Characterization of *Lactobacillus plantarum* Lp27 isolated from Tibetan kefir grains: A potential probiotic bacterium with cholesterol-lowering effects

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ABSTRACT

Lactobacillus plantarum Lp27 was isolated from Tibetan kefir grains. The Lp27 isolate survived a 3-h incubation at pH 2.0 and grew normally in 0.3% oxgall. In addition, the Lp27 isolate exhibited an adhesion ratio of $9.5 \pm 2.5\%$ with Caco-2 cells. Antibiotic susceptibility tests indicated that the Lp27 isolate was sensitive to gentamicin, tetracycline, erythromycin, and chloramphenicol, and was resistant to vancomycin with a minimum inhibitory value of 23 $\mu\text{g/mL}$. The Lp27 isolate inhibited cholesterol absorption through downregulation of Niemann-Pick C1-like 1 (NPC1L1) expression in Caco-2 cells. The Lp27 isolate was fed to hypercholesterolemic rats at a dose of 10^9 cfu/d for 4 wk. The Lp27 feeding significantly lowered serum total cholesterol, low-density lipoprotein cholesterol, and triglycerides concentrations, but no change was observed in the serum high-density lipoprotein cholesterol concentrations. In addition, liver total cholesterol and triglycerides were decreased in the Lp27-fed group. The expression of NPC1L1 in the duodenum and jejunum was significantly decreased following Lp27 feeding. These results indicate that Lp27 might be an effective cholesterol-lowering probiotic and a possible mechanism for the cholesterol-reducing effects of probiotics.

Key words: *Lactobacillus plantarum* Lp27, Tibetan kefir grains, cholesterol reduction, Niemann-Pick C1-like 1

INTRODUCTION

Hypercholesterolemia and elevated concentrations of dietary cholesterol are both associated with an increased risk of cardiovascular disease. A reduction as small as 1% in serum cholesterol concentrations has been shown to decrease the risk of coronary heart disease in human subjects by 2 to 3% (Manson et al., 1992). Endogenously synthesized cholesterol, the absorption of dietary cholesterol, and the reabsorption of biliary cholesterol

in the small intestine contribute to the regulation of plasma cholesterol concentrations (Wilson and Rudel, 1994). Moreover, reducing the intestinal absorption of dietary and biliary cholesterol can decrease plasma cholesterol concentrations (Temel et al., 2007, 2010). The beneficial effects of lactic acid bacteria (LAB) in the intestinal tract and the potential of particular strains as probiotics have been reported in numerous studies (Franz et al., 2010). Specifically, the potential of probiotic strains to lower the cholesterol level is gaining increased attention (Liong and Shah 2005, 2006; Ooi and Liong, 2010). However, the mechanism of the hypocholesterolemic effects of probiotics has not been fully elucidated.

Niemann-Pick C1-like 1 (NPC1L1) has been identified as a critical protein for intestinal cholesterol absorption; NPC1L1 is highly expressed in the small intestine, especially at the surface of enterocytes (Altmann et al., 2004). Mice deficient in NPC1L1 show dramatic reductions in dietary cholesterol absorption (Altmann et al., 2004) and are completely resistant to both diet-induced hypercholesterolemia and apolipoprotein E deficiency-induced atherosclerosis (Davis et al., 2004, 2007). In rats, the expression level of NPC1L1 along the length of the small intestine has been correlated with the efficiency of cholesterol absorption, with the highest expression found in the proximal intestine (duodenum and jejunum) and the lowest expression found in the distal intestine (ileum; Altmann et al., 2004).

Tibetan kefir grain (TK) is a natural starter for fermented milk used in Tibet, China, which contains LAB, acetic acid bacteria, and yeasts (Farnworth and Mainville, 2003). Tibetan kefir can be considered a probiotic resource because it can provide a variety of health benefits in addition to its nutritional status (Urdaneta et al., 2007). Many studies that have investigated the biological activities of TK identified antimicrobial, immunomodulating, anti-inflammatory, and hypocholesterolemic activities (Diniz et al., 2003; Vinderola et al., 2005; Liu et al., 2006; Silva et al., 2009). Various LAB present in TK have been isolated and include *Lactobacillus acidophilus* (Angulo et al., 1993), *Lactobacillus plantarum* (Garrote et al., 2001), and *Lactobacillus kefirifaciens* (Chen et al., 2012).

