Characterization of *Lactobacillus plantarum* Lb41, an isolate from kimchi and its application as a probiotic in cottage cheese

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Abstract *Lactobacillus plantarum* Lb41 was determined probiotic properties and applied to cottage cheese. *L. plantarum* Lb41 showed high viability (>80%) in artificial gastric (pH 2.5, 0.3% pepsin for 3 h) and bile (0.3% oxgall for 24 h) acids, and adhered strongly to HT-29 cells (7.5% adhesion). It did not produce β -glucuronidase and was resistant to several antibiotics. *L. plantarum* Lb41 did not inhibit proliferation of normal MRC-5 cells, but showed antiproliferative effects on AGS, HT-29, and LoVo cells, based on 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays. In addition, *L. plantarum* Lb41 reduced nitric oxide production by macrophages. Cottage cheese containing this strain did not show significant differences in physicochemical properties, but the number of lactic acid bacteria was maintained longer than that in control cheese. These results indicate that *L. plantarum* Lb41 could potentially be used as a probiotic in foods.

Keywords: probiotics, *Lactobacillus plantarum*, probiotic carrier, cottage cheese

Introduction

Priorities of food consumers are changing from flavor and quantity to health benefits. Probiotic foods have been important for decades, but are currently receiving more attention, as the market for these foods is rising dramatically (1). The global market value of probiotic products has increased from \$14.9 billion in 2007 to \$23.1 billion in 2012, with a compound annual growth rate (CAGR) of 6.2%. The market value in 2018 is expected to be as much as \$36.7 billion (2). Probiotics are defined as “live microorganisms which when administered in adequate amounts, confer a health benefit on the host” (3). Probiotics have valuable characteristics such as antibacterial, antidiabetic, anti-inflammatory, antioxidant, and anticancer effects (4,5). To exert their effects, probiotics must overcome physical and chemical obstacles in the human body (6). In addition, probiotic bacteria must have the ability to adhere to and colonize the intestinal epithelium to exert health benefits (7).

Most strains of the genera *Lactobacillus*, *Bifidobacterium*, *Pediococcus*, and *Streptococcus*, as well as some *Bacillus* and yeast, are probiotics. Among them, *Lactobacillus plantarum* has been used as a probiotic starter strain or in fermented foods such as meat, vegetables, milk, and coffee. *L. plantarum* can be found in various ecological niches such as the gut of humans and other animals (8). For human consumption, *L. plantarum* is generally recognized as safe (GRAS)

(9,10). *L. plantarum* has diverse beneficial features such as the production of plantaricin, a bacteriocin with antimicrobial activity, and ability for uptake and utilization of different sugars and peptides. These properties aid in adaptation to diverse conditions (11,12). In addition, *L. plantarum* has verified immunoregulatory properties, an anticolitis effect, and potential beneficial effects on inflammatory bowel disease *in vitro* (13).

Yogurt and fermented milk are regarded as representative probiotic foods. In addition, fermented vegetable-based foods such as kimchi and sauerkraut have been used as probiotic carriers (14). Among probiotic foods, cheese is regarded as an ideal carrier for delivering probiotic strains, based on properties such as high fat content, outstanding buffering capacity, and high pH (15). In the human gastrointestinal tract, foods can function as buffer to facilitate the survival of bacteria. In addition, functional ingredients in foods can interact with probiotics to improve their effects (14).

The aim of this study was to investigate the probiotic properties of *L. plantarum* Lb41 and to identify its potential as a probiotic in cottage cheese.

Materials and Methods

Bacterial strains and culture conditions *L. plantarum* Lb41, isolated

from kimchi, was grown in MRS broth at 37°C. *L. plantarum* KCTC 10782BP and *L. acidophilus* KCTC 11906BP obtained from Cell Biotech (Gimpo, Korea) were used as control strains for probiotic characterization. *Lactococcus lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* were used as starter cultures for the production of cottage cheese. These strains were obtained from New England Cheese Making Supply Co. (South Deerfield, MA, USA) in freeze-dried form.

Identification of isolated strain *L. plantarum* Lb41 was identified by following the 16S rRNA sequencing method of Macrogen Inc. (Seoul, Korea). The sequencing results were compared to the Basic Local Alignment Search Tool (BLAST) database of the GENBANK data library (<http://blast.ncbi.nlm.nih.gov>).

