Functional Probiotic Characterization and In Vivo Cholesterol-Lowering Activity of Lactobacillus helveticus Isolated from Fermented Cow Milk

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We characterized the probiotic properties of Lactobacillus helveticus strains KII13 and KHI1 isolated from fermented cow milk by in vitro and in vivo studies. The strains exhibited tolerance to simulated orogastrointestinal condition, adherence to Caco-2 cells, and antimicrobial activity. Both L. helveticus strains produced bioactive tripeptides, isoleucyl-prolyl-proline and valyl-prolyl-proline, during fermentation of milk. KII13 showed higher in vitro cholesterol-lowering activity (47%) compared with KHI1 (28%) and L. helveticus ATCC 15009 (22%), and hence, it was selected for in vivo study of cholesterol-lowering activity in atherogenic diet-fed hypercholesterolemic mice. For the study, mice were divided into four groups (viz., normal diet control group, atherogenic diet control group (HCD), KII13-atherogenic diet group (HCD-KII13), and Lactobacillus acidophilus ATCC 43121-atherogenic diet group (HCD-L.ac) as positive control). The serum total cholesterol level was significantly decreased by 8.6% and 7.78% in the HCD-KII13 and HCD-L.ac groups (p < 0.05), respectively, compared with the HCD group. Low-density lipoprotein cholesterol levels in both HCD-KII13 and HCD-L.ac groups were decreased by 13% and 11%, respectively, compared with the HCD group (both, p < 0.05). Analysis of cholesterol metabolism-related gene expression in mice liver showed increased expression of LDLR and SREBF2 genes in mice fed with KII13. By comparing all the results, we conclude that L. helveticus KII13 could be used as a potential probiotic strain to produce antihypertensive peptides and reduce serum cholesterol.

Keywords: Probiotics, cholesterol lowering, Lactobacillus helveticus, antihypertensive, bioactive peptide

Introduction

Lactobacillus helveticus is a thermophilic bacterium belonging to the Lactobacillus delbrueckii group, which produces high amounts of lactic acid in milk and is used as a starter culture in the dairy industry [22]. It has been widely used in cheese making owing to its superior proteolytic system, which enhances the flavor, reduces the bitterness of cheese, and also reduces the cheese ripening time [22]. L. helveticus strains are also used as probiotics, which are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host [26]. The reported health benefits of L. helveticus include immunomodulatory activity, antimicrobial activity, pathogen antagonism and prevention of gastrointestinal infections, improvement of gut microbial composition and intestinal health, enhancement of bioavailability of nutrients, cholesterol-lowering activity, and antihypertensive activity [1, 47].

Cholesterol-lowering activity is one of the most important beneficial effects of probiotic bacteria, followed by antihypertensive activity. Hypercholesterolemia and hypertension are the main risk factors for cardiovascular...
disease and considered as the leading cause of death all over the world [25, 33]. The World Health Organization (WHO) predicted that about 23.6 million people will be affected by cardiovascular disease by the year 2030 [1]. Very few studies have demonstrated the cholesterol-lowering activity of L. helveticus strains in vitro [1, 5] and in human subjects with different cholesterol levels [6] and in rabbit [11].

The antihypertensive activity of L. helveticus-fermented sour milk has been extensively studied, which is due to the production of angiotensin-I converting enzyme inhibitory (ACE-I) tripeptides such as isoleucyl-prolyl-proline (IPP) and valyl-prolyl-proline (VPP) from the milk protein casein by the proteolytic activity of L. helveticus strains [34, 52]. The antihypertensive activity of L. helveticus-fermented sour milk has been demonstrated in spontaneously hypertensive rats [53], and the long-term blood pressure-lowering effects were demonstrated in hypertensive human subjects [41].

Other beneficial effects of L. helveticus include improvement of cognitive functioning in healthy older adults [14]. Moreover, it was reported that the ethanol extract of L. helveticus-fermented milk induced a strong decrease of beta-amyloid level by improving amyloid precursor protein processing in Alzheimer’s disease [42]. Although, L. helveticus strains possess several health promoting properties, they are less studied for cholesterol-lowering activity compared with other Lactobacillus sp.

Therefore, in our present work, we evaluated the in vitro probiotic properties of two L. helveticus strains, isolated from traditional fermented cow milk, according to FAO-WHO guidelines [21] and demonstrated the in vivo cholesterol-lowering activity of the strain with most potential, L. helveticus KII13.