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In the present study, we identified and characterized a new strain of *Lactobacillus* from TK and evaluated its probiotic properties, including acid and bile salt tolerance, Caco-2 cell binding, and antibiotic susceptibility properties, in addition to hypocholesterolemic activities. The in vitro uptake of cholesterol and the in vivo effects of the *Lactobacillus* strain on serum cholesterol levels in rats were determined. The mechanisms by which the strain removed cholesterol in vitro and in vivo were also analyzed. Our goal is to develop a new LAB that can serve as a probiotic to lower cholesterol levels in humans.

MATERIALS AND METHODS

TK

The TK was collected from Tibet, China, and was evaluated. The grains were cultured in sterile 10% reconstituted skim milk at 20°C for 20 h. Tibetan kefir grains were then filtered and stored at 4°C.

Isolation of LAB and Cultivation from TK

The TK was recovered from the mother culture after reaching the fermentative end-point. Ten grams of TK was suspended in 90 g of sterile saline buffer (0.85%) and homogenized with a Stomacher (Laboratory Blender Stomacher 400, Seward, UK) for 20 min. Serial dilutions of the suspended samples were used for microbial enumerations and isolation on de Man, Rogosa, Sharpe (MRS) agar (Difco, Detroit, MI) under both aerobic and anaerobic conditions and on LM17 agar [M17 agar (Difco) with 0.5% (wt/wt) lactose (Sigma, St. Louis, MO)] under aerobiosis. In addition, 200 µg/ml of cycloheximide (Sigma) was added to both the MRS and LM17 agars to inhibit the growth of yeasts. The plates were incubated at 30°C for 7 d. After 7 d, yellowish colonies typical of lactobacilli were selected for morphological examination under a microscope. Pour plating was also performed with dilutions of 1:10⁷ and 1:10⁸, and the submerged colonies were selected for morphological examination using Gram staining. The putative *Lactobacillus* isolates were further subjected to catalase testing and molecular typing methods. Standard bacterial cultures, namely *Lb. acidophilus* ATCC 4356 and *Lb. plantarum* ATCC 8014, were obtained from ATCC (Rockville, MD).

Identification of *Lactobacillus* Isolates by PCR

Genomic DNA was extracted from cultures grown at 37°C for 16 to 18 h in MRS broth using the protocol described by Pospiech and Neumann (1995). A PCR

reaction was performed in a total volume of 50 µL that included 0.5 µM of each primer, 1.25 units of Taq DNA polymerase, 20 ng of DNA template, and 200 µM of each deoxynucleotide triphosphates and PCR buffer containing Tris-HCl, KCl, and MgCl₂. The amplification conditions were as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing for 30 s at 55°C, and a final extension of 10 min at 72°C. The PCR-amplified DNA fragments were resolved by agarose gel electrophoresis and stained with ethidium bromide (0.5 mg/mL) followed by visualization under UV light.

Genus-specific PCR assays were performed using the forward primer LbLMA-1 (5'-CTCAAACTA-AAGTTTC-3') and the reverse primer R-161 (5'-CTTGTACACACCGCCCGTTCA-3'; Dubernet et al., 2002). Sequencing of the 16S ribosomal RNA genes was performed to validate the phenotypic characterization of the *Lactobacillus* strains. The 16S ribosomal DNA of the selected strains were amplified by PCR using 2 universal primers: 27f and 1492r (Weisburg et al., 1991). The PCR products obtained were cloned, sequenced, and then compared using the NCBI BLAST sequence database (<http://www.ncbi.nlm.nih.gov/nucleotide/JQ236617.1>) to identify the species of each strain. Sequencing was performed by Bioasia Co. (Shanghai, China).

Acid Tolerance

The MRS broth was used to simulate the acidic conditions of the gut and was adjusted to different pH values (1.0, 2.0, and 3.0) with 1.0 N HCl; broth was adjusted to neutral pH (7.0) to serve as a control. Tubes of broth adjusted to the different pH values were inoculated (at 10⁹ cfu/mL) with cultures of lactobacilli grown overnight and incubated at 37°C for 24 to 48 h. One milliliter of culture was immediately taken from each tube (0 h), and 10-fold serial dilutions were prepared in 0.1% peptone water; pour plating was performed using MRS agar. Similarly, 1 mL of culture was taken from each tube after intervals of 1, 2, 3, and 4 h, followed by plating. The plates were incubated at 37°C for 24 to 48 h and the colony-forming units were counted.

