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Probiotic Properties of *Lactobacillus plantarum* NK181 Isolated from *Jeotgal*, a Korean Fermented Food

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Abstract Strain NK181 was isolated for probiotic use from *jeotkal* and based on results of API 50 CHL kit and 16S rDNA sequencing was tentatively named *Lactobacillus plantarum* NK181. *L. plantarum* NK181 was highly resistant to artificial gastric juice (pH 2.5) and bile acid and demonstrated strong adherence to Caco-2 cells. In test using API ZYM kit, eight enzymes were produced. Supernatant of *L. plantarum* NK181 exhibited about 30% 1,1-diphenyl-2-picyryl hedrazyl (DPPH) radical-scavenging activity and reduced cholesterol by 70%. These results demonstrate potential use of *L. plantarum* NK181 as health-promoting probiotic.

Keywords: probiotics, *jeotgal*, *Lactobacillus plantarum*, identification, Caco-2 cell, antioxidative activity, cholesterol-lowering activity

Introduction

At present, much effort is given to the maintenance of health through various approaches such as exercise and natural foods. Use of probiotics, in demand for improved well-being, in humans and animals has been widely discussed (1). Most common bacteria associated with probiotic activity are lactobacilli and bifidobacteria, although non-pathogenic organisms such as certain strains of *Escherichia coli* and non-bacterial organisms such as *Saccharomyces boulardii* have also been used (2, 3). Probiotics can survive gastric conditions and colonize the intestine, reduce lactose intolerance, prevent antibiotic-induced diarrhea and colon cancer, and stimulate the immune system (4).

Lactic acid bacteria (LAB) are generally considered as Gram-positive and catalase-negative bacteria that grow under microaerophilic to strictly anaerobic conditions, and do not form spores. They are common microflora in various fermented foods such as dairy products and processed vegetables, and show a wide variety of cell types and physiological and biochemical behavior. In addition, LAB have been shown to inhibit the *in vitro* growth of many enteric pathogens and have been used in both humans and animals to treat a broad range of gastrointestinal disorders (5). This inhibition may be due to the production of organic acids such as lactic and acetic acid, hydrogen peroxide, bacteriocins, bacteriocin-like substances and possibly biosurfactants (6).

The objectives of this study were to identify the strain NK181 in probiotics from *jeotgal*, a Korean fermented fish food, using carbon source, 16S rDNA sequencing. Characteristics such as tolerance of gastric juice and bile acid, adherence to intestinal epithelial cells, the antioxidative, and cholesterol-lowering effects were investigated.

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Materials and Methods

Bacterial strain and culture media Strain NK181 was isolated from *jeotgal* in LBS medium (Lactobacillus selective medium, BBL, Cockeysville, MD, USA). The strain was incubated in lactobacilli MRS broth (Difco, Detroit, MI, USA) as the growth medium at 37°C, and stored as stock solutions in 20% (v/v) glycerol at -70°C.

Identification of strain NK181 Cell morphology, Gram-staining, API 50 CHL medium, and 16S rDNA were analyzed for identification of the strain NK181. API 50 CHL medium (BioMerieux, Lyon, France) was used to study the carbohydrate use of the strain. 16S rDNA and chromosomal DNA extracted using a Wizard Genomic DNA purification kit (Promega, Wisconsin, WI, USA) were analyzed by polymerase chain reaclion (PCR) using universal primer (7). Amplification products were separated by electrophoresis and purified using a Wizard PCR Preps DNA purification system (Promega). The purified PCR product was inserted into topo vector (Invitrogen, Carlsbad, CA, USA) and sequenced. The sequence was then compared with that of *Lactobacillus* in KRIBB (Korea Research Institute of Bioscience and Biotechnology, Daejon, Korea) database.

Tolerance of artificial gastric juice and artificial bile acid Analysis of artificial digestive fluid tolerance followed the method of Kobayashi *et al.* (8) and Lee *et al.* (9). Initially, cells were harvested by centrifugation at 3860× g rpm at 4 for 10 min. *L. plantarum* NK181 was then suspended in MRS broth containing 1% pepsin, adjusted to pH 2.5 with 0.1 N HCl and cultured for 2 hr at 37°C. Artificial bile acid tolerance was determined by cultivating cells treated with artificial gastric juice. The cells were

incubated for 24 hr at 37°C in artificial bile acid consisting of MRS broth containing 0.1% oxgall (Difco). Viable cells were measured by incubating aliquots on MRS agar plates for 24 hr at 37°C.