Materials and Methods

Strains, Culture Media, and Biochemical Tests

The two L. helveticus strains, KII13 and KHI1, used in this study were isolated from traditional fermented cow milk called thayir (Language: Tamil) or dahi (Language: Hindi). Thayir samples were collected from the Indian state of Tamil Nadu, where thayir is prepared each day by fermenting heat-treated cow milk with a starter culture from the previous day’s thayir. This has been practised for several years, which makes thayir a good source of potential probiotic microbes. The samples were serially diluted and plated on de Man, Rogosa, Sharpe (MRS) agar medium, and the LAB strains were isolated and characterized as reported previously [16]. Furthermore, multiplex PCR analysis of the two strains was performed by amplifying species-specific marker peptidoglycan hydrolase genes Lhv_0190 and Lhv_0191 to differentiate L. helveticus from L. gallinarum, according to a previous report [29]. The carbohydrate fermentation pattern and enzyme activity of the strains were studied using the API 50 CHL kit and API ZYM kit (bioMérieux, USA), respectively. Pathogenic microbes used in this study such as Salmonella gallinarum KCTC 2931, Salmonella choleraesuis KCTC 2932, Escherichia coli K99 KCTC 2617, Bacillus cereus KACC 11240, Shigella boydii KACC 10792, Yersinia enterocolitica subsp. enterocolitica KACC 15520, Listeria monocytogenes KACC 10764, and Staphylococcus aureus KCCM 11335 were obtained from the Korean Collection for Type Cultures (KCTC), Korean Agricultural Culture Collection (KACC), and Korean Culture Center of Microorganisms (KCCM), Republic of Korea. MRS agar and Luria-Bertani (LB) agar media were used for the routine culture of the Lactobacillus strains and pathogens, respectively. The bacterial cultures were stored as 20% glycerol stocks at ~80°C. The probiotic strain L. rhamnosus GG (ATCC 53103) and type strains L. acidophilus ATCC 43121, and L. casei KACC 12413 and L. helveticus KACC 12418 were obtained from the American Type Culture Collection (ATCC) and KACC, respectively, and used as reference strains for the comparison of certain probiotic characteristics.

Determination of Minimum Inhibitory Concentration (MIC) of Antibiotics

The MIC of antibiotics (listed in Table 1) for strains KII13 and KHI1 were determined by the two-fold broth microdilution method [51]. The MIC cut-off values of various antibiotics, given by the European Food Safety Authority (EFSA) for L. helveticus [19], were used to determine the antibiotic susceptibility/resistance profile of the L. helveticus strains used in this study.

In Vitro Orogastrointestinal Transit (OGT) Tolerance Assay

The ability of L. helveticus strains KII13 and KHI1 to tolerate OGT was determined according to a previous report [16].

Table 1. Minimum inhibitory concentration (MIC) of L. helveticus strains for antibiotics.

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>KII13 MIC (mg/L)</th>
<th>KHI1 MIC (mg/L)</th>
<th>Recommended MIC value by EFSA, 2012</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloramphenicol</td>
<td>S (4)</td>
<td>S (4)</td>
<td>4</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>S (0.5)</td>
<td>S (0.5)</td>
<td>1</td>
</tr>
<tr>
<td>Tetracycline hydrochloride</td>
<td>S (0.5)</td>
<td>S (0.5)</td>
<td>4</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>S (2)</td>
<td>S (2)</td>
<td>2</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>S (8)</td>
<td>S (8)</td>
<td>16</td>
</tr>
<tr>
<td>Kanamycin sulfate</td>
<td>S (16)</td>
<td>S (16)</td>
<td>16</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>S (16)</td>
<td>S (16)</td>
<td>16</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>S (0.5)</td>
<td>S (0.5)</td>
<td>1</td>
</tr>
<tr>
<td>Clindamycin hydrochloride</td>
<td>S (0.5)</td>
<td>S (0.5)</td>
<td>1</td>
</tr>
</tbody>
</table>

S, Susceptible.
**Table 2. Antagonistic activity of L. helveticus strains against intestinal and foodborne pathogens.**

<table>
<thead>
<tr>
<th>Pathogens</th>
<th>Zone of inhibition in diameter</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>KII13</td>
</tr>
<tr>
<td><em>Salmonella gallinarum</em> KCTC 2931</td>
<td>+++</td>
</tr>
<tr>
<td><em>Salmonella choleraesuis</em> KCTC 2932</td>
<td>++</td>
</tr>
<tr>
<td><em>Shigella boydii</em> KACC 10792</td>
<td>++</td>
</tr>
<tr>
<td><em>Yersinia enterocolitica</em> ssp. <em>enterocolitica</em> KACC 15320</td>
<td>+++</td>
</tr>
<tr>
<td><em>Listeria monocytogenes</em> KACC 10764</td>
<td>+++</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> KCCM 11335</td>
<td>+++</td>
</tr>
<tr>
<td><em>Escherichia coli</em> K99 KCTC 2617</td>
<td>+</td>
</tr>
<tr>
<td><em>Bacillus cereus</em> KACC 11240</td>
<td>+</td>
</tr>
</tbody>
</table>

+++ (diameter > 20 mm); ++, (diameter 15–20 mm); +, (diameter 11–14 mm).