Bile Salt Tolerance

The MRS broth supplemented with 0.2 and 0.3% (wt/vol) oxgall was used to mimic the approximate levels of bile salts in the intestinal tract; MRS broth without bile salt was used as a control. A fresh overnight culture of Lp27 was inoculated at a final concentration of 1% (vol/vol) into each media, incubated at 37°C for 18 h, and monitored for growth hourly by spectrophotometry

at 620 nm. The time required to increase the absorbance at 620 nm by 0.3 units was measured in MRS broth with and without oxgall. The difference in time (h) between the 2 culture media was considered to be the lag time during which the bile salt tolerance effect was expressed.

Caco-2 Cell Adhesion Assay

The adhesion capability of the isolates was assayed according to the method described by Jacobsen et al. (1999). Initially, 1×10^5 Caco-2 cells were seeded into each well of a 6-well tissue culture plate. Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 10% (vol/vol) heat-inactivated (30 min, 56°C) fetal bovine serum, 100 U/mL of penicillin, and 100 mg/mL of streptomycin was used for culturing. The medium was replaced with fresh medium every other day and an adhesion assay was performed after 20 d postconfluency. The cells were then washed twice with 3 mL phosphate-buffered saline (pH 7.4). Two milliliters of DMEM without serum or antibiotics was added to each well and incubated at 37°C for 30 min. Approximately 10^9 cfu/mL of bacterial culture was suspended in 1 mL of DMEM (without serum or antibiotics) and added to the different wells. The plate was incubated at 37°C for 2 h in a 5% CO₂, 95% air atmosphere. The monolayer was washed with sterile PBS and the cells were detached by trypsinization. One milliliter of 0.25% trypsin-EDTA solution (Sigma) was added to each well of a 6-well plate, the plate was then incubated for 15 min at room temperature. The cell suspension was plated onto MRS agar by serial dilution to determine the number of adherent bacterial cells. The plate was incubated for 24 to 48 h at 37°C and the colonies were counted. The number of bacterial cells initially added to each well of the 6-well plates were also counted by serial dilution and plating onto MRS agar. The results of the adhesion assay were expressed as the adhesion percentage (i.e., the ratio between adherent bacteria and added bacteria per well). Caco-2 cells of the same passage were used for 3 independent experiments ($n = 3$) with 2 replicates in each experiment.

Antibiotic Susceptibility Testing

The MIC of gentamicin, tetracycline, erythromycin, chloramphenicol, and vancomycin (Sigma) was determined for Lp27 using the procedure as previously described (Rossetti et al., 2009). According to FEEDAP (2005), MIC breakpoint values for gentamicin, tetracycline, erythromycin, chloramphenicol, and vancomycin were 8, 8, 4, 4, and 4 µg/mL, respectively.

Bacteria, Cell Lines, and Coculture

The reference strains *Lactobacillus plantarum* ATCC 8014 and *Lactobacillus acidophilus* ATCC 4356 were obtained from ATCC. For all experiments, Lp27 was precultured for 6 to 8 h at 37°C on a rotating platform (225 rpm). Precultures were then added to prewarmed MRS medium (dilution 1:20). The cultures were cultivated overnight before use. One OD_{600nm} (optical density at 600 nm) of bacteria culture was equivalent to 1×10^8 Lp27/mL. The required amount of Lp27 was resuspended in an appropriate volume of the respective prewarmed medium without antibiotics. The coculture was prepared by washing Caco-2 cells with warm PBS. The cells were incubated with Lp27 or with medium alone. The experiment was terminated by thoroughly washing the plates with ice-cold PBS.

Cholesterol Uptake Assay

Caco-2 cells were seeded onto 6-well plates at a density of 1×10^5 cells/well were incubated with Lp27, *Lb. plantarum* ATCC 8014 or *Lb. acidophilus* ATCC 4356 for 6 h. For comparison, nontreated Caco-2 cells were also included. One hour before harvesting the cells, 0.15 mL of a micellar solution containing 6.6 mM sodium taurocholate, 74,000 Bq (2 mCi) [³H] cholesterol, 1 mM oleic acid, 0.5 mM monoolein, 0.1 mM unlabeled cholesterol, and 0.6 mM phosphatidylcholine was added to the Caco-2 cells. At the end of the incubation, the unincorporated, radiolabelled cholesterol was removed by washing the cells 4 times with 1.5 mL of cold DMEM. The cellular lipids were extracted with 1.5 mL of hexane:isopropyl alcohol:water (3:2:0.1, vol/vol/vol). The radioactivity in the cellular lipid extract was estimated by counting with a Packard liquid scintillation counter.

