheese composition. Therefore, *L. plantarum* C37 could be considered as a good candidate for potential application in probiotic cheese and other functional foods.

Acknowledgments

The financial support for this work from The Earmarked Fund for Modern Agro-industrial Technology Research Systems in China (CARS-37), Natural Science Foundation for the Youth (31201394), National Public Benefit Research (Agriculture) Foundation (201303085), and Natural Science Foundation of China (31071574), and Jilin Agricultural Innovation Project (C32120302).

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