Preliminary probiotic and technological characterization of *Pediococcus pentosaceus* strain KID7 and *in vivo* assessment of its cholesterol-lowering activity

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The study was aimed to characterize the probiotic properties of a *Pediococcus pentosaceus* strain, KID7, by *in vitro* and *in vivo* studies. The strain possessed tolerance to oro-gastrointestinal transit, adherence to the Caco-2 cell line, and antimicrobial activity. KID7 exhibited bile salt hydrolase activity and cholesterol-lowering activity, *in vitro*. *In vivo* cholesterol-lowering activity of KID7 was studied using atherogenic diet-fed hypercholesterolemic mice. The experimental animals (C57BL/6J mice) were divided into 4 groups viz., normal diet-fed group (NCD), atherogenic diet-fed group (HCD), atherogenic diet- and KID7-fed group (HCD-KID7), and atherogenic diet- and *Lactobacillus acidophilus* ATCC 43121-fed group (HCD-*L.ac*) as positive control. Serum total cholesterol (T-CHO) level was significantly decreased by 19.8% in the HCD-KID7 group (*P* < 0.05), but not in the HCD-*L.ac* group compared with the HCD group. LDL cholesterol levels in both HCD-KID7 and HCD-*L.ac* groups were decreased by 35.5 and 38.7%, respectively, compared with HCD group (both, *P* < 0.05). Glutamyl pyruvic transaminase (GPT) level was significantly lower in the HCD-KID7 and HCD-*L.ac* groups compared to HCD group and was equivalent to that of the NCD group. Liver T-CHO levels in the HCD-KID7 group were reduced significantly compared with the HCD group (*P* < 0.05) but not in the HCD-*L.ac* group. LDL cholesterol levels in both HCD-KID7 and HCD-*L.ac* groups were decreased by 35.5 and 38.7%, respectively, compared with HCD group (both, *P* < 0.05). Glutamyl pyruvic transaminase (GPT) level was significantly lower in the HCD-KID7 and HCD-*L.ac* groups compared to HCD group and was equivalent to that of the NCD group. Liver T-CHO levels in the HCD-KID7 group were reduced significantly compared with the HCD group (*P* < 0.05) but not in the HCD-*L.ac* group. Analysis of expression of genes associated with lipid metabolism in liver showed that low-density lipoprotein receptor (*LDLR*), cholesterol-7α-hydroxylase (*CYP7A1*) and apolipoprotein E (*APOE*) mRNA expression was significantly increase in the HCD-KID7 group compared to the HCD group. Furthermore, KID7 exhibited desired viability under freeze-drying and subsequent storage conditions with a combination of skim milk and galactomannan. *P. pentosaceus* KID7 could be a potential probiotic strain, which can be used to develop cholesterol-lowering functional food after appropriate human clinical trials.

Keywords: *Pediococcus*, probiotic, oro-gastrointestinal transit, cholesterol-lowering, bile salt hydrolase, LDL-receptor, cholesterol 7α-hydroxylase, storage viability
Introduction

High blood cholesterol (hypercholesterolemia) is a risk factor for cardiovascular diseases, which remains one of the largest causes of death worldwide (Ishimwe et al., 2015). It has been shown that a 1% reduction in serum cholesterol is associated with an estimated reduction of 2–3% in the risk of coronary heart disease (CHD) (Ishimwe et al., 2015). Statin drugs are predominantly prescribed for the reduction of serum cholesterol level and in turn the risk of CHD. There is an increasing interest in non-drug therapies for lowering serum cholesterol and the risk of CHD due to several adverse side effects of statin drugs reported in the literature and by patients (Sultan and Hynes, 2013). An alternative is probiotics with cholesterol-lowering activity. Probiotics are defined as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” (Hill et al., 2014). Recently, several studies have shown that probiotics could be used as alternative supplements to exert cholesterol-lowering effects in humans (Trautvetter et al., 2012; Fuentes et al., 2013; Guardamagna et al., 2014). Probiotics have been suggested to reduce cholesterol via various mechanisms (Ishimwe et al., 2015). Available literature suggests that probiotics with bile salt hydrolase (BSH) activity show cholesterol-lowering activity in vivo (Kumar et al., 2011; Jones et al., 2012, 2014; Pavlovic et al., 2012; Degirolamo et al., 2014). Other mechanisms such as cholesterol adsorption to cell surface, cholesterol assimilation into bacterial cell membrane (Liong and Shah, 2005a) and co-precipitation with deconjugated bile acids (Liong and Shah, 2005b) are also proposed/demonstrated in vitro without evidence of their occurrence in vivo.

Several species and strains belonging to the orders Lactobacillales and Bifidobacteriales are widely recognized, approved and used as probiotics with some yeast and Bacillus sp. as well. The beneficial effects of probiotics include but are not limited to gastro-intestinal microbial balance, suppression of pathogens, immunomodulatory activity, hypcholesterolemic activity, and alleviation of certain conditions such as diarrhea, allergy, lactose intolerance, irritable bowel syndrome, inflammatory bowel disease (IBD), and colon cancer (reviewed in Nagpal et al., 2012). However, a single probiotic microbe cannot provide all of these beneficial effects and efficacy and the activity of probiotic strains vary considerably. For example, certain probiotic strains show efficacy against antibiotic-associated diarrhea but there is less evidence for their efficacy against IBD.

Since, the probiotic property of microbes differs from one strain to another, new strains must be assessed for their putative probiotic properties according to FAO/WHO guidelines (FAO-WHO, 2002). The FAO/WHO guideline recommends certain testing methods to establish the health benefits of a microbe to be called probiotic. The testing methods include in vitro and in vivo study of oro-gastrointestinal transit tolerance, production of antimicrobial substances, beneficial probiotic characters such as cholesterol-lowering activity, anti-hypertensive activity, anti-diabetic activity etc., and adherence to human intestinal cells, before testing the microbe in human clinical trials (FAO-WHO, 2002). The guidelines also insist on the characterization of a putative probiotic microbe for its safety that the strain should not possess any transferrable antibiotic resistance (FAO-WHO, 2002). Additionally, any probiotic microbe should maintain its viability and probiotic activity during industrial manufacturing practices such as drying, and storage in various products.