Adherence assays The Caco-2 cell line (KCLB 30037) was obtained from the Korean Cell Line Bank (KCLB, Korea). The cells were cultured in Eagle's minimum essential medium (EMEM) supplemented with 20% fetal bovine serum, penicillin G Na (100 units/mL), streptomycin sulfate (100 mg/mL), L-glutamine (292 mg/L), and sodium bicarbonate (2,200 mg/L). For the adhesion assay, monolayers of the Caco-2 cells were placed in 24-well corning tissue culture plates. Cells were seeded at $1\text{-}2\times10^5$ cells to obtain confluence. The culture medium was changed daily, and cultures at post-confluence after 7 days of culture were used. All experiments and maintenance of cells were carried out at 37°C in a 5% CO_2/95% air atmosphere.

The Caco-2 cells were plated onto a 96-multiwell tissue culture plate (Corning, NewYork, NY, USA), incubated for 15 days as mono-layers, and logarithmic phase *L. plantarum* NK181 were washed with phosphate buffered saline (PBS) (pH 7.2). For the adherence assay, 100 μL *L. plantarum* NK181 were mixed with 0.4 mL incomplete Dulbeccos' modified Eagles' medium (DMEM, without streptomycin/penicillin), and incubated for 2 hr at 37°C in 5% CO_2/95% air atmosphere. After incubation, the medium and nonadherent bacteria were removed by washing four times with PBS. Two hundred microliters of 1% Triton X-100 (Sigma) solution was mixed for 10 min, and then 800 μL of 0.85% saline was added. *L. plantarum* NK181 was counted using 1 mL sample.

Enzyme activity The API ZYM kit (BioMerieux, Lyon, France) was used to study enzyme activity. *L. plantarum* NK181 was grown overnight at 37°C on MRS agar. Sediment from centrifuged broth culture was used to prepare the suspension at 10^5 cfu/mL. After inoculation, cultures were incubated for 4 hr at 37°C. Placing a surface-active agent (ZYM A reagent) in the cupules facilitated solubilization of the ZYM B reagent in the medium. Color was allowed to develop for at least 5 min, and values from 0-5 corresponding to the colors developed, were assigned. The approximate number of free nmol hydrolyzed substrate was determined based on the color strength: 0, negative reaction; 1, 5 nmol; 2, 10 nmol; 3, 20 nmol; 4, 30 nmol; 5, 40 or higher.

Spectrum of antimicrobial activity *L. plantarum* NK181 was examined for antimicrobial activity against indicator organisms on MRS agar plates using the modified deferred method. After spotting 3 μL *L. plantarum* NK181 on an MRS agar plate (1.5% agar), the plate was incubated at 37 °C for 24 hr. Five milliliters of the indicator strain's growth media (0.75% agar), containing about 10^7 cells of the indicator strain, was overlaid on MRS agar plates, and after 24 hr incubation at the indicator strain's optimal growth temperature, a clearly visible inhibition halo was obtained. The strength of the antimicrobial activity was expressed based on the diameter (mm) of the inhibition zone, and results are the means of duplicate tests.

Scavenging effect on DPPH radicals (Electron-donating ability) The antioxidative activities of *L. plantarum* NK181 were assessed on the basis of the radical scavenging effect of the stable 1,1-diphenyl-2-picyryl hedrazyl (DPPH) free radical (10). One milliliter of 100 μM DPPH ethanol solution was added to 200 μL sample solutions of different concentrations and allowed to react at room temperature. After 10 min, the absorbance was measured at 528 nm using a spectrometer and converted into the percentage antioxidant activity (AA) using the following formula;

EDA = [1-(absorbance of sample at 528 nm)/(absorbance of control at 528 nm)] × 100.

Each value is the mean of triplicate measurements.

Ability of bacteria to reduce cholesterol *L. plantarum* NK181 was incubated at 37°C for 24 hr. Samples were collected at 0, 2, 4, 6, 8, 10, and 12 hr to measure the cholesterol concentration, and viable bacteria on MRS agar was measured using a spectrometer. For assay of cholesterol, a sample (1 mL) of culture was centrifuged (1710×g, 10 min) and washed with demineralized water (1 mL). The concentrations of total cholesterol in supernatants were analyzed enzymatically using the BCS total cholesterol kit (Bio Clinical System Co., Korea).

Results and Discussion

Identification of strain NK181 as a probiotic strain Strain NK181 was isolated from *jeotgal* using LBS medium. The strain NK181 was Gram-positive, nonmotile, and of the bacillus type as revealed by scanning electorn microscopy (SEM) (Table 1 and Fig. 1). The strain had 99% similarity to those of *L. plantarum* (Table 1), with a 1,462 bp 16S rDNA sequence, which was compared against that in the same region in the genus *Lactobacillus*. Strain NK181 was identified as *L. plantarum* (data not shown), and was tentatively named *L. plantarum* NK181.