Animal Feeding and Grouping

Animal experiments were conducted in compliance with the Guide for Care and Use of Laboratory Animals from the National Institutes of Health (Yin et al., 2010). Twenty male Sprague-Dawley rats (4 wk of age) were obtained from the National Animal Breeding and Research Center, Beijing, China. The rats were fed a commercial chow (Kangqiao Inc., Beijing, China), which included 32% protein, 5% fat, 2% fiber, 1.8% Ca, 1.2% P, and 59% N-free extract for 1 wk. Rats were individually housed in metal cages at a controlled temperature ($23 \pm 2^\circ\text{C}$) and humidity ($55 \pm 6\%$) under a 12-h light-dark cycle. After this adaptation period, rats were divided into 2 groups consisting of 10 rats each; the initial average BW were similar between the

2 groups. Each group was assigned one of the following diet regimens: (1) high-cholesterol diet (HC diet group) or (2) high-cholesterol diet + *Lb. plantarum* Lp27 (HC Lp27 group). The high-cholesterol diet contained 1% (wt/wt) cholesterol, 10% lard, 5% sucrose, 0.3% sodium cholate, 0.2% propylthiouracil, and 78.5% commercial chow (Aoboxing Biotech Co. Ltd., Beijing, China). Each day of the study period, the HC Lp27 group received 2 mL (10^9 cfu/mL) of an *Lb. plantarum* Lp27 solution intragastrically. The HC diet group received an equivalent amount of normal saline. The rats were fed for 4 wk, during which time BW and food intake were recorded daily. After the feeding period, the rats were fasted overnight and used for subsequent testing.

Assay for Serum Lipids

Blood samples were collected from the tail veins of the rats under diethyl ether anesthesia on d 0, 7, 14, 21, and 28. Approximately 1 mL of blood was taken from each rat, transferred to sterile tubes and kept on ice for 30 min. The tubes were then centrifuged at $2,000 \times g$ for 20 min at 4°C. Serum samples were analyzed to determine serum total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and triglyceride (TG) concentrations using a commercial kit (Biosino Biotechnology and Science, Beijing, China).

Assay for Liver TC and TG

After 4 wk on the appropriate diet, all of the rats were euthanized. The livers were removed, rinsed with normal saline solution, blotted dry with filter paper and weighed. Liver TC and TG levels were determined according to the method of Chiu et al. (2006).

Preparation of Samples for RNA Measurements

After the 4-wk feeding period, rats were euthanized with diethyl ether. The small intestines were removed, flushed with ice-cold PBS, and cut into 3 sections of equal length. The sections were slit lengthwise, and the mucosas were gently scraped and frozen in liquid N₂ and stored at -80°C. Total RNA was extracted from the tissue samples using RNA STAT-60 (Tel-Test, Friendswood, TX). Total protein was recovered from the organic phase that remained after RNA isolation by precipitating with isopropanol, washing with 0.3 M of guanidine hydrochloride in 95% ethanol, and resuspending the protein pellet in 1% SDS and 50 mM of Tris-HCl, pH 8.8 (Banerjee et al., 2003). The RNA concentration was determined by absorbance at 260 nm,

and the protein concentration was determined using the Bio-Rad Protein Assay Kit (Hercules, CA).

Real-Time Quantitative PCR

The RNA was prepared using a Qiagen RNeasy Mini Kit (Germantown, MD) according to the manufacturer's protocol, and cDNA was synthesized using the cDNA Synthesis Kit from Invitrogen (Carlsbad, CA), according to the manufacturer's protocol. Transcripts were detected using SYBR green-based (Applied Biosystems, Foster City, CA) real-time PCR. Forward and reverse primers were mixed in equal proportions and used at a final concentration of 0.2 mM. Human NPC1L1 (forward: 5'-TATGGTCGCCCCGAAGCA-3', reverse: 5'-TGCGGTTGTTCTGGAAATACTG-3') and human β -actin (forward: 5'-CCTGGCACCCAGCACAAT-3', reverse: 5'-GCCGATCCACACGGAGTACT-3') transcripts were measured. Additionally, rat NPC1L1 (forward: 5'-AACAGCGAGAGGCTCACATT-3'; reverse: 5'-AGTGGCGTTCATGCCTGCCT-3') and rat β -actin (forward: 5'-ATTGTGATGGACTCCG-GAGA-3'; reverse: 5'-CAGCTCATAGCTCTTCTC-CA-3') transcripts were measured. β -Actin served as a housekeeping gene to normalize the expression of target genes. Relative expression was calculated according to the $2^{-\Delta\Delta C_t}$ method as previously described (Livak and Schmittgen, 2001). Each experiment was performed with duplicate samples, and the mRNA levels of each sample were determined in triplicate. Real-time PCR was performed using the ABI 7500 System for data acquisition, and the data were analyzed using the ABI 7500 System Sequence Detection software (both from Applied Biosystems). The data are presented as mean values with standard deviations.