The objective of this study was to establish the various probiotic properties and cholesterol-lowering activity of a Pediococcus pentosaceus strain KID7, through in vitro and in vivo studies and technological characterization of strain KID7 for its ability to maintain desired viability during manufacturing and storage condition.

Materials and Methods

Microorganisms and Culture Conditions
Strain KID7 was isolated from fermented finger millet (Eleusine coracana) gruel obtained from a household in Yongin, Korea. The gruel was serial-10-fold diluted and 10−4 to 10−5 dilutions were plated on de Man Rogosa and Sharpe (MRS) agar medium and incubated at 37°C for 24 h to pick as single colony of strain KID7. The probiotic strains Lactobacillus rhamnosus GG and Lactobacillus acidophilus ATCC 43121 were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and the type strain Pediococcus pentosaceus KACC 12311 was obtained from the Korean Agricultural Culture Collection (KACC), Republic of Korea. The strains were cultured in MRS agar medium and incubated at 37°C for 24 h before being used for experiments. The strains were stored as glycerol stocks (20% glycerol in MRS broth) at −80°C. The probiotic strains and type strain P. pentosaceus KACC 12311 were used as reference strains for comparison of probiotic and biochemical characteristics, respectively.

Pathogenic microbes used in this study were obtained from the Korean Collection for Type Cultures (KCTC), KACC and Korean Culture Center of Microorganisms (KCCM), Republic of Korea. The pathogenic strains were routinely cultured in Luria-Bertani (LB) agar medium and stored as glycerol stocks (20% glycerol in LB broth, v/v) at −80°C.

Strain Identification and Biochemical Test
KID7 was identified by gram staining, microscopic examination and the API 50 CHL kit (Biomerieux S.A., La Balme les Grottes, France). In addition, the fermentation pattern of KID7 was identified using homo- and hetero-fermentation (HHD) medium (Mcdonald et al., 1987). Enzyme activities such as β-galactosidase, β-glucosidase and protease activity were studied following previous reports (Vidhyasagar and Jeevaratnam, 2013; Lee et al., 2014). KID7 was studied for its growth in the presence of NaCl (1–10%, w/v) in MRS broth. Genomic DNA of strain KID7 was isolated using a genomic DNA isolation kit (GeneALL, Seoul, South Korea), following the manufacturer’s protocol. PCR amplification of the 16S rRNA gene from KID7 was performed with the primers 27F and 1492R (Borges et al., 2013) and sequencing of the PCR product was done with 27F and 785F (5′-GGATTAGATACCCGTGTA-3′) primers to get a partial sequence of the 16S rRNA gene. Sequencing service was provided by Solgent Co. Ltd. (Seoul, South Korea). The
sequence was searched for similarities in the EzTaxon database using the BLAST program. A phylogenetic tree was constructed using the closely related sequences by multiple alignment using Clustal X followed by neighbor-joining phylogenetic tree construction using MEGA 6 software (Tamura et al., 2013).

**In vitro Study of Probiotic Properties**

**Safety assessment**

KID7 was subjected to safety assessment such as biogenic amine production, hemolytic activity, and degradation of type III mucin from porcine stomach (Sigma, St. Louis, MO, USA). The biogenic amine production was tested according to the procedure described by Bover-Cid and Holzapfel (1999) using decarboxylase agar medium with or without amino acids such as L-phenylalanine, L-lysine, L-tryptophan, L-tyrosine, L-arginine, L-ornithine, or L-histidine. Hemolytic activity and mucin degradation were tested by petri dish-based methods described by Borges and Teixeira (2014) and Zhou et al. (2001), respectively. The minimum inhibitory concentration (MIC) of various antibiotics for strain KID7 was tested by 2-fold broth microdilution method (Wiegand et al., 2008). Susceptibility of the strain to a particular antibiotic was determined according to the cut-off MIC values given by European Food Safety Authority (EFSA, 2012).

**Oro-gastrointestinal transit assay**

Oro-gastrointestinal transit (OGT) tolerance assay was performed according to the method described by Bove et al. (2012) with modification. The assay mimic the physiological conditions of oral, gastric and intestinal stress. The strain KID7 was subjected to oral stress for 10 min in electrolyte solution (sodium chloride, 6.2 g; potassium chloride, 2.2 g; calcium chloride, 0.22 g; sodium bicarbonate, 1.2 g) (Marteau et al., 1997) with 150 mg/L lysozyme (Sigma). This was followed by gastric stress for a 30-min incubation in electrolyte solution of pH 3 + 0.3% pepsin (Sigma) and another 30-min incubation in electrolyte solution of pH 2 + 0.3% pepsin. Finally, the cells were subjected to a 120-min incubation in intestinal electrolyte solution (sodium chloride, 5 g; potassium chloride, 0.6 g; calcium chloride, 0.25 g; pH 7) (Marteau et al., 1997) with 0.3% bile oxxgal (BD) and 0.1% pancreatin (Sigma-Aldrich). The strains in PBS without stress were used as control. The cells were separated from the stress solution after each stress by centrifugation at 5000 rpm for 5 min and either subjected to next stress or used for viability analysis. The viability was calculated from colony-forming units (cfu) of appropriate dilutions from the control and stress-treated bacterial cells plated on MRS agar medium incubated for 48 h at 37°C. The bacterial viability at the end of each stress was also monitored using the LIVE/DEAD BacLight bacterial viability kit (Invitrogen, Oregon, USA) following the manufacturer’s protocol. The live and dead bacteria were visualized as green and red fluorescent cells, respectively under a fluorescence microscope (Olympus BX50, Tokyo, Japan) and photomicrographs were captured using a digital camera (Olympus DP70, Tokyo, Japan).