Tolerance of artificial gastric juice and bile acid Small intestine and colons of humans and animals contain relatively high concentrations of bile acid, which can inhibit growth of many bacteria. For probiotic bacteria to be effective they must survive the harsh environments in the stomach (low pH) and intestinal track, which contain

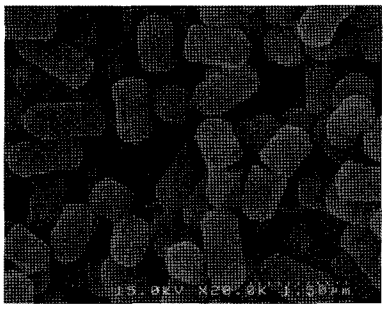

Fig. 1. Scanning electron microscopic (SEM) observation of strain NK 181.

Table 1. Microbiological identification of strain NK181 by carbon source utilization patterns

Carbohydrates	Strain NK181	Carbohydrates	Strain NK181
Morphology	Bacillus	Esculine	-
Gram staining	+	Salicine	+
Glycerol	+[1)]	Cellobiose	+
Erythritol	-	Maltose	+
D-Arabinose	-	Lactose	+
L-Arabinose	-	Melibiose	+
Ribose	+	Saccharose	+
D-Xylose	+	Trehalose	+
L-Xylose	-	Inulin	-
Adonitol	-	Melezitose	+
β-Methylxyloside	-	D-Raffinose	+
Galactose	+	Amidon	-
D-Glucose	+	Glycogen	-
D-Fructose	+	Xylitol	-
D-Mannose	+	β-Gentiobiose	+
L-Sorbose	-	D-Turanose	+
Rhamnose	+	D-Lyxose	-
Dulcitol	-	D-Tagatose	-
Inositol	-	D-Fucose	-
Mannitol	+	L-Fucose	-
Sorbitol	+	D-Arabitol	-
α-Methyl-D-mannoside	+	L-Arabitol	-
α-Methyl-D-glucoside	-	Gluconate	+
N-Acetylglucosamine	+	2-Ketogluconate	-
Amygdaline	+	5-Ketogluconate	+
Arbutine	+		

[1)]Data obtained by API 50 CHL kit. +, positive; -, negative.

Table 2. Survival of *L. plantarum* NK181 in artificial gastric juice for 2 hr at 37°C and artificial bile acid after treatment with artificial gastric juice for 2 hr at 37°C

Control (cfu/mL)	Artificial gastric juice (cfu/mL)	Artificial bile acid (cfu/mL)
$6.4\pm0.70\times10^8$	$3.4\pm0.06\times10^8$	$7.04\pm0.12\times10^8$

bile acid (11). At pH 2.5, viability of *L. plantarum* NK181 did not decrease during 2 hr incubation (Table 2). In addition, the *L. plantarum* NK181 strain tested in the present study was either resistance or tolerant, after 24 hr incubation in MRS broth supplemented with 0.1% oxgall (Table 2). Similarly, relative tolerance of these lactic acid bacteria in acidic environments was found to be dependent on the strain of bacteria. *L. acidophilus, L. bulgaricus, L. rhamnosus*, and *Streptococcus thermophilus* were able to grow well at low pH (12, 13). In addition, *L. acidophilus* was reported to be unaffected by bile acid (12). *Galactomyces geotrichum* SJM-59, isolated from Korean feces was reported the relative viability of 81.62% in pH 2 for 24 hr and over 60% in 900 ppm bile salt for 24 hr (14).

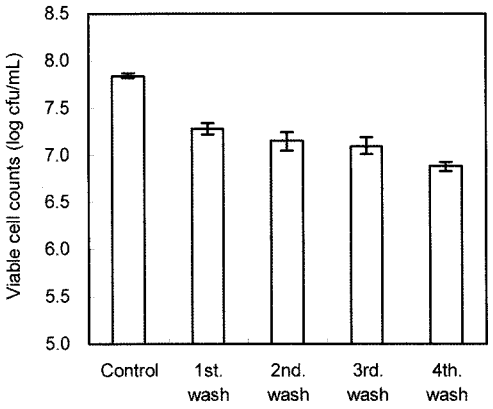

Fig. 2. Mean *L. plantarum* NK181 concentration (expressed as log cfu/mL) in samples cultured from four consecutive serial washings.