Characterization of probiotic *L. plantarum* Lb41 The resistance of *L. plantarum* Lb41 to artificial gastric acid and bile salts was determined according to a modified protocol by Lee *et al.* (16). The resistance to artificial gastric acid was determined using MRS broth containing 0.3 µg/mL pepsin (Sigma-Aldrich, St. Louis, MO, USA), adjusted to pH 2.5 with 0.1 M HCl at 37°C for 3 h. The resistance to bile salts was determined in MRS broth containing 0.3 µg/mL oxgall (BD BBL, Franklin Lakes, NJ, USA) at 37°C for 24 h. Viable cell counts were determined by incubating aliquots on MRS agar plates.

Enzyme activity was assessed using the API ZYM kit (BioMerieux, Lyon, France). *L. plantarum* Lb41 (10^6 CFU/mL) was inoculated in each of 20 cupules. After incubation for 4 h, ZYM A and ZYM B reagents were sequentially added to each cupule. The level of enzyme activity was determined by substrate hydrolysis assessed by color intensity.

The adhesive ability of *L. plantarum* Lb41 was examined using HT-29 cells (human colon adenocarcinoma cell line, KCLB 30038). HT-29 cells were seeded at a concentration of 1×10^5 cells/well in 24-well tissue culture plates and incubated at 37°C (17). After incubation for 24 h, *L. plantarum* Lb41 (1×10^7 CFU/well) was added to HT-29 cells and incubated for 2 h. The non-adherent cells were removed by washing thrice with phosphate-buffered saline (PBS; Gibco Life Technologies, Invitrogen, Carlsbad, CA, USA). Triton X-100 (Sigma-Aldrich) solution was used to detach the bacteria. The number of *L. plantarum* Lb41 was counted using the viable cell count method.

The disk diffusion method was performed to determine antibiotic susceptibility of *L. plantarum* Lb41 to eight kinds of antibiotics including ampicillin (0.2 mg/mL), gentamicin (0.2 mg/mL), kanamycin (0.6 mg/mL), streptomycin (0.2 mg/mL), tetracycline (0.6 mg/mL), ciprofloxacin (0.1 mg/mL), chloramphenicol (0.6 mg/mL), and doxycycline (0.6 mg/mL), according to the protocol of Haghshenas *et al.*, with modifications (18). *L. plantarum* Lb41 was spread evenly at a concentration of 1×10^5 CFU/mL on MRS agar plates, and paper discs containing antibiotics were applied. Inhibition zones were measured after incubation for 24 h at 37°C.

Cytotoxicity against tumor cells MRC-5 cells (human lung cell line,

KCLB 10171), LoVo cells (human colon adenocarcinoma cell line, KCLB 10229), HT-29, and AGS cells (human stomach adenocarcinoma cell line, KCLB 21739) were used to evaluate the cytotoxicity of *L. plantarum* Lb41. LoVo, HT-29, and AGS cell lines were cultured in RPMI 1640 containing 10% fetal bovine serum (FBS; Gibco) and 1% streptomycin/penicillin (Gibco). MEM containing 10% FBS and 1% streptomycin/penicillin was used for culturing MRC-5 cell lines. All cell lines were cultured at 37°C in an atmosphere of 5% CO₂ and 95% air.

For the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, cell lines were plated at concentration of 2×10^4 cells/well in the 96-well plate (19). After 24 h, *L. plantarum* Lb41 (1×10^7 CFU/well) were inoculated with and further incubated for 44 h. MTT solution (0.5 mg/mL) was added to the each well. Adsorption of the dissolved formazan crystals was measured at 570 nm in an ELISA plate reader (Molecular Devices, Sunnyvale, CA, USA). The values were used to determine cell viability.

Nitric oxide (NO) production in LPS-stimulated RAW 264.7 cells RAW 264.7 cells (murine macrophage cell line, KCLB 40071) were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco) containing 10% FBS and 1% streptomycin/penicillin.

RAW 264.7 cells, previously seeded at a concentration of 2×10^5 cells/well in 96-well tissue culture plates, were stimulated with lipopolysaccharide (LPS, 2 µg/mL) and *L. plantarum* Lb41 (10^7 cells/well) were added. After 24 h incubation, the NO concentration was determined by measuring the amount of nitrite in the cell culture supernatant, using the Griess reagent. A microplate reader (Molecular Devices) was used and absorbance was measured at 540 nm. NO production was calculated by comparison to a calibration curve produced using sodium nitrate as a standard.