**Caco-2 cell culture and adhesion assay**

Caco-2 cell line was obtained from KCTC (Korea) and routinely cultured in minimum essential medium (MEM) high-glucose medium (Gibco) supplemented with 20% (v/v) inactivated fetal bovine serum and 100 U of antibiotics penicillin-streptomycin. The culture was incubated at 37°C with 5% CO2. For the adhesion assay, the Caco-2 cell line was used between the 39th and 41st passage, which phenotypically resembled the enterocytes and expressed tight junction. The Caco-2 cells were seeded at the concentration of 2 × 10^5 cfu/ml in 12-cell culture plates and incubated for 21 days to get polarized monolayer and 100% confluence. The medium was changed every day.

The adhesion of lactic acid bacteria (LAB) strains to Caco-2 cells was determined by the method described by Lee et al. (2014) with minor modification. Briefly, the Caco-2 cells were replaced with non-antibiotic-supplemented MEM medium 2 h prior to the adhesion assay. 0.5 ml of 18-h bacterial suspension in PBS was mixed with an equal volume of non-antibiotic-supplemented cell culture medium. The final concentration of bacteria was 1 × 10^8 cfu/ml, added to each well of the tissue culture plate containing the Caco-2 monolayer, and incubated at 37°C in 5% CO2 for 2 h. After incubation, the number of adhered bacterial cells was determined according to Lee et al. (2014).

**Antimicrobial activity**

The antimicrobial activity of strain KID7 was determined against human, animal and food-borne pathogens listed in Table 2. Strain KID7 was cultured in MRS broth for 48 h at 37°C and the culture filtrate was concentrated 10-fold by lyophilization. One hundred microliter of concentrated crude culture filtrate was loaded on to an 8-mm paper disc (ADVANTEC, Japan) and dried. The disc was placed on the pathogen- spread LB agar plate and incubated for 18 h and the zone of inhibition was measured. The entire assay was performed in triplicates to check the reproducibility. Hydrogen peroxide production by the LAB strains was determined according to the method of Saito et al. (2007).

**Bile salt hydrolase activity**

Bile salt hydrolase activity of KID7 was detected by thin-layer chromatography (TLC). The reaction mixtures were prepared by a method described by Guo et al. (2011) with slight modification. MRS reaction mixture was prepared with 5 mM taurodeoxycholic acid (TDCA), or 5 mM glycocholic acid (GCA), or 5 mM taurocholic acid (TCA) in sodium phosphate buffer (pH 7.4). One microliter of 18-h culture was mixed with an equal volume of phosphate-buffered MRS + conjugated bile acid and incubated for 36 h at 37°C. After incubation, the mixture was lyophilized, dissolved in 1 ml of methanol and centrifuged to remove precipitates. Three microliter of sample was spotted on TLC (silica gel 60, 20 × 20 cm) and developed using a mobile phase of hexane:methyllethlyketone:glacial acetic acid (56:36:8, v/v/v) (Chavez and Krone, 1976). After development, the plates were dried with hot air, sprayed with 10% sulfuric acid in distilled water, and heated at 110°C for 10 min in a hot air oven to detect the free cholic acid, deoxycholic acid, and conjugated bile acids. The spots were visualized under UV illumination.
Determination of cholesterol-lowering activity
The LAB strains were studied for cholesterol-lowering activity in MRS liquid broth supplied with cholesterol. MRS broth was initially prepared with 0.05% cysteine-HCl to create microaerophilic condition and autoclaved, followed by addition of cholesterol (CHO) dissolved in ethanol:Tween 80 (3:1, v/v) to a final concentration of 500 µg/ml, w/v, in the MRS medium. The ethanol concentration should not exceed 5%. The MRS-CHO broth was inoculated with log phase bacterial culture (2% v/v of A<sub>600</sub> = 0.5 OD culture) and incubated at 37°C for 24 h. After the incubation, bacterial cells were pelleted out by centrifugation at 1500 × g at 4°C for 10 min. The cholesterol concentration in spent medium was estimated by enzymatic method using the BCS total cholesterol kit (Bioclinical System, Seoul, South Korea) following the manufacturer's protocol and compared with uninoculated MRS-CHO broth as control and the percentage cholesterol removal was calculated. The cholesterol-lowering activity of LAB was also studied in the presence of 0.3 and 0.5% bile. Additionally, growth of the LAB strains was also measured in MRS-CHO, and MRS-CHO-bile (0.3 and 0.5%) broth.

In vivo Study of Cholesterol-Lowering Activity of KID7 Experimental animals
Male C57BL/6j mice (7–8 weeks-old) were purchased from Orient Bio (Sung-Nam, South Korea). The mice were housed under temperature (23 ± 3°C) and humidity-controlled conditions (40 ± 6%) with a 12-h light/dark cycle and were given free access to food and water throughout the experiment. After acclimatization, the mice were fed an atherogenic diet (HCD, 40 kcal % fat, 1.25% cholesterol and 0.5% cholic acid, Cat #101556; Research Diets, NJ, USA) for 4 weeks to induce hypercholesterolemia before the oral administration of LAB. One group was continued with a normal diet (NCD). After 4 weeks, HCD-fed mice were assigned to one of the following three groups for 4–5 weeks: HCD-control (Con); HCD administered with 3 × 10<sup>8</sup> cfu/ml of KID7 (HCD-KID7); or HCD administered with 3 × 10<sup>8</sup> cfu/ml of <i>L. acidophilus</i> ATCC 43121 (HCD-L.ac). KID7 and <i>L. acidophilus</i> ATCC 43121 were mixed with distilled water and administered by oral gavage once daily for 32 days and the control NCD and HCD groups were administered distilled water alone. The body weight was measured once per week for 6 weeks. Food intake (g/mouse/day) was determined by subtracting the remaining food weight from the initial food weight of the previous feeding day, and dividing by the number of mice housed in the cage. The experimental design was approved by the Ethical Committee of the Myongji Bioeffeciency Research Center in Myongji University (Yongin-Si, Gyeonggi-do, Republic of Korea), and the mice were maintained in accordance with standard guidelines.

