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ORIGINAL ARTICLE

Screening of lactic acid bacteria with cholesterol-lowering and triglyceride-lowering activity in vitro and evaluation of probiotic function

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Abstract

To screen the lactic acid bacteria with cholesterol-lowering and triglyceride-lowering activity in vitro and evaluate their probiotic function. By plate separating, cholesterol-lowering and triglyceride-lowering activity in vitro were determined; and by evaluating the probiotic functions, including tolerances to simulated gastric and intestinal juice, the antibacterial spectrum, and the adhesion ability to Caco-2 cells, the probiotic strains with cholesterol-lowering and triglyceride-lowering activity in vitro were screened, and then were identified by phenotypical and physiological tests and 16Sr DNA. Finally, the cholesterol-lowering and triglyceride-lowering activity in vivo of the strains were evaluated using male Sprague-Dawley rats. Two strains L2-16 and L2-73 with stronger cholesterol-lowering and triglyceride-lowering activity in vitro, stronger tolerance to simulated gastric and intestinal juice and adhesion ability to Caco-2 cells, and wider antibacterial spectrum were screened from traditional Chinese fermented cucumber and were identified as *Lactobacillus acidophilus* and *Enterococcus faecalis*, respectively. Compared with a hyperlipidemia diet without lactic acid bacteria, the diet supplemented with *Lactobacillus acidophilus* L2-16 and *Enterococcus faecalis* L2-73 significantly reduced serum total cholesterol, triglycerides, and low-density lipoprotein cholesterol levels, and liver total cholesterol and triglyceride levels in rats ($P < 0.05$). Moreover, the diet supplemented with *Lactobacillus acidophilus* L2-16 and *Enterococcus faecalis* L2-73 significantly increased the fecal elimination of bile acids ($P < 0.05$). *Lactobacillus acidophilus* L2-16 and *Enterococcus faecalis* L2-73 may have application prospect in the production of some fermented foods such as fermented vegetables, milk, or meat, and probiotic preparations with the function to lower the serum lipid and liver lipid levels.

Keywords Probiotics · Cholesterol-lowering · Triglyceride-lowering · Screen

Introduction

Lactic acid bacteria are live microorganisms that can produce lactic acid, hydrogen peroxide, and bacteriocin. Lactic acid bacteria are generally regarded as safe and can impose health benefits on the host including the production of antimicrobial substances, adhesion to intestinal epithelium, and lowering the adhesion of pathogenic microorganisms (Sleator and Hill 2008). Probiotics are described as live microorganisms which confer health benefits to the host when they are administered in adequate amounts (FAO/WHO 2001). Several preliminary requirements to the microorganism as probiotics include strong resistance to acidity, bile, and pepsin; antimicrobial activity against intestinal pathogenic microorganism; and good adhesion to the intestinal epithelium (Vinderola and Reinheimer 2003). The most important benefit of probiotic microorganism is to maintain intestinal flora balance in the host (Sleator and Hill 2008). Moreover, some probiotic microorganisms have been proven having other benefits such as assimilating cholesterol and improving the resistance and immunity to diseases (Nissen et al. 2009). Some lactic acid bacteria especially *Bifidobacterium* and *Lactobacillus* genera have been proven to possess strong probiotic function (Izquierdo et al. 2008; Todorov et al. 2012).

Too much of cholesterol and triglyceride in the daily diet and blood is the major risk for cardiovascular and cerebrovascular

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diseases (Szapary and Rader 2004; Nissen et al. 2009). At present, drug therapies are not regarded as optimal solutions for this condition because the cost and side effects are still relatively high. Therefore, lowering the concentrations of cholesterol and triglyceride in the daily diet and blood by some natural safe foods and microorganisms is an effective way. Recently, some lactic acid bacteria have been proven to possess the potential benefit of lowering cholesterol (Nguyen et al. 2007; Kumar et al. 2011; Jones et al. 2012). These probiotic lactic acid bacteria mainly belong to *Bifidobacterium*, *Lactobacillus,* and *Enterococcus*, and are generally isolated from animals or human (Ha et al. 2006; Kumar et al. 2010; Zanotti et al. 2015). However, the lowering-triglyceride function of lactic acid bacteria has been seldom reported. Moreover, relatively less work has been conducted on probiotic lactic acid bacteria isolated from fermented foods including fermented meat and vegetables. Lactic acid bacteria with probiotic function originating from fermented foods may have the potential to be used as starter and may be exploited in probiotic preparations and function foods.

This paper reported two lactic acid bacteria with cholesterol-lowering and triglyceride-lowering activity in vitro isolated from fermented cucumber and the evaluation of their probiotic functions including acid and bile tolerance, antimicrobial activity against intestinal pathogenic microorganisms, and adhesion to Caco-2 cells.

Materials and methods

Screening of lactic acid bacteria with cholesterol-lowering and triglyceride-lowering activity in vitro

Serial dilutions of fermented cucumber samples were spread on MRS agar supplemented with calcium carbonate (5 g/l) and incubated at 37 °C for 24–48 h. Those colonies with clear zones around were randomly selected, transferred to fresh MRS tube slants, and incubated at 37 °C for 24–48 h. These colonies were streaked on MRS agar plates for purification and were preliminarily identified by gram staining and catalase activity test.