Application in cottage cheese as a probiotic carrier Cheese was manufactured according to standard procedures using skim milk produced by Seoul milk (Seoul, Korea). Control cheese was used starter cultures of New England CheeseMaking Supply (Deerfield, MA, USA), while probiotic cheeses were starter culture of New England CheeseMaking Supply with *L. plantarum* Lb41. When the temperature of the milk reached 37°C, the cheese starter culture was inoculated and left for 30-45 min for ripening. Subsequently, rennet was added and left to ripen. The resulting curd was cut into pieces with a width of approximately 1 cm and was heated slowly to 42°C until the pH reached 4.7 to 4.6. The curds were washed with cold water and the whey and water were drained through a cheesecloth. The cheese samples were wrapped under a vacuum and stored for 4 weeks.

Samples of cottage cheese were assayed for fat, protein, total solids, and ash following association of official analytical chemists (AOAC) methods (20). The nitrogen-to-protein conversion factor used 6.38. pH value was measured using a pH meter. All analyses were done after 0, 1, 2, 3, and 4 weeks of storage.

The number of lactic acid bacteria in cottage cheese was counted using the viable cell counting method. Aliquots were plated on plate count agar with bromocresol purple, using 10-fold dilutions, and incubated for 24 h at 37°C. Cell counting was performed after 0, 1, 2, 3, and 4 weeks of storage.

Statistical analysis The data for each treatment were obtained in triplicate, and analysis of variance (ANOVA) and Duncan's multiple range tests were performed using SPSS 18 software (SPSS Inc., Chicago, IL, USA).

Results and Discussion

Tolerance to artificial gastric acid and bile salts The tolerance of *L. plantarum* Lb41 to artificial gastric acid (pH 2.5, 0.3 µg/mL pepsin, for 3 h) and artificial bile salt (0.3 µg/mL oxgall, for 24 h) was assessed to evaluate the probiotic potential of this strain. *L. plantarum* Lb41 experienced a 0.06 log reduction in artificial gastric acid for 3 h and showed a high resistance to acidic conditions (Table 1). *L. plantarum* Lb41 was able to survive with a 1.36 log reduction in bile salt conditions after 24 h. Compared with *L. plantarum* Lb41, *L. plantarum* and *L. acidophilus* showed resistance to artificial gastric acid with a 0.15 and 1.03 log reduction after 3 h, respectively. Further, these strains showed less resistance to artificial bile salts than *L. plantarum* Lb41, with a 2.71 and 2.48 log reduction in cell growth, respectively. Bile salts are toxic for living cells because they compromise the structure of the cell membrane. In general, the bile salt concentration in the human gastrointestinal tract is approximately 0.3% (w/v) based on previous studies (21). *L. plantarum* WLPL04 exhibited tolerance to acidic conditions but displayed an approximate 2 log reduction in growth in bile salt (0.3 µg/mL oxgall, after 24 h) conditions (13). *L. plantarum* Lb41 revealed superior tolerance to acidic conditions and higher survival in artificial bile salts. Therefore, *L. plantarum* Lb41 should be capable of colonizing the human intestine.

Production of enzymes Procarcinogens are converted to carcinogens in the intestine by bacterial β-glucuronidase (22). β-Glucuronidase, regarded as a carcinogenic enzyme was not produced by *L. plantarum* Lb41 (data not shown). However, *L. plantarum* Lb41 produced

leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-galactosidase, and β-glucosidase.

Bacterial adhesion to HT-29 cells To colonize the human intestinal epithelium, adherence is regarded as an essential property of probiotics. Adhesion of *L. plantarum* Lb41 was confirmed by the plate method (Table 1). The initial inoculum of *L. plantarum* Lb41 was 8.68±0.16 log CFU/well. After 2 h, the number of bacteria adhering to HT-29 cells was 7.57±0.06 log CFU/well. Upon the addition of 8.65±0.03 log CFU/well of *L. plantarum*, the number of adherent bacteria was 7.58±0.09 log CFU/well. *L. acidophilus* showed lower adherence than *L. plantarum* Lb41. Inoculation of *L. acidophilus* at a concentration of 7.94±0.14 log CFU/well resulted in attachment at a concentration of 6.55±0.07 log CFU/well. *Lactobacillus* spp. were capable of attaching to human intestinal cell line from 2 to 10%. Previously, *L. lactis* ATCC 11454, *L. paracasei* 34, *L. paracasei* ATCC 27092, and *L. casei* ATCC 393 were shown to have adherence rates of 2-4% to Caco-2 cells (23). In addition, when *L. plantarum* SY11 and SY12 were plated at approximately 8 log CFU/well, they adhered at a concentration of 7.11±0.02 and 6.86±0.11 log CFU/well, respectively (24). Therefore, the adhesion rate of *L. plantarum* Lb41 (7.5% adhesion) was superior and survival was sufficient to exhibit probiotic properties.