Collection of tissues
At the end of the 32-days treatment period, the mice were sacrificed by cervical dislocation, and the liver and white adipose tissue (WAT) were excised immediately, rinsed, weighed, frozen in liquid nitrogen, and stored at −80°C until analysis.

Serum biochemical analysis
For biochemical analyses of serum, blood samples were collected from tail vein at 16-h-fasted state. Serum was obtained from the blood by centrifugation (3000 rpm, 10 min, 4°C) and was stored at −80°C until analysis. Serum total cholesterol (T-CHO), triglyceride (TG), HDL-cholesterol, glucose, glutamyl oxaloacetic transaminase (GOT) and glutamyl pyruvic transaminase (GPT) levels were determined by using commercial assay kits (Asan Pharm, Seoul, Korea). Low-density lipoprotein (LDL) cholesterol was calculated using the Friedewald formula (Friedewald et al., 1972).

Liver and fecal lipid contents
Lipids from the isolated liver and feces was extracted using the Folch method (Folch et al., 1957). The extracted lipid fraction was used to measure the T-CHO and TG levels using commercial kit mentioned above. Fecal cholic acid content was measured following the method of Kumar et al. (2011).

Total RNA isolation and gene expression analysis
Total RNA was extracted from liver using the RNeasy mini kit (Qiagen Korea, Seoul, South Korea) according to the manufacturer's protocol. Total RNA (1 µg) was reverse-transcribed to cDNA with the PrimeScript RT reagent kit (Takara, Shiga, Japan). The mRNA expression levels of cholesterol metabolisms-related genes were evaluated by real-time PCR using iQTM SYBR Green Supermix and the real-time PCR iQTM5 system (Bio-Rad Laboratories, Hercules, CA, USA). Relative gene expression levels were calculated by the difference in Ct value after normalization with β-actin (ACTB) and were expressed as the fold-change relative to the HCD control group. The primers used in the experiments are listed in Table 1.

Technological Characterization
Viability of strain KID7 under freeze-drying and storage conditions
KID7 was grown in MRS broth at 37°C for 30 h (late log-phase culture) and the cells were harvested by centrifugation at 3000 × g for 15 min at 4°C. The cell pellet was washed and resuspended in PBS (control) and additives to give a final cell concentration of 1.8 × 10<sup>8</sup> cfu/ml. The additives used were 1% (w/v) skim milk, 1% (w/v) galactomannan (Sigma-Aldrich), 1% (w/v) wheat bran hydrolysate, 0.5% (w/v) skim milk + 0.5% (w/v) galactomannan, and 0.5% (w/v) galactomannan + 0.5% (w/v) wheat bran hydrolysate, prepared separately in sterile distilled water. Wheat bran hydrolysate was prepared following the method of Wang et al. (2009). The cell suspension in PBS and additives were frozen at −80°C. The frozen suspensions were subjected to freeze-drying using a lab-scale freeze dryer (IlShin lab Co., ltd, Korea), pre-cooled to −80°C. The samples were dried under reduced pressure (5 mbar) for 24 h. The freeze-dried samples were rehydrated using sterile distilled water and serially diluted and plated on MRS agar to determine the viable count. Alternatively, the freeze-dried samples were stored at three different temperature viz., −20, 4, and 25°C for up to 6 months. The viable cell count present in each sample was determined as described above. The number of
colonies on MRS agar was calculated and the total cfu was calculated for each sample stored at various temperatures and time points.

Statistical analysis
Data are expressed as means ± SEM. The differences among treatment groups were analyzed by One-Way ANOVA, followed by Duncan test, using Origin 7 Software (MicroCal Software, Northampton, MA, USA). Values of P < 0.05 were considered to indicate statistical significance.

Results
Identification of Strain KID7 Based on 16S rDNA Sequence and Biochemical Test
BLAST search of the EzTaxon database showed that the 16S rDNA sequence of KID7 (Genbank accession number KJ810576) (1508 bp) showed 99.3% (difference of 10/1499 bp) similarity with P. pentosaceus type strain DSM 20336. All other Pediococcus species showed less than 98.5%, which is the cut-off value for species-level comparison studies (Stackebrandt, 2011). Phylogenetic analysis of the 16S rRNA gene sequence of KID7 with that of various Pediococcus species confirmed its close relatedness to P. pentosaceus DSM 20336 (Figure S1). Hence, P. pentosaceus KACC 12311 (=DSM 20336) was used for comparative biochemical test with KID7. The biochemical tests of strain KID7 and the closely related strain P. pentosaceus KACC 12311 are shown in Table S1. The strain KID7 possesses various enzymatic activities such as protease, β-galactosidase and β-glucosidase similar to that of P. pentosaceus KACC 12311. It showed difference in the carbohydrate utilization pattern, which is considered as strain-level difference between KID7 and P. pentosaceus KACC 12311. Strain KID7 grew well in the presence of NaCl, however, a reduction in cell growth was observed with increasing concentration of NaCl (Figure S2).

In vitro Probiotic Properties of KID7

Survival of KID7 under simulated oro-gastrointestinal transit condition
The strain KID7 showed good tolerance to oral, gastric and intestinal stress based on the results of fluorescence microscopic analysis for live and dead/damaged cells (Figure 1A) and plate count obtained after each stress treatment (Figure 1B). We observed a high amount of red fluorescent cells (indicating dead/damaged cells) in the gastric stress (pH 2) and intestinal stress in the fluorescence microscopic analysis (Figure 1A). However, we observed that these were only damaged cells, as they recovered during culture on MRS agar, which was evidenced by the number of colony-forming units in the gastric stress and intestinal stress (Figure 1B). Overall, a reduction of 1 log unit cfu (P < 0.05) was observed at the end of the OGT assay compared to the initial count (Figure 1B).