Lowering-cholesterol effects in vitro of the strains isolated from fermented cucumber were determined using the method described by Mandal et al. (2009). Firstly, the strains were grown overnight at 37 °C in MRS broth [3% (*v*/*v*)], and then were inoculated into MRS broth supplemented with cholesterol (100 μg/ml, Sigma, Shanghai, China). The MRS broth supplemented with cholesterol but uninoculated strain was used as control. After being incubated without agitation at 37 °C for 72 h, the amount of cholesterol in cell-free supernatant was analyzed by the o-phthalaldehyde method (Rudel and Morris 1973).

Analyses of lowering-triglyceride effect were conducted in the strains in which cholesterol-lowering rates were more than 35%. Firstly, the strains grew overnight at 37 °C in MRS broth [3% (*v*/*v*)], and then were inoculated into MRS-triglyceride broth. The MRS-triglyceride broth was prepared according to the methods described as follows. At first, 20 ml of polyvinyl alcohol aqueous solution (2%) and 50 ml of triglycerides (Sigma, 99%) were mixed in a high-speed blender (PRISM, Thermo Fisher Scientific Company) for 20 min. Then, 3% (*v*/*v*) of the mixture was added to MRS broth, adjusted to pH 6.5, sterilized at 121 °C for 15 min, and cooled. The MRS-triglyceride broth without strain was used as control. After being incubated at 37 °C for 72 h, the amount of triglyceride in the cell-free supernatant was determined at 500 nm using Triglyceride Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Evaluation of tolerance to simulated gastric and intestinal juice

The tolerance to simulated gastric and intestinal conditions was carried out according to the methods described by Saraniya and Jeevaratnam (2015). After being cultivated in the MRS broth at 37 °C for 18 h, the cells were collected by centrifugation (6000*g*, 15 min, 4 °C), washed with saline (0.85%), suspended in saline, adjusted to pH 2.0 containing pepsin (3 mg/ml) for gastric tolerance, and in saline adjusted to pH 8.0 containing pancreatin (3 mg/ml) and bile salt (0.3%) for intestinal tolerance. Cell suspended in saline with pH 7.0 was used as the control. After being incubated at 37 °C for 0, 2, and 4 h, the viable colony counts were determined.

Determination of antibacterial spectrum and adhesion ability to Caco-2 cells

Antibacterial spectrum was determined using cell-free supernatant. The indicatory lactic acid bacteria were cultivated without agitation in the MRS broth at 37 °C for 18 h and other bacteria were cultivated in nutrient broth at 37 °C and 100 r/min for 12 h. At last, diameters of inhibition zones of cell-free supernatant of the strain were measured by the agar diffusion assay method (Ennahar et al. 2000). Fifteen milliliters of MRS agar was used as the lower layer of double-plate in the plate (*Φ* 90 mm). Five milliliters of MRS soft agar (0.75% agar) inoculated with 100 μl cell suspension of indicator strain cultured overnight was used as the upper layer. One hundred microliters of cell-free supernatants of the strain was placed into wells (6.0 mm in diameter) on MRS soft agar plates seeded with the indicator strains. After incubation at 30 °C for 18 h, the diameters of inhibitory zones were determined.

Adhesion ability of the strain to Caco-2 cells was assayed according to the methods described by Greene and Klaenhammer (1994). Caco-2 cells (ATCC HTB-38) were

cultivated in Dulbecco's modified Eagle's minimal essential medium (DMEM) (Solarbio Biotechnology Limited Company, Shanghai, China) with 100 U/ml of penicillin and streptomycin (Sigma, Shanghai, China), and 10% (*v*/*v*) sterilized fetal bovine serum (Sigma, Shanghai, China). After being cultivated in an atmosphere containing 10% (*v*/*v*) CO_2 at 37 °C for 15–20 days, Caco-2 cells (1×10^5 cells per well) were transferred to 24-well tissue culture plates (MB Limited Company, Shanghai, China) and continually cultivated with fresh medium replaced every other day. After cultivation for about 15 days until formation of a monolayer of Caco-2 cells, these cells were washed twice with PBS (pH 7.2). After overnight cultivation in the MRS broth, 1 ml culture of the tested probiotic strain was collected by centrifugation (6000*g*, 15 min), suspended in DMEM medium (100 ml). Then, 0.1 ml of the Caco-2 cell suspension and 0.9 ml of the culture suspension (1×10^8 CFU/ml) were added to each hole of 24-well tissue culture plates. After cultivation in the atmosphere containing 10% (*v*/*v*) CO_2 at 37 °C for 1 h, the Caco-2 cells were washed three times using 2 ml of PBS (pH 7.2), re-suspended in the same PBS (0.8 ml), supplemented with 200 μl of 1% (*v*/*v*) Triton X-100 (Sigma, Shanghai, China), and vortexed for 15 min. One hundred microliters of appropriate dilutions in sterile saline (0.85%) of the suspension was spread on MRS agar plates and cultivated at 37 °C for 48 h. Adherence rate was calculated using the formula: Adherence rate (%) = (viable cell count/initial cell number) × 100.