Antibiotic susceptibility of *L. plantarum* Lb41 All bacteria used for starter and probiotic cultures must be assessed for antibiotic resistance and monitored to block the transfer of antibiotic resistance genes to other microorganisms, especially pathogenic bacteria (25). Through conjugative plasmids and transposons, antibiotic resistance genes are delivered to other bacteria (26). Tetracycline, streptomycin, erythromycin, or chloramphenicol resistance genes are encoded by R-plasmids in *Lactobacillus* spp. Antibiotic resistance genes such as *tet* (M), *ermAM*, *cat*, and *aphA-3* are encoded by AR genes in the X chromosome of LAB (27). *L. plantarum* Lb41 had resistance to four of eight antibiotics, gentamicin, kanamycin, streptomycin, and ciprofloxacin (data now shown). However, *L. plantarum* Lb41 was inhibited by ampicillin, tetracycline, chloramphenicol, and doxycycline. Previous studies have reported that lactobacilli have high resistance to these antibiotics (26). For *L. plantarum* Lb41, similar results were observed. In addition, *L. plantarum* Lb41 was accepted in CLSI (Clinical and Laboratory Standards Institute) guideline.

Table 1. Probiotic characterization of lactic acid bacteria

Treatment	Cell no. (Log CFU/mL)		
	<i>L. acidophilus</i>	<i>L. plantarum</i>	<i>L. plantarum</i> Lb41
Tolerance to artificial gastric acid and bile salts			
Initial cell no.	9.02 ^{c1} ±0.02	8.62 ^b ±0.01	8.54 ^a ±0.05
pH 2.5, 0.3% (w/v) pepsin, 3 h	7.99 ^a ±0.01	8.47 ^b ±0.03	8.48 ^b ±0.01
0.3% (w/v) oxgall, 24 h	6.55 ^a ±0.07	5.91 ^b ±0.13	7.18 ^c ±0.07
Adhesion to HT-29 cell			
Initial cell no.	7.94 ^a ±0.10	8.65 ^b ±0.02	8.68 ^b ±0.12
Adhesion cell no.	6.55 ^a ±0.08	7.58 ^b ±0.08	7.56 ^b ±0.10

^{1)a-c} Different superscript letters in the same row indicate statistical differences ($p < 0.05$). Values are expressed as mean±SD ($n=3$).

Table 2. Cytotoxicity of *L. plantarum* Lb41 in normal and cancer cells

Cell	Origin	Cytotoxicity (%)		
		<i>L. acidophilus</i>	<i>L. plantarum</i>	<i>L. plantarum</i> Lb41
Normal cell				
MRC-5 cell	Lung	ND ^{1) 2)}	ND ^a	17.19 ^a ±6.63
Cancer cell				
AGS cell	Stomach	94.77 ^c ±1.29	88.80 ^b ±2.23	84.33 ^a ±2.24
HT-29 cell	Colon	99.25 ^b ±1.29	98.51 ^b ±1.29	82.09 ^a ±6.72
LoVo cell	Colon	94.77 ^c ±1.29	81.34 ^a ±3.42	88.06 ^b ±3.42

^{1)a-c}Different superscript letters in the same row indicate statistical differences ($p<0.05$). Values are expressed as mean±SD ($n=3$).

²⁾Not detected.

Fig. 1. The inhibitory effect of *L. plantarum* Lb41 on NO production in RAW 264.7 cells stimulated by lipopolysaccharide. A, non-lipopolysaccharide-stimulated; B, lipopolysaccharide-stimulated; C, *L. plantarum* Lb41 treated in lipopolysaccharide-stimulated. Values are expressed as mean±SD ($n=3$). Different superscript letters in the same row indicate statistical differences ($p<0.05$).

Anticancer activity of *L. plantarum* Lb41 The cytotoxicity of *L. plantarum* Lb41 was assessed against normal and cancer cells through MTT assays (Table 2). *L. plantarum* Lb41 (10^7 CFU/well) induced a superb anti-proliferative effect on AGS and HT-29 cells with 84.33 and 82.09% cell death, respectively. Proliferation of colon adenocarcinoma LoVo cell was inhibited effectively at 88.06%. In contrast, the cytotoxicity of *L. plantarum* Lb41 on normal MRC-5 cells was 17.19%. Therefore, this strain did not inhibit the proliferation of MRC-5 cells, and can be regarded as non-toxic. When *L. plantarum*

SBL was applied to AGS and HT-29 cells, this strain showed a cytotoxic effect of less than 80% against both cell lines. (28). At a concentration of 10^8 CFU/mL, *L. acidophilus* 606 showed cytotoxic effects of approximately 80, 65, 50, and 55% against HT-29, CX-1, WiDr, and DLD-1 cells, respectively (29). These results demonstrate that *L. plantarum* Lb41 has anticancer effects and can inhibit the survival and proliferation of cancer cells effectively.