**Antimicrobial Activity of L. helveticus Strains**

The antimicrobial activity of *L. helveticus* strains KII13 and KHI1 was determined using the various pathogens listed in Table 2. The bacterial spent culture filtrate was concentrated to 10-fold by lyophilization. One hundred microliter of concentrated crude culture filtrate was used for the disk diffusion assay using LB agar plates at 37°C for 12–24 h. The plates were examined for the zone of inhibition of bacterial growth. The entire assay was performed in triplicates to check the reproducibility. Hydrogen peroxide production by strains KII13 and KHI1 was detected by the presence or absence of blue coloration around the bacterial colony on Prussian blue agar medium [40].

**Caco-2 Cell Line and Culture**

The Caco-2 cell line was cultured and maintained according to our previous report [16]. The Caco-2 cell line, obtained from KCTC, was cultured in MEM high glucose medium (Gibco) supplemented with 20% (v/v) inactivated fetal bovine serum and 100 U/ml of penicillin-streptomycin. The culture was incubated at 37°C in a CO₂ incubator supplied with 5% CO₂. For the adhesion assay, the Caco-2 cells were seeded at the concentration of 2 × 10⁵/ml in 12-well cell culture plates and incubated for 21 days to obtain well-differentiated and polarized cells with 100% confluence.

**Adherence Assay**

For the adhesion assay, 1 × 10⁷ CFU/ml of bacterial cells was used and the adhesion assay was performed [49] and the percentage adherence was calculated following a previously reported method [49]. Additionally, the Caco-2 monolayer was washed four times with PBS to remove unattached bacterial cells, fixed with 10% formalin, stained with crystal violet, and observed under an inverted microscope (Olympus IX71; Japan) to examine the adherence of strains KII13 and KHI1 to the Caco-2 cell monolayer. The experiments were repeated three times to check the reproducibility.

**Qualitative Analysis of Bile Salt Hydrolase (BSH) Activity**

The BSH activity of the *L. helveticus* strains was detected by TLC analysis. The BSH reaction mixture was prepared and extracted according to our previous report [16]. For TLC analysis, the TLC plate (silica gel 60, 10 × 20 cm) was spotted with 3 μl of sample and separated using a mobile phase of hexane:methylthylethylketone:glacial acetic acid (56:36:8 (v/v/v)) [13] for 1 h. The plates were dried and sprayed with 10% phosphomolybdic acid. The plates were immediately heated at 80°C for 10 min in a hot air oven for the detection of free and conjugated bile acids. The experiments were repeated three times to check the reproducibility.

**Quantitative Analysis of IPP and VPP Production by L. helveticus Strains**

The *L. helveticus* strains were grown in MRS broth for 18 h and the bacterial cells were washed by centrifugation and resuspended in PBS (pH 7.2). The bacterial suspension (1% (v/v)) was inoculated into 2.5%, 7.5%, or 10% skim milk or 2.5% skim milk+2.5% casein (v/v) and incubated at 37°C for 24 h. After incubation, the fermented skim milk preparations were centrifuged at 13,000 × g for 10 min. The supernatant was collected and applied to a centrifugal ultrafiltration system of 10 kDa molecular weight cutoff (MWCO) (Sartorius, France) and centrifuged at 10,000 × g for 10 min. The flow-through was used for analysis of IPP and VPP content by UPLC-MS/MS.

UPLC-MS/MS analysis was performed using an Acquity UPLC I-class system (Waters, USA) connected to a Xevo TQ-S MS/MS system (Waters, UK). Peptides were separated in an Acquity UPLC BEH C18 RP column (1.7 μm, 2.1 × 100 mm; Waters) using 0.1% formic acid in acetonitrile and 0.1% formic acid in Milli-Q water as the mobile phases with the gradient condition described previously [27]. LC-MS/MS analysis was performed as described previously [27]. Pure standards of the tripeptides IPP and VPP (obtained from Peptron Inc., Korea) were used as calibration standards.
In Vitro Cholesterol Assimilation Activity

The cholesterol-lowering activity of *L. helveticus* strains was studied in MRS broth supplied with 100 μg/ml cholesterol (MRS-CHO broth). The MRS-CHO broth was inoculated with a 2% (v/v) bacterial culture of OD 0.6 at A600 nm and incubated at 37°C for 24 h. Then, the suspension was centrifuged at 3,000 xg for 10 min at 4°C and the cholesterol concentration in the spent medium was extracted and estimated by a previously described method [28]. The experiments were repeated three times to check the reproducibility.