Strain identification

The strain was firstly identified by phenotypical and physiological tests including cell morphology and growth ability at different temperatures and NaCl concentrations, as well as carbohydrate fermentation patterns (Kandler and Weiss 1986). By 16S rDNA sequence analysis, the strain was further identified. Forward primer for PCR is 5′-AGAG TTTGATCCTGGCTCAG-3′ and reverse primer is 5′-GTGT GACGGGCGGTGTGTAC-3′ (Takara Bio., Dalian, China). PCR reaction was performed in a 50 μl mixture consisting of 10 ng of genomic DNA, 0.5 μmol/l primer, 1.5 mmol/l MgCl2, and 200 μmol/l each deoxynucleoside triphosphate, and 5 μl of 10 × PCR amplification buffer (500 mmol/l KCl, 100 mmol/l Tris-HCl, pH 8.3). Amplification was conducted by initial denaturation for 5 min at 94 °C, followed by 30 cycles of denaturing at 94 °C for 1 min, primers annealing at 55 °C for 1 min, extension for 1.5 min at 72 °C, and the final polymerization at 72 °C for 5 min. The PCR product was sequenced after purification using Agarose Gel DNA Purification Kit (Takara Bio., Dalian, China). Sequence homologies were determined after comparing the sequences of the strain with those in the databases (http://www.ncbi.nlm.nih.gov/BLAST).

Evaluation of cholesterol-lowering and triglyceride-lowering activity in vivo using male Sprague-Dawley rats

Animal studies in this experiment were carried out strictly according to the rules of the Animal Welfare and Research Ethics Committee of Jilin University (Changchun, China), and the permit number was 20090719-1. Meanwhile, all the animal experiments were conducted according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (National Institutes of Health 1996). During animal experiment, all efforts were made to decrease the suffering of the rats, and all surgeries were carried out under anesthesia (pentobarbital sodium). Eighteen male Sprague-Dawley rats, aged 4 weeks, were purchased from the Animal Center of Harbin Medical University (Harbin, China). These rats were individually housed at 22 °C and a 12 h light-dark cycle (06:00–18:00 for light) and were fed a commercial diet (Xietong Medicine Biotechnology Limited Company, Nanjing, China) for 1 week. After that, the rats were randomly divided into three groups, with six rats in each group. Each group had similar initial average body weight. These three groups were fed and equilibrated according to the following: (1) (HP), (2) hyperlipidemia diet + *Lactobacillus acidophilus* L2-16 (HP-L2-16), and (3) hyperlipidemia diet + *Enterococcus faecalis* L2-73 (HP-L2-73). The hyperlipidemia diet contained 5% (*w*/*w*) corn germ oil, 5% lard oil, 1% cholesterol, 0.5% sodium deoxycholate, 5% sucrose, 5% dried egg yolk, 0.2% propylthiouracil, and 78.3% commercial diet (Xietong Medicine Biotechnology Limited Company, Nanjing, China). During a 4-week study period, the HP-L2-16 and HP-L2-73 groups obtained 2 ml (10^9 CFU/ml) of *L. acidophilus* L2-16 and *E. faecalis* L2-73 each day intragastrically. The HP diet groups received of 2 ml of normal saline intragastrically. Food consumption of each rat was monitored each day and body weight was determined each week.

After 4 weeks of feeding, the rats were deprived diet for 12 h and were bled from the tail vein under anesthesia (pentobarbital sodium). Approximately 1 ml of blood of each rat was withdrawn, cooled on ice for 30 min, and centrifuged (2000*g*, 20 min, 4 °C). Serum total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) were analyzed using the commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

After blood collection, the rat livers were taken out, washed with normal saline, blotted dry using filter paper, and weighted. The lipids in the liver were extracted and treated using the method described by Folch et al. (1957), and liver TC, TG, LDL-C, and HDL-C were analyzed using the commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

The feces of each rat were collected at 27 and 28 days, and were dried at 60 °C. After the remaining contaminants were removed by air blower, the feces were ground by an analytical mill, and the cholic acid level was analyzed using the method described by Locket and Gallaher (1989).

Statistical analysis

All experiments in this study were replicated three times and in duplicate. Data was analyzed using SAS software (version 8.1, SAS Institute Inc., Cary, NC, USA). Significant differences between means were checked using one-way ANOVA and significance level was set at $P < 0.05$. Multiple comparisons between sample means were performed by critical difference values.

Results

Screening of lactic acid bacteria with cholesterol-lowering and triglyceride-lowering activity in vitro

A total of 200 isolates with clear zones around were isolated from traditional fermented cucumber and were cultivated in the MRS broth containing cholesterol, and the 32 strains with more than 45% cholesterol-lowering rate were obtained. After cultivation in the MRS broth containing triglyceride, triglyceride-lowering rate of five strains was more than 35%, and their cholesterol-lowering rate and triglyceride-lowering rate were listed in Table 1. Among the five strains, strain L2-16 exhibited the most cholesterol-lowering rate (64.11 ± 0.58%) and strain L2-73 exhibited the most triglyceride-lowering rate (41.38 ± 0.26%).