Adherence of KID7 to Caco-2 cell monolayer
The adherence of KID7, L. rhamnosus GG, and L. acidophilus ATCC 43121 to Caco-2 cell monolayer was calculated as the percentage of cells that remained attached to the Caco-2 monolayer after two washes compared to the initial amount of LAB added (Figure 2). Plate count of the attached bacterial cells revealed high adherence of KID7 cells (34.7%) (Figure S4B) and the reference strain L. acidophilus ATCC 43121 (5%) (Figure S4A) compared to L. rhamnosus GG (14.2%) and L. acidophilus ATCC 43121 (5%) (Figure 2).

Antimicrobial activity of strain KID7
The concentrated culture filtrate from KID7 showed a broad-spectrum antimicrobial activity against human, animal and food-borne pathogens used in this study (Table 3). The antimicrobial activity was observed against both gram-positive and gram-negative pathogenic bacteria, whereas no antimicrobial activity was observed against LAB strains tested. We observed that KID7 (Figure S4A) and the reference strain P. pentosaceus KACC 12311 (Figure S4B) did not produce H₂O₂, whereas, L. rhamnosus GG produces H₂O₂, which is observed as a zone of blue color around the colony on Prussian blue agar (Figure S4C).

Bile salt hydrolase and cholesterol-lowering activity of KID7
The strain KID7 possessed bile salt hydrolase activity that deconjugated TDCA, GCA and TCA (Figure 3A). We
In vitro assessment of oro-gastrointestinal transit tolerance of strain KID7 under simulated conditions. (A) Fluorescence photomicrograph of KID7 cells after various stages of simulated oro-gastrointestinal transit: (a) initial, (b) oral stress, (c) gastric stress pH 3, (d) gastric stress pH 2, and (e) intestinal stress. The live, and dead/damaged cells were identified by using the LIVE/DEAD BacLight bacterial viability kit after oral, gastric and intestinal stress treatment. Green color indicates viable bacteria; red color indicates damaged or dead bacteria. (B) Viable count of KID7 after each stages of simulated oro-gastrointestinal transit. The viable count of KID7 was determined by plating the cells from each stage on MRS agar and incubation at 37°C for 48 h. Error bar represents standard error of mean of three independent experiments. *** Significantly different from initial count at P < 0.005.

Adherence of P. pentosaceus strain KID7, L. rhamnosus GG, and L. acidophilus ATCC 43121 to Caco-2 cell monolayer. The error bars indicate standard deviation of three independent experiments.

Antibiotic susceptibility of P. pentosaceus KID7.

| Antibiotics          | Minimum inhibitory concentration (MIC) (mg/L) | EFSA cut-off (MIC) (mg/L) |
|----------------------|-----------------------------------------------|--------------------------|
| Chloramphenicol      | 1 (S)                                         | 4                        |
| Tetracyclin hydrochloride | 4 (S)                                | 8                        |
| Erythromycin         | 0.25 (S)                                      | 1                        |
| Streptomycin sulfate | 256 (R)                                       | 64                       |
| Gentamycin C         | 8 (S)                                         | 16                       |
| Clindamycin          | 0.5 (S)                                       | 1                        |
| Ampicillin           | 1 (S)                                         | 4                        |
| Kanamycin B sulfate  | 1024 (R)                                      | 64                       |

Bacterial susceptibility to antibiotic was determined according to the cut-off values given by EFSA (2012). S, Susceptible; R, Resistant.

Observed a 40% decrease in in vitro cholesterol concentration in MRS-CHO broth after the growth of KID7 for 24 h. However, this reduction level was decreased in the presence of bile oxgall—0.3% (33% cholesterol reduction) and—0.5% (17% cholesterol reduction) (Figure 3B). The percentage of cholesterol reduction by KID7 was comparatively higher than that of P. pentosaceus KACC 12311 (Figure 3B). The reduction in cholesterol-lowering activity of KID7 and P. pentosaceus KACC 12311 in the presence of 0.3 and 0.5% bile oxgall may be due to reduced cell growth in the presence of bile compared to bile- non-supplied MRS-CHO broth treatment.

In vivo Cholesterol-Lowering Activity of KID7

Effects of KID7 and L. acidophilus on body weight gain, food intake, and organ weight

The body weight gain, food intake, and organ weight are shown in Table 4. Body weight gain was not significantly different among all groups. Food intake of the NCD group was higher than that of the HCD control group (P < 0.005), but there was no significant difference in food intake among the HCD control and HCD-LAB groups. WAT weight did not differ among all groups. Liver weights in the NCD group were reduced vs. that of the...
TABLE 3 | Antimicrobial activity of culture filtrate of strain KID7.

| Test strains | Zone of inhibition (mm)* |
|--------------|--------------------------|
| **GRAM POSITIVE PATHOGENS** | |
| Staphylococcus aureus KCCM 11335 | 15 |
| Staphylococcus epidermidis KCTC 1917 | 12 |
| Listeria monocytogenes KACC 10764 | 20 |
| Staphylococcus aureus KCCM 40510 (Methicillin-resistant) | 11 |
| Bacillus cereus KACC 11240 | 10 |
| **GRAM NEGATIVE PATHOGENS** | |
| Salmonella typhi KCTC 2514 | 12 |
| Salmonella choleraesuis KCTC 2932 | 15 |
| Pseudomonas aeruginosa KCCM 11802 | 18 |
| Yersinia enterocolitica ssp. enterocolitica KACC 15320 | 20 |
| Salmonella gallinarum KCTC 2931 | 26 |
| Shigella boydi KACC 10792 | 14 |
| Escherichia coli O138 KCTC 2615 | 11 |
| E. coli O1 KCTC 2441 | 12 |
| **LACTIC ACID BACTERIA (GRAM POSITIVE)** | |
| Enterococcus durans KACC 10787 | – |
| Lactobacillus fermentum KACC 11441 | – |
| Lactobacillus plantarum ssp. plantarum KACC 11451 | – |
| Lactobacillus brevis KACC 11433 | – |
| Lactobacillus paracasei KACC 12361 | – |

*Concentrated crude culture filtrate of strain KID7 was used for the antimicrobial assay. –, indicates no zone of inhibition.