Anti-inflammatory activity of *L. plantarum* Lb41 The anti-inflammatory activity of *L. plantarum* Lb41 was measured in RAW 264.7 cells, and the inflammatory response was induced with LPS (Fig. 1). NO production decreased in the group cultured with *L. plantarum* Lb41 (2.76 μmol) compared to that in the control LPS-stimulated group (44.71 μmol). Therefore, *L. plantarum* Lb41 had an anti-inflammatory effect.

Composition and viability of LAB during cottage cheese storage

The physicochemical properties of cottage cheese during 4 weeks of storage are presented in Table 3. Two types of cottage cheese did not show any significant differences in total solid, ash, protein, fat, and pH ($p<0.05$). The viable cell number of LAB in control and probiotic cheeses was increased at first, but decreased after the second and third week, respectively. The general shelf life of cottage cheese at 3–4°C is known to be approximately 10–12 days. The number of bacteria in cottage cheese was increased by approximately 2–3 log

Table 3. Change in composition and the number of lactic acid bacteria in cottage cheese during 4 weeks of refrigerated storage at 4°C

Cheese types	Storage period (wk)	Composition					Cell no. (Log CFU/mL)
		Total solid (%)	Ash (%)	Protein (%)	Fat (%)	pH	
Control	0	48.68 ^{c1} ±0.96	1.23 ^a ±0.01	23.97 ^a ±1.31	13.65 ^a ±0.29	4.42 ^b ±0.01	8.27 ^a ±0.11
	1	47.66 ^c ±0.86	1.97 ^c ±0.23	27.39 ^c ±0.14	15.46 ^{bc} ±0.27	4.37 ^a ±0.00	9.12 ^e ±0.02
	2	45.38 ^b ±0.14	1.52 ^b ±0.12	25.79 ^b ±0.14	15.59 ^c ±0.13	4.46 ^c ±0.00	8.97 ^d ±0.03
	3	44.36 ^{ab} ±0.01	1.27 ^a ±0.01	24.61 ^a ±0.29	15.21 ^b ±0.01	4.72 ^e ±0.01	8.66 ^c ±0.08
	4	44.08 ^a ±0.25	1.25 ^a ±0.01	26.55 ^b ±0.04	15.31 ^{bc} ±0.08	4.57 ^d ±0.04	8.54 ^b ±0.09
Probiotic	0	45.70 ^b ±1.95	1.2 ^a ±0.29	28.30 ^b ±1.83	15.45 ^c ±0.21	4.37 ^b ±0.01	8.49 ^a ±0.01
	1	45.65 ^b ±0.97	1.97 ^c ±0.24	23.83 ^a ±0.58	13.95 ^a ±0.03	4.33 ^a ±0.01	8.89 ^b ±0.03
	2	41.91 ^a ±0.24	1.55 ^b ±0.07	25.07 ^a ±0.68	14.5 ^b ±0.25	4.45 ^c ±0.01	9.18 ^d ±0.06
	3	42.78 ^a ±0.25	1.42 ^{ab} ±0.05	24.46 ^a ±0.15	14.63 ^b ±0.06	4.6 ^e ±0.00	9.12 ^{cd} ±0.07
	4	42.15 ^a ±0.20	1.31 ^{ab} ±0.01	25.55 ^a ±0.26	14.66 ^b ±0.08	4.54 ^d ±0.01	9.04 ^c ±0.06

^{1)a-c}Different superscript letters in the same column indicate statistical differences ($p<0.05$). Values are expressed as mean±SD.

CFU/g during 12 days of storage (30). Similarly, the viable cell number of LAB in control cheese was increased until 2 weeks, after which it began to decrease. However, the number of LAB in cottage cheese with the probiotic strain increased until the third week. The rate of decline of viable cells in probiotic cheese was slower than that in control cheese. These results proved that the addition of probiotic bacteria has a positive effect on cottage cheese storage. From these results, we conclude that *L. plantarum* Lb41 has potential for use in cottage cheese as a probiotic carrier.

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