Table 1 Cholesterol-lowering rate and triglyceride-lowering rate of the strains selected from traditional fermented cucumber

Strain	Cholesterol-lowering rate (%)	Triglyceride-lowering rate (%)
L1-24	58.24 ± 0.46^{b}	35.56 ± 0.47^{c}
L1-85	55.13 ± 0.68^{c}	36.15 ± 0.62^{c}
L2-16	64.11 ± 0.58^{a}	38.27 ± 0.40^{b}
L2-41	59.85 ± 0.68^{b}	36.23 ± 0.38^{c}
L2-73	63.40 ± 0.54^{a}	41.38 ± 0.26^{a}

Presented values are means of triplicate determinations; ± indicates standard deviation from the mean. Mean values within the same column followed by different superscript letters differ significantly ($P < 0.05$)

Evaluation of tolerance to simulated gastric and intestinal juice

To exert beneficial effects, a probiotic strain is required to tolerate the gastrointestinal environments. Table 2 showed the survival of the five strains over exposure to simulated gastric juice in pH 2.0 containing pepsin (3 mg/ml). When exposed to simulated gastric juice for 2 h, the differences of viable colony counts of strains L1-24, L1-85, L2-16, and L2-73 were not significant ($P > 0.05$), but the viable colony counts of strain L2-41 were significantly less than these four strains ($P < 0.05$). When exposed to simulated gastric juice for 4 h, the differences of viable colony counts of strains L1-85, L2-16, and L2-73 were not significant ($P > 0.05$), but the viable colony counts of strain L1-24 and strain L2-41 were significantly less than those of these three strains ($P < 0.05$). When exposed to simulated gastric juice for 2 h, the decreases of viable colony counts of strains L2-16 and L2-73 were not significant ($P > 0.05$). When exposed to simulated gastric juice from 2 to 4 h, the decreases of viable colony counts of these two strains were still not significant ($P > 0.05$).

Table 3 showed the survival of the five strains over exposure to simulated intestinal condition in saline adjusted to pH 8.0 containing pancreatin (3 mg/ml) and bile salt (0.3%). When exposed to simulated intestinal condition for 2 h, the survival of the five strains was similar to that of those exposed to simulated gastric juice. When exposed to simulated intestinal condition for 4 h, the difference of viable colony counts of strains L2-16 and L2-73 was not significant ($P > 0.05$), but the viable colony counts of strains L1-85, L1-24, and L2-41 were significantly less than those of these two strains ($P < 0.05$). When exposed to simulated intestinal juice for 2 h, the decrease of viable colony counts of all the strains was significant ($P < 0.05$), but the decreases of the strains L2-16 and L2-73 were less, and were 5.44 and 4.07%, respectively. But the decreases of viable colony counts of these two strains were not significant when exposed to simulated gastric juice from 2

Table 2 Survival (log CFU/ml) of the strains over exposure to simulated gastric juice

Strains	0 h	2 h	4 h
L1-24	8.83 ± 0.40$^{a\ a}$	8.35 ± 0.26$^{a\ b}$	7.64 ± 0.26$^{b\ c}$
L1-85	9.04 ± 0.36$^{a\ a}$	8.53 ± 0.32$^{a\ b}$	8.32 ± 0.42$^{a\ b}$
L2-16	8.79 ± 0.41$^{a\ a}$	8.48 ± 0.35$^{a\ a}$	8.29 ± 0.31$^{a\ a}$
L2-41	8.92 ± 0.28$^{a\ a}$	7.82 ± 0.31$^{b\ b}$	7.34 ± 0.40$^{b\ c}$
L2-73	8.80 ± 0.34$^{a\ a}$	8.44 ± 0.43$^{a\ a}$	8.26 ± 0.29$^{a\ a}$

Presented values are means of triplicate determinations; ± indicates standard deviation from the mean. Mean values within the same column followed by the first different superscript letters differ significantly ($P < 0.05$); mean values within the same row followed by the second different superscript letters differ significantly ($P < 0.05$)

Table 3 Survival (log CFU/ml) of the strains over exposure to simulated intestinal juice

Strains	0 h	2 h	4 h
L1-24	$8.90 \pm 0.42^{a\ a}$	$8.17 \pm 0.31^{a\ b}$	$7.52 \pm 0.41^{b\ c}$
L1-85	$8.94 \pm 0.31^{a\ a}$	$8.06 \pm 0.30^{a\ b}$	$7.65 \pm 0.34^{b\ c}$
L2-16	$8.82 \pm 0.40^{a\ a}$	$8.34 \pm 0.29^{a\ b}$	$8.06 \pm 0.37^{a\ b}$
L2-41	$8.85 \pm 0.36^{a\ a}$	$7.55 \pm 0.34^{b\ b}$	$7.12 \pm 0.23^{c\ c}$
L2-73	$8.83 \pm 0.35^{a\ a}$	$8.37 \pm 0.33^{a\ b}$	$8.13 \pm 0.36^{a\ b}$

Presented values are means of triplicate determinations; ± indicates standard deviation from the mean. Mean values within the same column followed by different superscript letters differ significantly ($P < 0.05$); mean values within the same row followed by the second different superscript letters differ significantly ($P < 0.05$)

Table 5 Adhesion assay of the five strains to Caco-2 cells

Strain	Adhesion rate (%)
L1-24	11.8 ± 0.4^{d}
L1-85	28.3 ± 1.0^{a}
L2-16	23.8 ± 1.2^{b}
L2-41	19.5 ± 0.8^{c}
L2-73	29.6 ± 0.6^{a}

Presented values are means of triplicate determinations; ± indicates standard deviation from the mean. Mean values within the same column followed by different superscript letters differ significantly ($P < 0.05$)

to 4 h ($P > 0.05$). Therefore, among the five strains, the strains L2-16 and L2-73 had the strongest tolerance to the gastrointestinal environments.