Statistical Analysis

Data are expressed as means with standard deviations. The statistical significance of the difference between 2 means was evaluated using a Student's *t*-test. For analyzing multiple mean values, Dunnett's test was used after an ANOVA. In these tests, values of $P < 0.05$ were considered to be significant.

RESULTS

Screening and Identification of *Lactobacillus* Isolates

A total of 38 typical *Lactobacillus* isolates recovered from kefir grain samples on MRS plates were initially selected for screening. Typical colonies of these *Lac*-

tobacillus isolates were randomly selected from MRS plates based on their morphological characteristics assessed by microscopic examination after Gram staining and from negative tests for the catalase reaction. For 12 of the isolates, a 250 bp fragment was amplified using 16S rRNA-based PCR primers specific for the genus *Lactobacillus*. Two standard probiotic cultures, *Lb. acidophilus* ATCC 4356 and *Lb. plantarum* ATCC 8014, were used as positive controls (data not shown) in the PCR assays.

Adherence of the putative probiotic organism to the gastrointestinal tract is crucial for the organism to have an extended residence time in the host. Therefore, the 12 isolates were subjected to an in vitro adherence test using the human carcinoma cell line Caco-2, which has been widely accepted as an in vitro model to assess the adhesion of potential probiotics to the intestinal epithelium. On qualitative evaluation, the isolate Lp27 most strongly adhered with Caco-2 cells and was selected for further characterization. The adhesion ratio of the Lp27 isolate to the Caco-2 cells was estimated to be $9.5 \pm 2.5\%$, which was comparable to the adhesion values of 6.8 ± 1.2 and $12.7 \pm 1.9\%$ of *Lb. plantarum* ATCC 8014 and *Lb. acidophilus* ATCC 4356, respectively, with Caco-2 cells. The 16S rDNA gene sequencing identified the Lp27 as an *Lb. plantarum* species.

Acid Tolerance

The 4-h survival curve of the Lp27 isolate at pH values of 7.0, 3.0, 2.0, and 1.0 is shown in Figure 1A. The viability of Lp27 increased when it was incubated at pH 7.0 and 3.0. As no viable count was observed after the fourth hour of incubation at pH 2.0 and after the second hour at pH 1.0, it was concluded that Lp27 could survive at pH 2.0 for 3 h and pH 1.0 for 1 h. At pH 3.0, Lp27 remained viable for more than 4 h.

Bile Salt Tolerance

As shown in Figure 1B, the time required to increase the A₆₂₀ nm reading by 0.3 units was 4.0 h when Lp27 was incubated in MRS broth without oxgall, whereas the times required for this increase were 5.8 and 6.2 h when Lp27 was grown in MRS broth containing 0.2 and 0.3% oxgall, respectively. The lag phase in 0.2 and 0.3% oxgall concentration was 1.8 and 2.2 h, respectively. Furthermore, no significant difference was observed between the lag times for the 2 oxgall concentrations. Maximum absorbance for Lp27 was acquired after 10 h in MRS broth without oxgall, whereas it required 11 and 14 h in MRS broth with 0.2 and 0.3% oxgall, respectively. However, the maximum value for absorbance at 620 nm was nearly the same for all treatments.

Antibiotic Susceptibility

The Lp27 isolate was found to be sensitive to tetracycline, erythromycin, chloramphenicol, and gentamicin, and resistant to vancomycin (MIC of 23 µg/mL), according to the FEEDAP (2005) breakpoint (vancomycin, 4 µg/mL; data not shown).