Animal and Feeding Type

Male ICR (Institute of Cancer Research) mice (3 weeks old, 30–45 g) were purchased from Oriental Bio (Korea). The mice were housed with a 12-h light/dark cycle under constant temperature (23 ± 3°C) and humidity (40 ± 6%). The mice were fed an atherogenic high cholesterol diet (HCD; 40 kcal % fat, 1.25% cholesterol, and 0.5% cholic acid; Cat #101556; Research Diets, USA) for 4 weeks to induce hypercholesterolemia. After 4 weeks, the HCD-fed mice were assigned to one of the following three experimental groups for 7 weeks: HCD-control (Con); HCD administrated with 1 ml of 3 × 10^8 CFU/ml *L. helveticus* KII13 (HCD-KII13); or HCD administrated with 1 ml of 3 × 10^8 CFU/ml *L. acidophilus* ATCC 43121 (HCD-Lac). One group was continued on a normal diet (NCD). The HCD-KII13 and HCD-Lac groups were orally administered with KII13 and Lac cells, respectively, in distilled water once daily for 7 weeks. Only distilled water was administered to the control groups. The body weight of mice was measured once weekly for 7 weeks. The food intake of each mouse (g/mouse/day) was calculated the day after feeding by subtracting the weight of food remained from the previous day’s feeding from the total weight of food given for the feeding, and divided by the number of mice in the cage. The ethical committee of the Myongji Bioefficiency Research Centre at Myongji University (Yongin, Korea) approved the experimental design and animal study (MJU-2016-03).

Tissue Collection and Serum Lipid Analysis

The mice were sacrificed at the end of the treatment period (7 weeks) by cervical dislocation. The organs, such as liver, and epididymal white adipose tissue were immediately excised, rinsed, and weighed. The blood samples were collected from the abdominal aorta after making a longitudinal incision up the abdomen to the xiphoid. The serum was collected by centrifugation of blood at 1,800 xg for 10 min at 4°C and stored at −80°C until analysis. Serum total cholesterol (T-CHO), triglycerides (TG), and high-density lipoprotein (HDL) cholesterol, were determined using commercial assay kits (Asan Pharm, Korea). Low-density lipoprotein (LDL) cholesterol was calculated according to the Friedewald formula [23].

Expression Analysis of Cholesterol Metabolism-Related Genes in Mice Liver

Mice liver total RNA was extracted using the RNeasy mini kit (Qiagen Korea, Korea) according to the manufacturer’s protocol. cDNA was synthesized from the total RNA (1 μg) using the PrimeScript II-1st strand cDNA synthesis kit (Takara, Japan). The expression levels of cholesterol metabolism-related genes were evaluated using SYBR Premix Ex Taq II (Tli RNase H Plus) (Takara Korea Biomedical Inc., Korea) in a Roche LightCycler 96 System (Roche Life Science, USA). The reaction mixture was incubated for an initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 5 sec, 60°C for 45 sec, and 72°C for 30 sec. Primers for mouse LDLR were F: 5’-GCC TAT CTG TCG ACA ACA CC-3’ and R: 5’-TGT CCA CAC CAT TCA AAC CC-3’, mouse SREBF2 were F: 5’-GCC TCC TTT TTT AAC CCC TT-3’ and R: 5’-CAC CAT TTA CCA GCC ACA GG-3’, mouse HMGCR were F: 5’-GTC GCA GAA AGA GGG AAA GG-3’ and R: 5’-CCG CCT TGT TTG CGT GTT GA-3’ [2], and mouse β-actin were F: 5’-TGT CCA CCT TCC AGA AGT GT-3’ and R: 5’-AGC TCA GTA ACA GTC CCG CTA GA-3’ [50]. Relative gene expression levels were calculated as previously described [37] using β-actin gene as the internal control and were expressed as the fold-change relative to the HCD-control group.

Statistical Analysis

Data are expressed as the mean ± standard deviation of three separate experiments, n = 3. The results of animal experiments were analyzed by one-way ANOVA (differences among treatment groups) followed by Duncan’s test, using Origin 7 software (MicroCal Software, USA). Values of p < 0.05 were considered to be significant.

Results

Strain Identification and Biochemical Test

The 16S rRNA gene sequencing of strains KII13 and KHI1 resulted in a 1,467 bp sequence for both strains. BLAST search of the EzTaxon database showed that the 16S rRNA gene sequence of both KII13 and KHI1 showed 99.52% similarity (difference of 7/1,467 bp) with *L. gallinarum*, followed by 99.39% similarity with *L. helveticus* (difference of 9/1,467 bp) (Fig. 1A). The cutoff value for species level identification is 98.5% and the 16S rRNA gene sequence similarity of KII13 and KHI1 with all other *Lactobacillus* strains was less than this cutoff value (Fig. 1A). Furthermore, to identify the strains to the species level, peptidoglycan hydrolase genes Lhv_0190 and Lhv_0191 were amplified using two sets of primers by multiplex PCR to differentiate the strains between *L. helveticus* and *L. gallinarum*. Amplification of the Lhv_0190 and Lhv_0191 genes from the genomic DNA of the two strains resulted in 542 and 747 bp amplicons, respectively, which corresponds to *L. helveticus* (Fig. 1B) according to Jebava et al. [29]. However, for *L. gallinarum*, 542 bp and an amplicon >1,500 bp were produced (Fig. 1B),
consistent with Jebava et al. [29]. The strains showed significant difference in carbohydrate fermentation pattern (Table S1) and enzyme activities (Table S2) compared with the type strains \textit{L. helveticus} KACC 12418 and \textit{L. gallinarum} KACC 12370.