Antibacterial spectrum and adhesion ability to Caco-2 cells

The antibacterial activity of the five strains listed in Table 4 showed that these strains could inhibit the tested indicators in various degrees. Among these five strains, the strains L2-16, L2-41, and L2-73 had stronger inhibitory activity against the indicators than the strains L1-24 and L1-85.

The results shown in Table 5 indicated that the strains of L2-73, L1-85 and L2-16 exhibited the stronger adhesion ability to Caco-2 cells than the strains L1-24 and L2-41, and the adhesion rates were 29.6 ± 0.6% for strain L2-73, 28.3 ± 1.0% for strain L1-85, and 23.8 ± 1.2% for the strain L2-16.

Strain identification

The results from Tables 1, 2, 3, 4, and 5 suggested that the strains L2-16 and L2-73 not only had stronger cholesterol-lowering and triglyceride-lowering activity in vitro, but also had stronger tolerance to simulated gastric and intestinal juice, adhesion ability to Caco-2 cells, and wider antibacterial spectrum. Thus, strains L2-16 and L2-73 were selected as probiotics with cholesterol-lowering and triglyceride-lowering activity in vitro for strain identification.

The strains L2-16 and L2-73 were preliminarily identified by morphological and physiological tests. Cells of strain L2-16 were long, rod-shaped gram-positive bacteria, and those of strain L2-73 were coccus-shaped gram-positive bacteria. The two strains were both able to grow in broth containing 6.5% (*m*/*v*) NaCl and at 10 °C and 45 °C. Strain L2-16 can metabolize glucose, sucrose, ribose, fructose, lactose, and mannose, but not xylose, rhamnose, cellobiose, mannitol, and sorbite. Strain L2-73 could metabolize turanose, L-arabinose, lactose, fructose, glucose, and xylose, but not mannose, rhamnose, cellobiose, mannitol, sucrose, and sorbite. For further identification, 16S rDNA nucleotide sequences of strains L2-16 and L2-73 were amplified. By comparing the nucleotide sequences of strains L2-16 and L2-73 with those in the databases of NCBI, the nucleotide sequence of strain L2-16 was 99% similar to that of *Lactobacillus acidophilus* ATCC 4356 (AB008203.1), and that of strain L2-73 was 99%

Table 4 Antibacterial activity of the five strains against different indicator

Indicator strains	L1-24	L1-85	L2-16	L2-41	L2-73
Staphylococcus aureus ATCC 63589	++	++	+++	+++	++
Bacillus subtilis ACCC 11060	+	++	++	++	+++
Listeria monocytogenes CMCC 54002	++	+	+++	+++	+++
Lactobacillus acidophilus ATCC 4356	++	++	+++	+++	+++
Salmonella typhimurium CMCC 47729	+	++	++	++	+++
Streptococcus thermophilus CICC 06038	+++	++	+++	+++	+++
Sarcina flava CMCC 29001	+	+++	++	++	+++
Shigella flexneri CMCC 51606	++	++	++	++	++
Escherichia coli ACCC 30005	+	+	+++	++	+++

[a] *ATCC*, American Type Culture Collection; *ACCC*, Agricultural Culture Collection of China; *CICC*, China Center of Industrial Culture Collection; *CMCC*, China Center of Medicine Culture Collection. Diameter of inhibition zone: +, 8.00–12.00 mm; ++, 12.00–16.00 mm; +++, more than 16.00 mm

similar to that of *Enterococcus faecalis* ATCC 19433 (NR_115765.1). Therefore, strains L2-16 and L2-73 were identified as *L. acidophilus* and *E. faecalis*, respectively. The GenBank accession number of strain L2-16 is KU922757, and that of strain L2-73 is KU922756.

Evaluation of cholesterol-lowering and triglyceride-lowering activity in rats

The results shown in Fig. 1(A) indicated that the serum TC, TG, and LDL-C levels among the HP group, HP-L2-16 group, and HP-L2-73 group all differed significantly ($P < 0.05$). Compared with the HP group, a reduction of 31.3% in TC, a reduction of 26.8% in TG, and a reduction of 40.8% in LDL-C ($P < 0.05$) were obtained by the HP-L2-73 group, whereas a reduction of 18.6% in TC, a reduction of 20.2% in TG, and a reduction of 34.2% ($P < 0.05$) in LDL-C of the serum were obtained by the HP-L2-16 group. Compared with the HC diet group, HDL-C levels among the HP group, HP-L2-16 group, and HP-L2-73 group differed insignificantly ($P > 0.05$).

Before the analysis of liver lipid levels, the average liver weights of the three groups were determined, and had no significant difference ($P > 0.05$). The liver lipid levels of the HP group, HP-L2-16 group, and HP-L2-73 group were shown in Fig. 2. The liver TC and TG levels of the HP-L2-16 group and HP-L2-73 group were both significantly lower than those of the HP group ($P < 0.05$). The liver TC levels of the HP-L2-16 group and HP-L2-73 group had no significant difference ($P > 0.05$), but the liver TG levels of the HP-L2-73 group were significantly lower than those of the HP-L2-16 group ($P < 0.05$).