Lp27 Inhibits NPC1L1 Expression in Caco-2 Cells

The colon carcinoma cell line Caco-2 was used to elucidate the effects of probiotics on NPC1L1 expression in colonocytes. Cells were stimulated for 6 h with Lp27, *Lb. plantarum* ATCC 8014, or *Lb. acidophilus* ATCC 4356 (Figure 2A). The Lp27 isolate significantly downregulated the expression of NPC1L1 mRNA; *Lb. plantarum* ATCC 8014 and *Lb. acidophilus* ATCC 4356

Figure 1. Survival curves of *Lactobacillus plantarum* Lp27 in media with different pH values (A) and growth curves in media with different bile salt concentrations (B). (A) Survival curve at pH 7.0 (control; ■), pH 3.0 (▲), pH 2.0 (●), and pH 1.0 (○). (B) Absorbance at 620 nm in de Man, Rogosa, Sharpe broth without bile salt (control; ■), with 0.2% oxgall (▲), and 0.3% oxgall (○). Error bars represent standard deviations and each data point is the average of 3 repeated measurements from 2 independently replicated experiments (n = 3).

were also able to downregulate NPC1L1 mRNA. Further characterization of NPC1L1 expression revealed that Lp27 downregulated NPC1L1 mRNA in a dose- and time-dependent manner (Figure 2B-C).

Lp27 Decreases Micellar Cholesterol Uptake

To address whether Lp27 interferes with the uptake of a micellar cholesterol solution, Caco-2 cells were incubated with Lp27, *Lb. plantarum* ATCC 8014, or *Lb. acidophilus* ATCC 4356 and were compared with an untreated control. One hour before harvesting the cells, 0.15 mL of a micellar solution was added to the medium, and the amount of cholesterol taken up by the cells was then measured (Figure 3). The uptake of cholesterol by cells incubated with Lp27 was approximately 1.5-fold lower than the uptake of cholesterol by the untreated control. The cells incubated with *Lb. plantarum* ATCC 8014 or *Lb. acidophilus* ATCC 4356 also exhibited decreased uptake of micellar cholesterol compared with the nontreated control.

BW and Food Intake

All rats appeared to be healthy throughout the feeding period. No significant differences were observed in BW gain, total food intake, or food intake efficiency ($P > 0.05$) between the 2 groups of rats (Table 1).

Blood Lipid Analysis

Table 2 shows the effects of dietary cholesterol and Lp27 on TC and TG levels in rats. The TC, LDL-C, and TG concentrations observed in the HC Lp27 group were significantly decreased compared with those observed in the HC diet group. However, the HDL-C concentrations did not show a significant difference between the groups.

Liver Lipid Analysis

Table 3 shows the data for weight and lipid content of the rat livers. Average liver weight was not significantly different between the 2 groups. However, liver TC and TG concentrations were significantly lower in the HC Lp27 group than in the HC diet group ($P < 0.05$).

Lp27 Inhibits NPC1L1 Expression in the Small Intestine

To address the mechanism underlying Lp27-mediated inhibition of cholesterol absorption, mRNA levels of NPC1L1 in the small intestines were assayed (Figure 4). The level of NPC1L1 mRNA varied in different seg-

Figure 2. Probiotics can downregulate Niemann-Pick C1-like 1 (NPC1L1) expression in Caco-2 cells. (A) The expression of NPC1L1, as determined by real-time PCR, in Caco-2 cells cocultured with Lp27, *Lactobacillus plantarum* ATCC 8014 (Lp.8014; 10^7 per mL) and *Lactobacillus acidophilus* ATCC 4356 (La.4356; 10^7 per mL) for 6 h was compared with the untreated (NT) control. The data are presented as the means and standard deviations. An asterisk represents standard error of $P < 0.01$ compared with the NT control (by Dunnett's test for multiple comparisons). (B) Expression analysis of NPC1L1 in Caco-2 cells after 6 h of stimulation with *Lb. plantarum* Lp27 at different concentrations. The data are presented as the means and SD. An asterisk represents a standard error of $P < 0.01$ compared with the NT control (by a Dunnett's test for multiple comparisons). (C) Time course of the effects of *Lb. plantarum* Lp27 (10^7 per mL) on NPC1L1 mRNA expression in Caco-2 cells. The data are presented as the means and SD. An asterisk represents a standard error of $P < 0.01$ compared with the NT control (by Student's t test).