Effects of KID7 and L. acidophilus on lipid content in liver and feces

Liver T-CHO levels and TG in the NCD group were lower than that of the HCD control group (P < 0.001 and P < 0.05, respectively) [Figure 5]. Liver T-CHO levels in the HCD-KID7 group were reduced significantly compared with the HCD control group (P < 0.05) [Figure 5A]. Liver TG levels tended to decrease in the HCD-KID7 group compared with the HCD control group, but not statistically significant [Figure 5B]. Fecal T-CHO levels tended to increase in the HCD-KID7 group compared with the HCD control group, but not statistically significant (P = 0.056) [Figure 5C]. A significant increase (P < 0.05) in fecal cholic acid content was observed in the HCD-KID7 group compared to HCD group. In the HCD-Lac group no significant change in liver total cholesterol, liver triglyceride content, fecal cholesterol and fecal cholic acid contents was noted compared to the HCD control group [Figures 5A–D].

Technological Characteristic of KID7

Viability of KID7 under freeze-drying and storage conditions

The viability of strain KID7 after freeze-drying condition in the presence of various food grade additives was given in Figure 7A. Among the tested additives, 0.5% skim milk + 0.5% galactomannan (SMG), protected the bacterial cells during freeze-drying condition and the viability was retained at 99.79%, which was better than in the presence of 1% skim milk alone (viability = 99.6%) (Figure 7A). Further, we tested KID7 cells in SMG combination for storage at different temperature conditions in freeze-dried form for up to 6 months. The viability of KID7 cells in SMG combination was high at 4°C followed by –20°C and 25°C (Figure 7B). After 6 months of storage, KID7 cells in SMG matrix retained >90% viability at 4°C and >89% viability at –20°C, which resulted in 1-log-order decrease in viable cells (Figure 7B) whereas at 25°C the viability was reduced to 68% with 2-log-order decrease in the viable cells (Figure 7B). These results suggest that KID7 combined with SMG matrix could be stored long term at 4°C in freeze-dried form and demonstrate the technological properties of the strain.
FIGURE 3 | In vitro cholesterol-lowering activity of *P. pentosaceus* KID7. (A) Thin-layer chromatography analysis of free cholic acid present in the culture filtrate of KID7 grown in MRS containing various conjugated bile salts; 1, MRS+5 mM TDCA; 2, MRS+5 mM TCA; 3, MRS+5 mM GCA; S, Standard mixture of TDCA, TCA, GCA, cholic acid (CA), and deoxycholic acid (DCA). (B) Cholesterol reduction activity of strain KID7 and *P. pentosaceus* KACC 12311 with or without bile-oxgall. (C) Growth of KID7 and *P. pentosaceus* KACC 12311 in MRS-CHO medium with or without bile-oxgall represented in log cfu/ml. The data are representation of mean values of three independent assays and error bars indicates ± standard deviation.

TABLE 4 | Effects of KID7 and *L. acidophilus* on body weight, food intake, and tissues weight in mice.

| Parameters                  | Dietary groups                  |
|-----------------------------|---------------------------------|
|                            | NCD    | HCD    | HCD + KID7 | HCD + *L. acidophilus* |
| **BODY WEIGHT (g)**        |        |        |            |                     |
| Initial                    | 21.77 ± 0.48 | 22.24 ± 0.49 | 20.89 ± 0.52 | 21.03 ± 0.37         |
| Final                      | 23.24 ± 0.49 | 22.01 ± 0.36 | 21.97 ± 0.50 | 22.64 ± 0.45         |
| Gain                       | 1.88 ± 0.35 | 1.56 ± 0.39 | 1.68 ± 0.40  | 1.8 ± 0.70           |
| Food intake rate (g/mouse/day) | 3.24 ± 0.18 | 2.50 ± 0.23 | 2.5 ± 0.21   | 2.42 ± 0.17          |
| **TISSUES WEIGHT (g)**     |        |        |            |                     |
| Liver                      | 0.9 ± 0.03*** | 1.29 ± 0.05 | 1.27 ± 0.04  | 1.21 ± 0.02          |
| Epididymal fat             | 0.16 ± 0.03 | 0.16 ± 0.019 | 0.17 ± 0.015 | 0.20 ± 0.033         |

NCD, Normal diet; HCD, high-cholesterol diet; KID7, *P. pentosaceus* KID7; L.ac, *L. acidophilus*. Data are expressed as means ± SEM (n = 8/group). *** Significantly different at P < 0.005 vs. the HCD group.

**Discussion**

*P. pentosaceus* has been approved as an animal feed additive in the United States, European Union, China, Thailand, Australia, and New Zealand (Lim and Tan, 2009). In recent years it has received increased attention and has been characterized for its possible utilization as probiotic in humans (Osmanyagaoglu et al., 2012; Zhao et al., 2012; Borges et al., 2013; Vidhyasagar and Jeevaratnam, 2013; Borges and Teixeira, 2014; Garcia-Ruiz et al., 2014; Lee et al., 2014).