The results of the fecal cholic acid analysis listed in Table 6 showed that the fecal cholic acid levels in the HP-L2-16 group and HP-L2-73 group were significantly higher than that in the HP group ($P < 0.05$). After 4 weeks of feeding, cholic acid concentration in the fecal of the HP-L2-73 group was the most (6.32 ± 0.27 μmmol/g), followed by the HP-L2-16 group (5.96 ± 0.24 μmmol/g), and that in the HP group was the least (2.15 ± 0.11 μmmol/g).

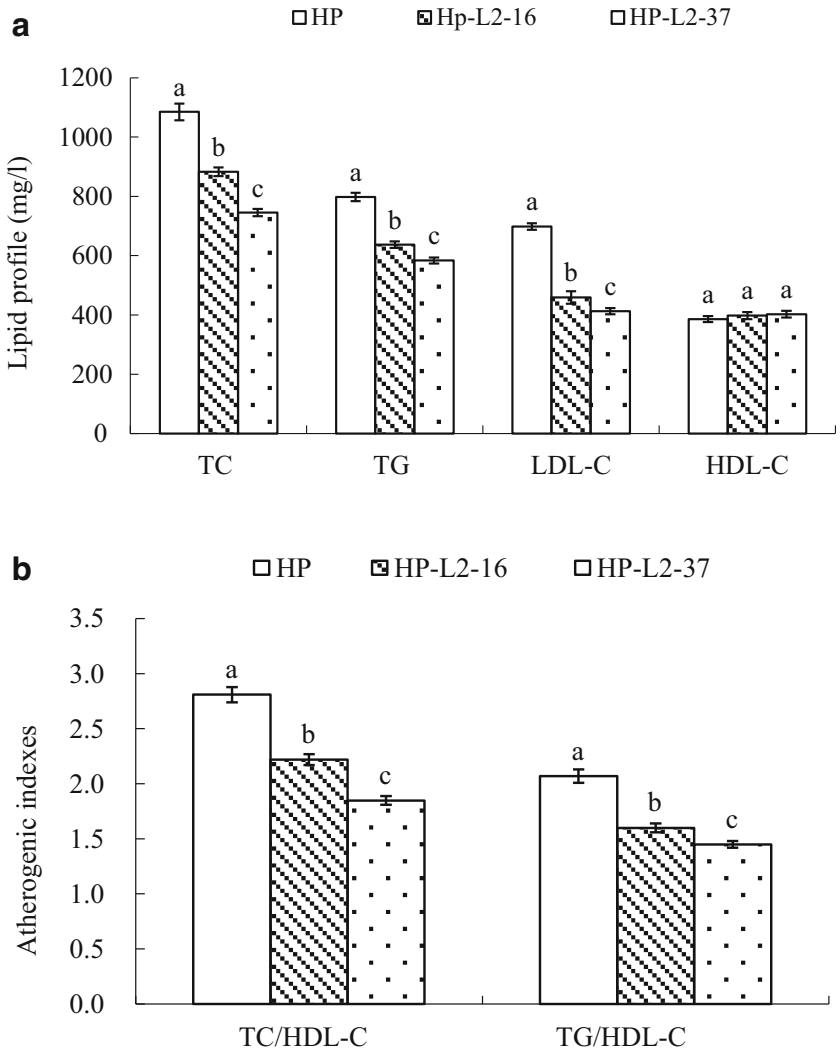

Fig. 1 Effects of the *Lactobacillus acidophilus* L2-16 (L2-16) and *Enterococcus faecalis* L2-73 (L2-73) on lipid metabolism in rats fed experimental diets. HP diet, hyperlipidemia diet; HP-L2-16, HP diet+L2-16; HP-L2-73, HPdiet+L2-73. (A) Serum total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) contents; (B) atherogenic indexes of rats. The data are shown as means ± standard deviation. Mean values with different letters (a–c) within each clustered group differ significantly ($P < 0.05$)

Discussion

It has been proven that too much of cholesterol and triglyceride in the daily diet and blood is the major risk for cardiovascular and cerebrovascular diseases (Nissen et al. 2009). Recently, studies have reported that some probiotics are effective in reducing the levels of serum triglycerides, cholesterol, and low-density lipoprotein cholesterol (Ooi et al. 2010; Watanabe et al. 2013). And these reports have led to the increasing interest to prevent coronary heart disease by the usage of these probiotics.

In this study, the strains L2-16 and L2-73, with not only stronger cholesterol-lowering and triglyceride-lowering activity but also stronger tolerance to simulated gastric and intestinal juice and adhesion ability to Caco-2 cells and wider antibacterial spectrum under in vitro conditions, were screened from fermented cucumber. The morphological and physiological properties of these two strains matched with those of *L. acidophilus* and *E. faecalis*. Moreover, the nucleotide sequences of these two strains were 99% similar to those of *L. acidophilus* ATCC 4356 and *E. faecalis* ATCC 19433. Therefore, these two strains were identified as *L. acidophilus* and *E. faecalis*, respectively.