**Antibiotic Susceptibility of the \textit{L. helveticus} Strains**

The \textit{L. helveticus} strains were tested for antibiotic susceptibility according to the cutoff value recommended by EFSA (2012). Both the \textit{L. helveticus} strains were found to be susceptible to all antibiotics tested (Table 1).

**Orogastrintestinal Transit Tolerance of the \textit{L. helveticus} Strains**

We studied the ability of \textit{L. helveticus} strains KII13 and KHI1 to tolerate simulated OGT condition in vitro (Fig. 2). Both \textit{L. helveticus} strains KII13 and KHI1 showed resistance to the lysozyme concentration of simulated oral condition (Fig. 2). The viability of KII13 and KHI1 was $8.20 \pm 0.23$ and $8.23 \pm 0.13$ Log CFU/ml, respectively, after oral stress compared with the initial number of cells subjected to the stress ($8.35 \pm 0.2$ and $8.39 \pm 0.11$ Log CFU/ml, respectively) (Fig. 2). The viability of KII13 and KHI1 was significantly reduced after exposure to gastric stress ($7.87 \pm 0.13$ and $7.20 \pm 0.16$ Log CFU/ml, respectively). However, intestinal stress did not significantly affect the viability of both strains KII13 and KHI1 ($7.43 \pm 0.14$ and $7.11 \pm 0.12$ Log CFU/ml, respectively) (Fig. 2). We observed an overall log reduction of $0.92 \pm 0.14$ and $1.28 \pm 0.12$ for strains KII13 and KHI1, respectively, at the end of the OGT assay.

**Antimicrobial Activity of the \textit{L. helveticus} Strains**

The \textit{L. helveticus} strains inhibited the growth of both gram-positive and gram-negative pathogens tested (Table 2).

![Fig. 1.](image1.png)

Fig. 1. Identification of strains KII13 and KHI1 isolated from fermented cow milk.

(A) Phylogenetic tree constructed based on 16S rRNA gene sequence of strains KII13 and KHI1. The strains showed 99% similarity with \textit{Lactobacillus helveticus} DSM 20075. The GenBank accession number is indicated in parenthesis. (B) Amplification of peptidoglycan hydrolase genes Lhv_0190 and Lhv_0191 by multiplex PCR to differentiate \textit{L. helveticus} and \textit{L. gallinarum}. \textit{L. helveticus}: Amplicons 542 bp and 747 bp correspond to Lhv_0190 and Lhv_0191, respectively. \textit{L. gallinarum}: Amplicons 542 bp and ~1,800 bp correspond to Lhv_0190 and Lhv_0191, respectively. 1, KII13; 2, KHI1; 3, \textit{L. helveticus} KACC 12418; 4, \textit{L. gallinarum} KACC 12370. M, GeneRuler DNA ladder mix 100–10,000 bp (Fermentas).

![Fig. 2.](image2.png)

Fig. 2. Determination of the viability of \textit{L. helveticus} strains subjected to simulated OGT condition. Error bars indicate the standard deviation of three independent experiments ($n = 3$).
The spent culture filtrate of KII13 and KHI1 inhibited *Y. enterocolitica* subsp. *enterocolitica*, *L. monocytogenes*, *S. aureus*, and *S. gallinarum* more significantly compared with *S. choleraesuis*, *S. boydii*, *E. coli* K99, and *B. cereus* (Table 2).

We observed the production of hydrogen peroxide by the *L. helveticus* strains in Prussian blue agar (Fig. S1).

**Adherence to Caco-2 Cell Line**

The *L. helveticus* strains KII13 and KHI1 showed an adherence capacity of 35% and 20%, respectively (Fig. 3), which is higher compared with *L. rhamnosus* GG (17%) (Fig. 3).

**Quantitative Analysis of IPP and VPP Production by *L. helveticus* Strains**

The production of IPP and VPP by the *L. helveticus* strains was significantly higher in the 2.5% skim milk + 2.5% casein combination compared with 2.5%, 7.5%, and 10% skim milk alone (Fig. 4). In the 10% skim milk, IPP and VPP production by KII13, KHI1, and *L. helveticus* ATCC 15009 was quite higher (16.50, 14.30, and 7.43 mg/l of IPP and 3.85, 7.29, and 2.19 mg/l of VPP, respectively) than 2.5% and 7.5% skim milk (Fig. 4). The IPP production of KII13, KHI1, and *L. helveticus* ATCC 15009 in the 2.5% skim milk + 2.5% casein combination was significantly higher (45.77, 23.80, and 20.42 mg/l, respectively), whereas the VPP production of KII13, KHI1, and *L. helveticus* ATCC 15009 in the same combination was significantly higher than the various concentrations of skim milk alone except KHI1, which produced significantly higher concentrations of VPP in 10% skim milk (7.29 mg/l) (Fig. 4).