Microbes used as probiotics should satisfy certain safety criteria such as being negative for mucin degradation activity (Abe et al., 2010), since microbes degrading mucin can translocate from the intestinal lumen to other body parts and cause bacteremia (Abe et al., 2010). Generally, members of LAB do not possess hemolytic activity. However, *Enterococcus* and *Bacillus* strains possess hemolytic potential and such strains should be adequately tested and confirmed for non-hemolytic activity (FAO-WHO, 2002). Testing for bioamine production is an important safety test, since most of the LAB have the ability to produce bioamines (Bover-Cid and Holzapfel, 1999). These are toxic substances generated from the decarboxylation of amino acids present in foods, the most important being diaminos, which are precursors of carcinogenic nitrosamines (Bover-Cid and Holzapfel, 1999). Bover-Cid and Holzapfel (1999) observed that pediococci do not show any ability to produce bioamines,
which is in accordance with our observation that KID7 did not produce bioamine on any of the amino acids tested in this study (Figure S2). Antibiotic susceptibility is another key requirement of a probiotic strain used for human or animal consumption (FAO-WHO, 2002; EFSA, 2012). Ingestion of probiotic bacteria with transmissible (plasmid or transposon-borne) antibiotic-resistant genes may lead to generation of new antibiotic-resistant pathogens in the host gut by horizontal gene
Transferring (EFSA, 2012). However, ingestion of probiotic bacteria containing non-transmissible drug resistance genes pose a low potential for horizontal transfer and generally may be used as feed-additive (EFSA, 2012). The strain KID7 was resistant to kanamycin and streptomycin. Pediococcus pentosaceus strains were shown to have MICs of 256 to >256 and up to 192 \( \mu \text{g/ml} \) for kanamycin and streptomycin, respectively (Danielsen et al., 2007), which are higher than the EFSA recommended cut-off values. Strains showing higher MIC than the EFSA cut-off values are considered resistant and must be studied for the genetic basis of resistance and must be of non-transmissible nature; otherwise, those strains should not be used as feed additives (EFSA, 2012). The aminoglycoside resistance gene \( \text{aac(6')Ie-aph(2')Ia} \) has been reported in some Pediococcus species (Ammor et al., 2007), which codes for the bifunctional aminoglycoside-modifying enzyme 69-N-aminoglycoside acetyltransferase-20-O-aminoglycoside phosphotransferase (Tenorio et al., 2001). The \( \text{aac(6')Ie-aph(2')Ia} \) gene confers high level of resistance to gentamicin in addition to other aminoglycoside antibiotics (Tenorio et al., 2001), however, KID7 was susceptible to gentamicin. The genetic basis of streptomycin and kanamycin resistance in strain KID7 is not known and needs further study to assess the nature of resistance to these aminoglycoside antibiotics.

Several previous reports on \( P. \) pentosaceus studied the acid and bile tolerance separately (Jonganurakkun et al., 2008; Osmanagaoglu et al., 2010; Vidhyasagar and Jeevaratnam, 2013; Garcia-Ruiz et al., 2014; Lee et al., 2014); however, in real situations, the consumed probiotic should pass through the physiological events of ingestion and digestion of the human gastrointestinal tract (GIT), which include passage through the oral cavity, stomach and the small intestine (Bove et al., 2012). Tolerance to the GIT is one of the selection criterion recommended by FAO-WHO (2002); hence, we studied the tolerance of KID7 to progressive oral, gastric, and intestinal stress (OGT assay) in order to mimic the physiological condition. Tolerance of \( L. \) plantarum strains to oral and gastric stress up to pH 3 was reported previously (Bove et al., 2012). However, when the pH reduced from 3 to 2 a drastic drop of viability was observed for all \( L. \) plantarum strains and \( L. \) acidophilus LA5 (Bove et al., 2012) and at the end of the OGT assay a tendency to recover viability was noticed for bacterial samples (Bove et al., 2012). In our study, we observed KID7 strain was able to recover from cell damage caused by the OGT transit (Figure 1), which is in accordance with Bove et al. (2012). Survival and tolerance of the strains are essential to express the probiotic functions in the intestine (Kumar et al., 2011).

Adherence and colonization of LAB strains in the intestine can be studied using human intestinal epithelial cell models such as Caco-2 and HT-29 cell lines, in vitro (Tuo et al., 2013). KID7 showed higher adherence to Caco-2 cells as compared to \( L. \) rhamnosus GG and \( L. \) acidophilus ATCC 43121. A good adherence capacity can promote gut residence time of a probiotic bacteria, aids in pathogen exclusion and interaction with host cells for the protection of epithelial cells and immune modulation (Lebeer et al., 2008). Several previous reports demonstrated that...
P. pentosaceus strains adhered to intestinal epithelial cells better than Lactobacillus strains (Turpin et al., 2012; Thirabunyanon and Hongwittayakorn, 2013; Garcia-Ruiz et al., 2014).

Antimicrobial activity of LAB against pathogens can be due to the production of organic acids, hydrogen peroxide (H$_2$O$_2$), and bacteriocins (Vesterlund, 2009). KID7 does not produce H$_2$O$_2$ (Figure S5). The antimicrobial activity of culture filtrate from KID7 against gram-positive and -negative pathogenic bacteria and not against LAB strains suggest that the antimicrobial activity could be due to the production of organic acids.

BSH enzymes deconjugate the conjugated bile acids into the amino acids (glycine or taurine) and free bile acids (cholic acid or deoxycholic acid) (Joyce et al., 2014). It has been reported that high-level expression of BSH activity by gut microbiota resulted in host weight reduction and cholesterol reduction (Joyce et al., 2014). Hence, BSH activity is an important selection criterion for probiotic microbes to be used for cholesterol reduction.

Furthermore, BSH activity is also among the selection criteria recommended by FAO-WHO (2002) for screening potential probiotic strains. Bile acids are synthesized in the liver using cholesterol as precursor. BSH activity of probiotics in the gut could modify the conjugated bile acids and excretion of the free bile acids, which results in de novo synthesis of conjugated bile acids from cholesterol to maintain hepatic bile acid pool (Herrema et al., 2010; Degirolamo et al., 2014). Thus, bacterial BSH activity in the gut acts as a sink for cholesterol (Joyce et al., 2014).