Figure 3. *Lactobacillus plantarum* Lp27 inhibits cholesterol uptake in Caco-2 cells. Caco-2 cells were cocultured with *Lb. plantarum* ATCC 8014 (Lp.8014) and *Lactobacillus acidophilus* ATCC 4356 (La.4356) as well as the untreated (NT) control. One hour before harvesting the cells, the medium was supplemented with 0.15 mL of a micellar solution. At the end of the incubation, the cells were washed thoroughly, and cellular lipids were extracted. The radioactivity in the cellular lipid extract was measured. Values shown are the means and standard deviations of 3 independent experiments. Statistically significant differences versus NT were determined by Dunnett's test (* = $P < 0.05$).

ments of the rat intestine, with the peak expression observed in the proximal jejunum. In the duodenal and jejunal segments, NPC1L1 mRNA levels detected in the HC Lp27 group were significantly lower than those detected in the HC diet group. In contrast, no significant difference was observed in NPC1L1 mRNA levels in the ileal segment.

DISCUSSION

To survive passage through the stomach, probiotic bacteria must be tolerant of acidic environments. Stresses to ingested microorganisms begin in the stomach, which has a pH between 1.5 and 3, and continue in the upper intestine, which contains a 0.03 to 0.3% (wt/vol) concentration of bile salts. For probiotic strains, survival at pH 3 for 2 h and in a bile concentration of 0.3% (wt/vol) for 12 h is considered optimal (Usman and Hosono, 1999). The Lp27 isolate survived at pH 3

Table 1. Body weight gain, total food intake, and food intake efficiency of rats fed a high-cholesterol (HC) diet for 4 wk¹

Item	HC diet ²	HC Lp27 ³
Body weight gain (g)	120.04 ± 2.89 ^a	118.52 ± 3.12 ^a
Total food intake (g)	589.76 ± 21.04 ^a	597.37 ± 17.21 ^a
Food efficiency ⁴ (%)	20.35 ± 0.37 ^a	19.51 ± 0.42 ^a

^aMean values within a row with different superscripted letters differ significantly ($P < 0.05$).

¹The data are shown as the mean ± SD.

²HC diet group = high-cholesterol diet.

³HC Lp27 group = high-cholesterol diet + *Lactobacillus plantarum* Lp27.

⁴Food efficiency (%) = (BW gain/food intake) × 100.

for 2 h and even grew well in 0.3% oxgall; thus, Lp27 exhibited good acid and bile salt tolerance.

One of the required properties of probiotic strains is that they are safe for human consumption. A qualified presumption of safety status has been granted to several *Lactobacillus* species; *Lb. plantarum* is included in the list of taxonomic units within the qualified presumption of safety status, provided that the lack of acquired antibiotic resistance is systematically demonstrated (EFSA, 2007). The Lp27 strain was tested for susceptibility to 5 antibiotics belonging to the clinically most relevant antibiotics. The antibiotic susceptibility tests indicated that Lp27 was only resistant to vancomycin. The results were as expected, as lactobacilli are known to be naturally resistant toward vancomycin and such resistance is usually intrinsic, chromosomally encoded, and not transmissible (Klein et al., 1998, 2000).

High levels of serum cholesterol have been associated with an increased risk of coronary heart disease. The use of probiotic bacteria to reduce serum cholesterol levels has attracted much interest in recent years (Ebringer et al., 2008). The results of the present study indicate that Lp27 was able to reduce cholesterol absorption by inhibiting NPC1L1 gene expression in Caco-2 cells. Our previous in vitro study indicated that *Lb. acidophilus*

Table 2. Serum total cholesterol (TC), low-density lipoprotein-cholesterol (LDL-C), and triglycerides (TG) levels in rats fed experimental diets¹

Item ²	Day 0	Day 7	Day 14	Day 21	Day 28
TC (mg/L)					
HC-diet	986.18 ± 14.5 ^a	996.46 ± 21.3 ^a	1,089.32 ± 31.2 ^a	1,187.36 ± 26.9 ^a	1,269.18 ± 31.53 ^a
HC-Lp27	982.85 ± 19.2 ^a	972.57 ± 15.4 ^a	866.15 ± 24.2 ^b	832.85 ± 19.2 ^b	797.43 ± 30.67 ^b
LDL-C (mg/L)					
HC-diet	278.21 ± 18.20 ^a	325.32 ± 17.26 ^a	493.46 ± 12.48 ^a	586.59 ± 16.63 ^a	687.24 ± 21.32 ^a
HC-Lp27	283.64 ± 15.38 ^a	278.47 ± 13.52 ^a	256.25 ± 16.52 ^b	245.37 ± 15.58 ^b	223.31 ± 18.03 ^b
TG (mg/L)					
HC-diet	421.08 ± 22.14 ^a	445.92 ± 12.89 ^a	561.47 ± 28.27 ^a	675.62 ± 37.43 ^a	734.52 ± 26.13 ^a
HC-Lp27	428.63 ± 12.58 ^a	419.65 ± 25.05 ^a	403.62 ± 21.23 ^b	361.55 ± 21.12 ^b	339.52 ± 24.07 ^b

^{a,b}Mean values within the same column followed by different superscript letters are significantly different ($P < 0.05$).