**BSH Activity and in vitro Cholesterol-Lowering Activity**

The strains KII13 and KHI1 did not deconjugate any of the conjugated bile acids tested in this study, such as taurocholic acid, glycocholic acid, and taurodeoxycholic acid, similar to that of *L. helveticus* ATCC 15009 (Fig. S2), which showed that *L. helveticus* strains are BSH negative.

The strains KII13, KHI1, and *L. helveticus* ATCC 15009 assimilated cholesterol in vitro. KII13 assimilated a higher amount of cholesterol (about 48%) compared with KHI1 and *L. helveticus* ATCC 15009, which assimilated 28% and 22%, respectively, in cholesterol-supplied MRS medium after 24 h (Fig. 5A). Hence, *L. helveticus* KII13 was used for in vivo study of its cholesterol-lowering activity.

**Fig. 3.** Adherence of the *L. helveticus* strains and *L. rhamnosus* GG to human intestinal epithelial like-Caco-2 cells. Error bar indicates ± SD of three independent experiments, *n* = 3.

**Fig. 4.** Production of IPP and VPP by strains KII13 and KHI1 and *L. helveticus* ATCC 15009. The *L. helveticus* strains were used for fermentation of various concentrations of skim milk and skim milk + casein. The concentrations of IPP and VPP in the fermented skim milk and skim milk + casein after 24 h were quantified using LC-MS/Ms.
In Vivo Cholesterol-Lowering Activity of *L. helveticus* KII13

Effects of KII13 feeding on body weight gain, food intake, and organ weight of atherogenic diet-fed ICR mice. The body weight gain, food intake, and organ weight of control and treated groups are shown in Table 3. We observed no significant difference in the body weight gain and food intake of all groups (Table 3). We found the liver weight and epididymal white adipose tissue weight of the NCD group were significantly lower than the HCD-control and HCD-LAB (HCD-KII13 and HCD-*L. ac*-*bacillus*) groups. However, there was no significant difference between the HCD-

![Graph](image-url)

**Fig. 5.** Cholesterol-lowering activity of the *L. helveticus* strains.  
(A) In vitro cholesterol-lowering activity of *L. helveticus* strains KII13, KHI1, and ATCC 15009 in MRS-CHO broth containing 100 μg/ml cholesterol. Error bar indicates ± SD of three independent experiments, *n* = 3. (B) Effect of *L. helveticus* strain KII13 on the plasma biochemical parameters total cholesterol (a), LDL-cholesterol (b), HDL-cholesterol (c), and triglyceride (d) of ICR mice after 7 weeks. NCD, Normal control diet-fed mice; HCD, atherogenic high cholesterol diet-fed mice; KII13, HCD mice administered 3 × 10^8^ CFU/ml of strain KII13; *L. ac*-*bacillus*, HCD mice administered with 3 × 10^8^ CFU/ml of *L. acidophilus* ATCC 43121. *p* < 0.05 vs. HCD group.
control and HCD-LAB groups (Table 3).

**Effects of KII13 feeding on the serum biochemical levels of atherogenic diet-fed ICR mice.** We observed that the T-CHO and LDL-CHO levels of the NCD group were significantly lower compared with the HCD-control group \((p < 0.05)\) and HCD-LAB group (Fig. 5B: a, b). T-CHO levels were significantly decreased by 8.6\% in the HCD-KII13 group and 7.78\% in the HCD-Lac group \((p < 0.05)\) compared with the HCD group (Fig. 5B: a). LDL-CHO levels were decreased by 13\% and 11\% in the HCD-KII13 and HCD-Lac groups, respectively, compared with the HCD-control group (both, \(p < 0.05\)) (Fig. 5B: b). We found no significant difference in HDL-CHO and TG level gain among all the groups (Fig. 5B: c, d).

**Effects of KII13 feeding on expression levels of genes associated with cholesterol metabolism in mice liver.** The LDLR, SREBF2, and HMGCR gene expression levels in mice liver were significantly increased in the NCD-group compared with those of the HCD group (Fig. 6). The LDLR expression level in mice liver was significantly increased in the HCD-KII13 \((p < 0.05)\) and in the HCD-Lac groups \((p < 0.005)\) compared with the HCD group (Fig. 6A). Expression levels of SREBF2 in mice liver increased significantly in the HCD-KII13 and HCD-Lac groups \((p < 0.005)\) compared with the HCD group (Fig. 6B). Meanwhile, no significant difference was observed in HMGCR expression in the HCD-KII13 group but there was a significant increase in the HCD-Lac \((p < 0.05)\) group compared with the HCD group (Fig. 6C).