In vitro, cholesterol-lowering rate and triglyceride-lowering rate of *L. acidophilus* L2-16 were 64.11 ± 0.58% and 38.27 ± 0.40%, respectively. Cholesterol-lowering rate and triglyceride-lowering rate of *E. faecalis* L2-73 were 63.40 ± 0.54% and 41.38 ± 0.26%, respectively. The cholesterol-lowering rate in vitro of *L. acidophilus* L2-16 was comparably higher than that of *Pediococcus acidilactici* LAB 5 (62%) (Mandal et al. 2009), *L. sake* C2 (53.2%) (Gao et al. 2012), *L. helveticus* MG2–1 (51.74%) (Bilige et al. 2009), and *L. fermentum* F1 (48.87%) (Zeng et al. 2011). The triglyceride-lowering ability in vitro has been seldom found in other lactic acid bacteria, but there are already few literatures available on triglyceride-lowering ability of lactic acid bacteria under in vivo conditions (Huang et al. 2012).

For probiotics, strong resistance to gastrointestinal stress condition including acidity, bile, and pepsin, antimicrobial activity against intestinal pathogenic microorganism, and good adhesion to the intestinal epithelium are regarded as preliminary requirements. Cultivated in simulated gastric juice condition in pH 2.0 containing pepsin (3 mg/ml) for 4 h, the viable cell counts of *L. acidophilus* L2-16 and *E. faecalis* L2-73 decreased from 8.79 ± 0.41 to 8.29 ± 0.31 log CFU/ml, and from 8.80 ± 0.34 to 8.26 ± 0.29 log CFU/ml, respectively (Table 2). Cultivated in simulated intestinal condition in pH 8.0 containing pancreatin (3 mg/ml) and bile salt (0.3%) for 4 h, the viable cell counts of *L. acidophilus* L2-16 and *E. faecalis* L2-73 decreased from 8.82 ± 0.40 to 8.06 ± 0.37 log CFU/ml, and from 8.83 ± 0.35 to 8.13 ± 0.36 log CFU/ml, respectively (Table 3). When exposed to simulated gastric juice from 0 to 2 h and from 2 to 4 h, the decreases of viable colony counts of *L. acidophilus* L2-16 and *E. faecalis* L2-73 were not significant ($P > 0.05$). When exposed to simulated intestinal juice for 2 h, the decreases of viable colony count of these two strains were significant ($P < 0.05$), but the decreases of viable colony count of these two strains were not significant when exposed to simulated gastric juice from 2 to 4 h ($P > 0.05$). After being exposed to the gastric juice and intestinal juice for 4 h, the decreases of the viable colony count of the two strains were both less than 1 log CFU/ml, the viable colony counts of the two strains were still more than 8 log CFU/ml. These results showed that these two strains have strong tolerance to the gastrointestinal environments.

The most important beneficial effect for probiotics has been generally considered as maintaining intestinal flora balance in the host by inhibiting pathogenic microorganisms. Results listed in Table 4 showed *L. acidophilus* L2-16 and *E. faecalis* L2-73 have a broad inhibitory spectrum. They could inhibit not only gram-positive bacteria including *Staphylococcus aureus* ATCC 63589, *Bacillus subtilis* ACCC 11060, *Listeria monocytogenes* CMCC 54002, *L. acidophilus* ATCC 4356, and *Streptococcus thermophilus* CICC 06038, but also gram-negative bacteria including *Salmonella typhimurium* CMCC 47729, *Sarcina flava* CMCC 29001, *Shigella flexneri* CMCC 51606, and *Escherichia coli* ACCC 30005. Moreover, *L. acidophilus* L2-16 and *E. faecalis* L2-73 are the strongest inhibitors to *L. monocytogenes* which is a ubiquitous pathogen generally recognized as a serious hazard to host health.

From a functional point of view, it is necessary to test whether the probiotic strain has the ability to adhere to intestinal mucosa. Results listed in Table 5 showed the adhesion rates of *L. acidophilus* L2-16 and *E. faecalis* L2-73 to Caco-2 cells were 23.8 ± 1.2% and 29.6 ± 0.6%, which were higher than those of *L. sake* C2 (15.2 ± 1.2%) (Gao et al. 2012), *Pediococcus pentosaceus* OZF (14.40 ± 0.81%) (Osmanagaoglu et al. 2010), *B. longum* BIF 53 (11.07%) (Izquierdo et al. 2008), *P. pentosaceus* BH105 (10.24%) (Uymaz et al. 2009), and *B. longum* NCC2705 (5.75%) (Izquierdo et al. 2008).

To confirm the cholesterol-lowering and triglyceride-lowering activity, *L. acidophilus* L2-16 and *E. faecalis* L2-73 were further examined in a rat model. The results in Figs. 1 and 2 showed that supplementations with *L. acidophilus* L2-16 and *E. faecalis* L2-73 were effective in reducing TC, TG, and LDL-C levels in the serum and liver of Sprague-Dawley rats fed a high-hyperlipidemia diet compared with those fed with the same high-hyperlipidemia diet but without LAB supplementation. In particular, these effects were more evident with *E. faecalis* L2-73 (serum TC, TG, and LDL-C reduced by 31.3%, 26.8%, and 40.8%, respectively). The reduction to serum TC and TG of *E. faecalis* L2-73 was higher than that of *L. plantarum* PH04 (Nguyen et al. 2007),