Several LAB strains have been reported to possess in vitro cholesterol assimilation/uptake activity. LAB exhibit mechanisms, such as cholesterol adsorption to cell wall, and cholesterol assimilation into cell membrane (Liong and Shah, 2005a) and co-precipitation with deconjugated bile acids (Liong and Shah, 2005b) in in vitro assays. Cholesterol removal from culture medium by KID7 could involve any of these mechanisms. Previous reports on cholesterol reduction by Pediococcus strains showed varying levels (58–73%) of cholesterol reduction in liquid medium supplied with 100–200 μg/ml of cholesterol in the presence of 0.3% bile (Vidhyasagar and Jeevaratnam, 2013). In the present study, we observed a reduction in cholesterol-lowering activity in the presence of 0.3 and 0.5% bile oxgall, dose-dependently (Figure 3B). This reduction in the cholesterol assimilation activity is due to reduction in cell growth of KID7 in 0.3 and 0.5% bile oxgall (Figure 3C). The reduction in cell growth in the presence of oxgall was also reported for a L. fermentum strain (Pereira et al., 2003), however, the cholesterol assimilation activity increased despite low growth rate. In our case, lower growth led to lower cholesterol assimilation by KID7 cells. Although, KID7 tolerated bile during brief exposure of 2 h and showed good viability in the OGT assay, growth of the strain was significantly affected during longer exposure to bile as is the case with cholesterol-lowering activity in the presence of bile. The type strain P. pentosaceus KACC 12311 also followed the same pattern as KID7; however, it showed relatively lower growth and cholesterol assimilation activity in the presence and absence of bile oxgall. The lower growth of the KID7 and P. pentosaceus KACC 12311 in bile-supplied medium could be due to bile salt-deconjugating activity of the strains, which leads to accumulation of free bile acids that are more toxic than conjugated bile (Pereira et al., 2003). However, these in vitro assays do not reflect the true nature of probiotic strains in cholesterol-lowering activity, since the conditions in vivo are completely different. Hence, the probiotic activity of any strain must be validated through in vivo studies.

We tested the strain KID7 in vivo to confirm the cholesterol-lowering activity in atherogenic diet-induced hypercholesterolemic mice in comparison with the previously reported cholesterol-lowering probiotic strain L. acidophilus ATCC 43121 as positive control. Supplementation of hypercholesterolemic mice with strain KID7 significantly lowered the serum total cholesterol level, LDL level and hepatic total cholesterol level. Reduction in the serum LDL level and cholesterol level correlated with our observation of increased expression of genes related to cholesterol metabolism in liver such as LDLR and ApoE (Figure 6). Increased expression of...
LDL-receptor will lead to increased absorption of LDL by hepatocytes. The absorbed LDL is degraded and the cholesterol is added to the cholesterol pool (Cohen, 2008). An increase in cholesterol pool will down regulate HMG CoA-reductase (HMGCR) and LDL receptor (LDLR) expression. In our study, we did not observe any significant down regulation in HMGCR expression in the HCD-KID7 group compared to HCD group, whereas LDLR expression and APOE expression increased in the HCD-KID7 group. This could be due to sinking cholesterol level in cholesterol pool due to expression of CYP7A1 (Figure 6), which codes for the enzyme cholesterol 7-α-hydroxylase, which catalyzes a rate-limiting step in the de novo synthesis of bile acids from cholesterol (Cohen, 2008). Bile salts are secreted into bile duct at a rate of ~24 g/day, but their synthesis is only a fraction of that rate, because most of the bile salts are recycled to the liver from the ileum (Cohen, 2008). Reduction in bile salt reabsorption due to bile salt hydrolase activity in the gut will cause bile synthesis from cholesterol to maintain the bile salts pool size. The increased expression of CYP7A1 and a decrease in hepatic cholesterol pool also correlated with an increase in total cholic acid excretion in feces of the HCD-KID7 group (Figure 5D). These results suggests that cholesterol-lowering activity of KID7 in vivo could be due to its bile salt hydrolase activity. We also observed a significant increase in the level of hepatic LDL mRNA expression in the HCD-Lac group, which is consistent with Park et al. (2007). In addition to the LDLR gene, we observed the expression of SREBF2, and APOE genes in the HCD-Lac group, which was not previously reported in studies using L. acidophilus ATCC 31432.

A previous study (Moon et al., 2014) on lipid-lowering effect of Pediococcus acidilactici strain M76 and P. acidilactici DSM 20284 reported a reduction in serum cholesterol level of 12 and 2.4%, respectively, and a reduction in total hepatic cholesterol level of 30.5 and 14.9%, respectively for the two P. acidilactici strains used in their study. Compared to those results, KID7 showed a 19.8% reduction in serum total cholesterol level, a 35.5% reduction in LDL cholesterol level, and a reduction in liver total cholesterol level of 19.7%. Our results indicated that KID7 could be used as a potential cholesterol-lowering probiotic that needs to be confirmed by clinical trials in humans.

Probiotic supplements are usually in the form of dry powders to reduce water activity and protect viability. The preferred drying method is freeze-drying as it preserves viability of the probiotic bacteria. However, not all strains tolerate drying and may show significant loss of viability (Ross et al., 2005). Hence, protective agents and stabilizing agents are added while freeze-drying (Ross et al., 2005). The combination of skim milk and galactomannan increased the viability of KID7 after freeze-drying and subsequent storage conditions. It was reported that skim milk acts as a cryoprotectant and prevents the cells from the damage during freeze-drying (Ross et al., 2005). Additionally, galactomannan from locust bean is a natural polysaccharide used as emulsifying, thickening, and stabilizing agent in the food industry (Cheow et al., 2014). It has been reported elsewhere that spray-drying of probiotic Lactobacillus paracasei NFBC in a milk medium supplemented with gum acacia provided better protection to the cells than milk powder alone (Desmond et al., 2002).

In conclusion, the P. pentosaceus KID7 reported in this study exhibited cholesterol-lowering activity in vivo and possessed the essential characteristics to become a potential probiotic. Furthermore, KID7 maintained good viability during freeze-drying and storage when formulated with a combination of skim milk and galactomannan, which can be utilized in the large-scale production of P. pentosaceus KID7-based probiotic products after validation of the probiotic ability in human clinical trials.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00768

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