**Discussion**

*L. helveticus* strains are homofermentative and are reported to be more frequently isolated from fermented dairy foods [47]. The presence of any potentially transferable antibiotic resistance genes needs to be analyzed prior to any other probiotic characterization of a newly isolated strain [3]. We did not observe resistance of *L. helveticus* strain KII13 and

**Table 3.** Effects of KII13 and *L. acidophilus* on the body weight, food intake, and tissues weight of ICR mice.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>NCD</th>
<th>HCD</th>
<th>HCD + KII13</th>
<th>HCD + L. ac</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial body weight (g)</td>
<td>40.17 ± 0.48</td>
<td>39.72 ± 2.57</td>
<td>40.29 ± 1.72</td>
<td>41.12 ± 2.21</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>41.79 ± 0.91</td>
<td>42.65 ± 1.16</td>
<td>41.52 ± 1.10</td>
<td>42.37 ± 1.63</td>
</tr>
<tr>
<td>Gain in body weight (g)</td>
<td>1.62 ± 0.43</td>
<td>2.93 ± 1.41</td>
<td>1.23 ± 0.62</td>
<td>1.25 ± 0.58</td>
</tr>
<tr>
<td>Food intake rate (g/mouse/day)</td>
<td>5.79 ± 1.34</td>
<td>4.57 ± 0.58</td>
<td>4.97 ± 0.67</td>
<td>4.78 ± 1.23</td>
</tr>
<tr>
<td>Liver (g)</td>
<td>2.20 ± 0.52***</td>
<td>2.80 ± 0.31</td>
<td>2.43 ± 0.12</td>
<td>2.47 ± 0.08</td>
</tr>
<tr>
<td>Epididymal fat (g)</td>
<td>0.28 ± 0.07***</td>
<td>0.42 ± 0.04</td>
<td>0.44 ± 0.03</td>
<td>0.41 ± 0.04</td>
</tr>
</tbody>
</table>

NCD: Normal diet; HCD: atherogenic high-cholesterol diet; KII13: *L. helveticus* KII13; Lac: *L. acidophilus*.

Data are expressed as means ± SEM \((n = 7–8/group)\). ***\(p < 0.005\) vs. the HCD group.

**Fig. 6.** Effects of KII13 and *L. acidophilus* ATCC 43121 on expression levels of cholesterol metabolism-related genes (A) LDLR (LDL receptor), (B) SREBF2 (sterol regulatory elements binding protein-2), and (C) HMGCR (HMG-CoA reductase) in mice liver. Data were normalized to \(\beta\)-actin mRNA expression levels and then compared with the HCD group. * and ** indicates significant difference at \(p < 0.05\) and 0.005, respectively, vs. HCD group.
KHI1 towards any of the tested antibiotics. The \textit{L. helveticus} are included in “Qualified Presumption of Safety” status by EFSA [20], due to lack of observation of transferable antibiotic resistance genes. Hence, \textit{L. helveticus} strains K113 and KHI1 isolated from fermented cow milk were subjected to probiotic characterization. Survival of candidate LAB strains in digestive enzymes, pH of gastric juice, and bile concentration of intestinal juice, and adherence to the intestinal epithelium are desired characteristics for the selection of potential probiotic strains [21]. The viability of strains K113 and KHI1 was slightly affected by the oral lysozyme, pepsin, and acidic pH of the gastric conditions, respectively, but they survived well in pancreatin and bile concentration of intestinal condition. However, their viability after OG1 condition was higher when compared with \textit{L. helveticus} BGRA43 [43]. A 5 log order decrease in cell viability of \textit{L. helveticus} BGRA43 without food additives after 90 min exposure to gastric juice followed by a further decrease to >1 log viable cells after 10 min exposure to 0.6% bile concentration was reported [43].

\textit{L. helveticus} strains were reported to possess antagonistic activity against various pathogens belonging to the family Enterobacteriaceae [47]. Both the strains K113 and KHI1 significantly inhibited the enteric pathogens. Similarly, Strahinic et al. [43] reported that the culture filtrate of \textit{L. helveticus} significantly inhibited the growth of enteropathogenic strains such as \textit{Yersinia enterocolitica}, \textit{Shigella sonnei}, and \textit{Shigella flexneri}. The antagonistic activity of lactic acid bacterial culture filtrate could be due to the production of bacteriocin-like molecules, hydrogen peroxide, and organic acids such as lactic acid [32, 36]. \textit{L. helveticus} strains were also reported to inhibit various human pathogens. Atassi et al. [7] reported that the vaginosis-associated pathogens such as \textit{Gardnerella vaginalis} and \textit{Prevotella bivia}, uropathogenic \textit{Escherichia coli}, and diarrhoeagenic \textit{Salmonella enterica} serovar. Typhimurium were significantly inhibited by co-culture with the \textit{L. helveticus} strains.