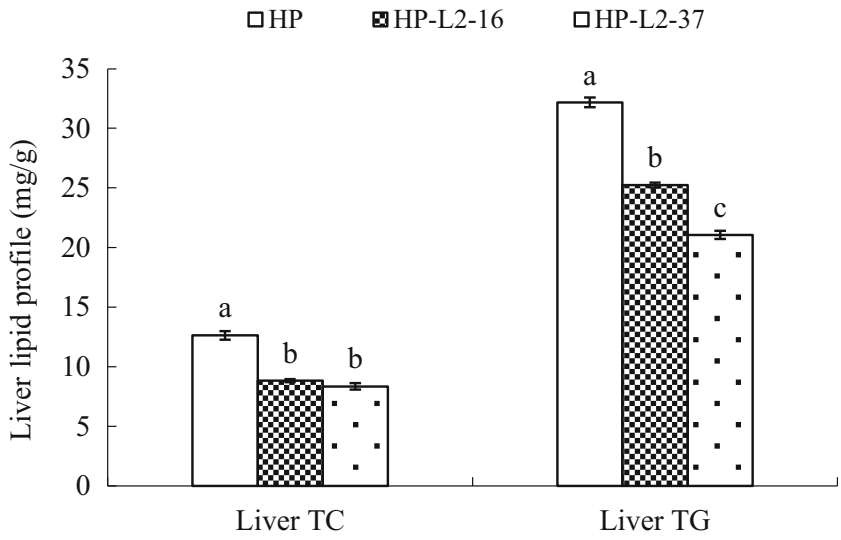

Fig. 2 Liver total cholesterol (TC) and triglyceride (TG) levels in rats fed experimental diets. HP diet, hyperlipidemia diet; HP-L2-16, HP diet+L2-16; HP-L2-73, HP diet+L2-73

L. plantarum 9-41-A (Xie et al. 2011), and *L. plantarum* Lp45 (Huang et al. 2012). LDL-C is the main ingredient in serum cholesterol, and lowering the LDL-C level is very important for reducing total cholesterol in serum (Szapary and Rader 2004). These two strains were predominant in lowering the serum LDL-C level (reduction of 40.8% by *E. faecalis* L2-73, and reduction of 34.2% by *L. acidophilus* L2-16). However, supplementation with *L. acidophilus* L2-16 and *E. faecalis* L2-73 did not significantly decrease the serum HDL-C level. These results were similar to the results of St-Onge et al. (2002), Ibrahim et al. (2005), and Xie et al. (2011).

Results in Fig. 2 indicated that rats supplemented with *L. acidophilus* L2-16 and *E. faecalis* L2-73 significantly reduced liver TC and TG levels. Compared with rats without LAB, liver TC and TG levels of the rats supplemented with *L. acidophilus* L2-16 reduced by 30.2 and 21.6%, and liver TC and TG levels of the rats supplemented with *E. faecalis* L2-73 reduced by 33.9 and 34.6%, respectively. *Lactobacillus plantarum* 9-41-A (Xie et al. 2011) and *L. plantarum* Lp45 (Huang et al. 2012) were also reported to have the similar effect.

Results in Table 6 showed greater amounts of cholic acids in the feces of rats supplemented with LAB, especially in the feces of the rats supplemented with *E. faecalis* L2-73. Several probiotic strains including *L. acidophilus* ATCC 43121 (Park et al. 2007), *L. plantarum* 9-41-A (Xie et al. 2011), and *L. plantarum* Lp45 (Huang et al. 2012) have reported the increase in fecal elimination of bile acids. These results indicated that the lowered cholesterol levels in serum and liver of the rat may be a result of more bile acid in the gastrointestinal tract that was precipitated or de-conjugated. This may partly explain the rats supplemented with *E. faecalis* L2-73 exhibiting lower cholesterol levels than the rats supplemented with *L. acidophilus* L2-16.

Table 6 Fecal cholic acid levels of the three rat groups

Group	Cholic acid (μmmol/g)
HP	2.15 ± 0.11^{c}
HP-L2-16	5.96 ± 0.24^{b}
HP-L2-37	6.32 ± 0.27^{a}

Presented values are means of triplicate determinations; ± indicates standard deviation from the mean. Mean values within the same column followed by different superscript letters differ significantly ($P < 0.05$)

Conclusion

Lactobacillus acidophilus L2-16 and *E. faecalis* L2-73 with stronger cholesterol-lowering and triglyceride-lowering activity in vitro, stronger tolerance to simulated gastric and intestinal juice and adhesion ability to Caco-2 cells, and wider antibacterial spectrum were screened from traditional Chinese fermented cucumber. Compared with HC diet without LAB, the diet supplemented with *L. acidophilus* L2-16 and *E. faecalis* L2-73 significantly reduced serum TC, TG, and LDL-C level, and liver TC and TG levels of rats. Moreover, the diet supplemented with *L. acidophilus* L2-16 and *E. faecalis* L2-73 significantly increased the fecal elimination of bile acids. Therefore, these two strains maybe have application prospect in the production of some fermented foods such as fermented vegetables, milk, or meat, and probiotic preparations with the function to lower the serum lipid and liver lipid levels.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. Animal studies in this experiment were carried out strictly according to the rules of the Animal Welfare and Research Ethics Committee of Jilin University (Changchun, China), and the permit number was 20090719-1.

Informed consent Informed consent was obtained from all individual participants included in the study.

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