¹The data are shown as the mean ± SD.

²HC diet group = high-cholesterol diet; HC Lp27 group = high-cholesterol diet + *Lactobacillus plantarum* Lp27.

Table 3. Liver cholesterol and triglycerides (TG) levels in rats fed experimental diets¹

Treatment group	Liver weight (g)	Liver cholesterol (mg/g)	Liver TG (mg/g)
HC-diet ²	9.08 ± 0.18 ^a	12.52 ± 0.23 ^a	36.28 ± 0.27 ^a
HC-Lp27 ³	9.06 ± 0.11 ^a	8.25 ± 0.12 ^b	25.16 ± 0.17 ^b

^{a,b}Mean values within the same column followed by different superscript letters are significantly different ($P < 0.05$).

¹The data are shown as the mean ± SD.

²HC diet group = high-cholesterol diet.

³HC Lp27 group = high-cholesterol diet + *Lactobacillus plantarum* Lp27.

4356 reduces cholesterol absorption by downregulating the expression of NPC1L1 (Huang and Zheng, 2010). Substantial downregulation of NPC1L1 expression in Caco-2 cells by *Lactobacillus rhamnosus* BFE5264 and *Lb. plantarum* has also been reported by Yoon et al. (2013). These results indicate that downregulation of NPC1L1 might be an activity of most *Lactobacillus* strains and may provide a possible molecular mechanism for the modulation of cholesterol concentrations. Moreover, Lp27 reduced the serum TC, LDL-C, and TG concentrations of rats fed a high-cholesterol diet, as reported earlier for other *Lb. plantarum* strains (Nguyen et al., 2007; Fazeli et al., 2010). No significant differences in HDL-C concentrations were observed in our study, which is in agreement with previous results

in rats and human subjects (Abd El-Gawad et al., 2005; Wang et al., 2009). Significant reductions in TC and TG concentrations were also observed in the livers of rats in the Lp27-fed group. These findings demonstrated that the serum TC and TG levels in HC Lp27 group were actually reduced rather than merely being redistributed from the blood to the liver.

Because cholesterol absorption primarily occurs in the duodenum and proximal jejunum, with little absorption by the ileal segment of the intestine (Borgstrom, 1960; Grundy, 1983), we investigated NPC1L1 mRNA expression along the duodenum-ileum axis. The level of NPC1L1 mRNA varied in the different segments of rat intestine, with the peak expression detected in the proximal jejunum; this result is in agreement with a

Figure 4. Real-time PCR of Niemann-Pick C1-like 1 (NPC1L1) mRNA in the small intestines of rats fed *Lactobacillus plantarum* Lp27 compared with NPC1L1 mRNA levels in the small intestines of the control group [high-cholesterol (HC) diet group]. The data are presented as the means and standard deviations. An asterisk represents mean values that differed significantly compared with the control group by Student's *t* test ($P < 0.05$).

study by Altmann et al. (2004). Moreover, NPC1L1 mRNA levels in the duodenal and jejuna segments of rats in the Lp27-fed group were significantly lower than those in the duodenal and jejunal segments of the control group. The present results indicate that Lp27 is able to reduce cholesterol absorption by inhibiting NPC1L1 mRNA transcription in the small intestine, which is in agreement with our previous results (Huang et al., 2010).

CONCLUSIONS

In summary, *Lb. plantarum* Lp27 isolated from TK exhibited efficient cholesterol-reducing ability in vitro and in vivo. The results of this study indicate that the probiotic potential of the Lp27 strain for the control of cholesterol is at least partially mediated by the downregulation of NPC1L1. Furthermore, these results indicate a possible mechanism for the cholesterol-reducing effects of probiotics. In future studies it will be necessary to test more animals, using varying doses of bacteria over long durations, to assess the long-term probiotic potential of Lp27.

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