The efficiency of adherence of a probiotic strain to the intestinal epithelial cell-like Caco-2 cell line will determine its colonization ability and proliferation in the intestinal tract [4]. \textit{Lactobacillus} sp. strains of dairy origin are reported to possess less adherent property as compared with \textit{Lactobacillus} sp. of human origin [12, 18]. The adherence property of \textit{Lactobacillus} sp. differs among the strains and it depends on the interaction of bacterial surface molecules to the intestinal epithelial receptors [10].

IPP and VPP are two well-studied ACE-I inhibitory tripeptides derived from bovine milk casein by proteolytic activity of \textit{L. helveticus}, which are reported to reduce blood pressure in spontaneously hypertensive rats and hypertensive human subjects [35, 39]. In addition to these tripeptides, other peptides from fermented milk, such as Tyr-Pro and Lys-Val-Leu-Pro-Val-Pro-Gln, were also shown to possess ACE-inhibitory activity [38, 54]. \textit{L. helveticus} DSM13137 produced IPP and VPP of 1.62 and 1.55 mg/dl after 24 h and 2.27 and 2.18 mg/dl after 48 h in fermented milk [24]. In the present study, \textit{L. helveticus} K113 and KHI1 produced significantly higher IPP in 2.5% skim milk and 2.5% casein combination after 24 h. However, the strains produced significantly lower amount of VPP in the same combination.

We observed that strains K113 and KHI1 did not show bile salt hydrolase activity. \textit{L. helveticus} strains of dairy and vegetable origin did not possess BSH activity [45]. However, BSH activity was observed in \textit{L. helveticus} strains of human fecal origin [30]. It has been reported that the bsh gene could be transferred between commensal bacteria in the intestine via horizontal gene transfer [8, 31]. Therefore, the absence of BSH activity in \textit{L. helveticus} strains K113 and KHI1 isolated from fermented milk concurs with previous studies.

Ahire et al. [1] reported that the strain \textit{L. helveticus} CD6 assimilated 97% cholesterol after 24 h and showed 100% assimilation after 48 h in MRS-cholesterol (3 mM)-supplied medium, which was significantly higher than that of the cholesterol assimilation activity of K113 and KHI1 strains used in our study. Furthermore, in the in vivo study using strain K113 in comparison with \textit{L. acidophilus} ATCC 43121, we did not observe any significant difference in food intake and body weight gain among control and treatment groups. Similarly, in a previous report, Wistar rats fed a high-fat diet and administered \textit{L. paracasei} NCC2461 for 11 weeks resulted in no significant difference in food consumption [46]. However, the authors observed a decrease in body weight gain and abdominal fat of the treatment group [46].

The \textit{L. helveticus} strain K113 isolated from fermented cow milk significantly reduced serum cholesterol and LDL levels in atherogenic diet-induced hypercholesterolemic ICR mice. Similarly, CS7/BL6 mice fed with a high-fat diet and administered with \textit{L. plantarum} 14 intragastrically were found to have lower adipose tissue weight, serum cholesterol, and leptin, and displayed no change in body weight gain and serum CLA concentration [44]. Furthermore, rabbits fed with \textit{L. helveticus} 416 in combination with \textit{Enterococcus faecium} CRL 183 in an aqueous soy extract for 60 days showed a reduction of total cholesterol, non-HDL cholesterol, and auto-antibodies against oxidized LDL with a significant increase in HDL cholesterol [11]. They also observed a

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reduced level of atherosclerotic lesion areas in aortic segments of the rabbits.

The hypocholesterolemic effect of *Lactobacillus* is due to various mechanisms, including bile salt hydrolase activity; however, *L. helveticus* KII13 did not possess bile salt hydrolase activity. Therefore, the reduction in serum LDL and total cholesterol level observed in our study could be due to other mechanisms, such as production of short-chain fatty acids, propionate and butyrate [48]. Increased expression of LDLR and SREBF2 genes in mice liver fed a high-fat diet plus KII13 indicates depletion of liver cholesterol [9]. Increased expression of LDLR leads to absorption of LDL by the liver from the circulating blood [15], which is the reason for the observed decrease in serum LDL and total cholesterol in this study. Production of butyric acid by gut microbiota was shown to inhibit synthesis of liver cholesterol, whereas production of propionate will eventually results in a reduction in the rate of cholesterol synthesis and reduction in plasma cholesterol levels [11]. Hepatic bile acid synthesis due to positive modulation of gut microbiota by probiotics has also been reported previously [17]. We hypothesize that the observed cholesterol-lowering activity of KII13 could be due to modulation of gut microbiota that results in production of sufficient butyrate.

In conclusion, the results of this study suggest that *L. helveticus* strain KII13 is a promising probiotic strain with in vivo cholesterol-lowering activity and production of antihypertensive tripeptides (IPP and VPP) from milk casein, which could be used to develop functional foods for hypertension and hypercholesterolemia after appropriate clinical trials.

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