Adherence to Caco-2 cells Probiotics pass the acidic stomach to reach the intestine. Caco-2 cells are the most generally used enteric cell line, and closely resemble enterocytes of the human small intestine (3). Caco-2 cells were treated with *L. plantarum* NK181 (7.85±0.01 log cfu/mL) *in vitro* (Fig. 2). The ability of *Lactobacillus* to adhere to Caco-2 cells varies with the species (5, 15, 16). Bernet *et al.* (15) reported that *L. gasseri* has high adherence to Caco-2 cells. Following the 1st, 2nd, 3rd, and 4th washings, the counts of *L. plantarum* NK181 were 7.28±0.06, 7.16± 0.10, 7.10±0.02, and 6.88±0.05 log cfu/mL, respectively. *Lactococcus lactis* NK24 showed high survival (17). These results indicate *L. plantarum* NK181 can pass through the small and large intestines without loss of viability.

The enzyme activity of *L. plantarum* NK181 The enzyme production from strain NK181 was one of the most important criteria in its selection, because carcinogenic enzyme such as β-glucuronidase could be produce by microorganisms (18). When carcinogenic substances such as a benzo(a)pyrene enter the human body, their poisonous effects are counteracted due to conjugation with glucuronic acid in the liver. If this conjugated product is excreted with bile acid in the intestine, cleavage β-glucuronidase can liberate these substances to become toxic once again. On the other hand, enzymes such as lipase, protease, and β-galactosidase have advantages for digestion and the treatment of lactose intolerance. *L. plantarum* NK181 did not produce enzymes such as alkaline phosphatase, esterase (C_4), esterase lipase (C_8), lipase (C_{14}), trypsin, cystine acrylamidase, α-chymotrypsin, α-galactosidase, β-glucuronidase, α-mannosidase, and α-fucosidase (Table 3). However, leucin acrylamidase, valine acrylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-galactosidase, α, β-glucosidase, and N-acetyl-β-glucosaminidase were detected. *L. plantarum* NK181 did not produce the carcinogen enzyme, β-glucuronidase, just 8 enzymes including useful enzyme produce in this study.

Antimicrobial spectrum of activity Probiotics, once they pass through the acidic stomach, are the first microorganisms encountered in the gastrointestinal tract (19). Probiotics can balance intestinal bacteria by

Table 3. Enzyme activities of *L. plantarum* NK181 by API ZYM kit analysis

Enzyme	*L. plantarum* NK181[1)]
Control	0
Alkaline phosphatase	0
Esterase (C_4)	0
Esterase lipase (C_8)	0
Lipase (C_{14})	0
Leucine acrylamidase	5
Valine arylamidase	5
Cystine acrylamidase	0
Trypsin	0
α-Chymotrypsin	0
Acid phosphatase	2
Naphthol-AS-BI-phosphohydrolase	3
α-Galactosidase	0
β-Galactosidase	4
β-Glucuronidase	0
α-Glucosidase	4
β-Glucosidase	2
N-Acetyl-β-glucosaminidase	2
α-Mannosidase	0
α-Fucosidase	0

[1)]0, 0 nmol; 1, 5 nmol; 2, 10 nmol; 3, 20 nmol; 4, 30 nmol; 5, ≥40 nmol.

producing organic acid, bacteriocins, and antimicrobial peptides (20). This may lead to a competitive displacement of intestinal pathogens, the engagement of cell membrane receptors, which activate signaling events leading to cytokine synthesis, including interferons, and cell resistance to viral attack. *L. plantarum* NK181 has a broad spectrum of antimicrobial activities (Table 4), inhibiting, among others, *L. delbrueckii* ATCC 4797, *Pediococcus acidilactici* KCTC 1626, *Bacillus cereus*, *B. pumilis*, *Listeria monocytogenes* ATCC 15313, and *Aeromonas hydrophila*. Moreover, the supernatant of *L. plantarum* NK181 did not cause loss of enzyme (protease IX, protease XIV, trypsin, papain, pepsin, proteinase K) (data not shown). These results show that antimicrobial activity of *L. plantarum* NK181 was not directed towards peptide (bacteriocin), but towards other antimicrobial substances such as organic acid(s). Some studies have been conducted on properties of bacteriocin produced by *L. plantarum* from *kimchi* (21-24).

Evaluation of DPPH radical-scavenging activity Proton radical-scavenging activity is an important mechanism of antioxidation (25). The supernatant of *L. plantarum* NK181 showed 30% DPPH radical-scavenging activity (Fig. 3), which indicates that the culture supernatant of *L. plantarum* NK181 functions as a good antioxidant.

Effect of *L. plantarum* NK181 on cholesterol During the 12 hr incubation, the bacterial number increased, whereas the cholesterol concentration decreased by over 70% (Fig. 4). While MRS medium alone was shown to undergo no decrease in cholesterol (data not shown). These results suggest *L. plantarum* NK181 has cholesterol-lowering effect caused by physiological actions of the end products of short-chain fatty acid fermentation (particularly

Table 4. Spectrum of the antimicrobial activity of *L. plantarum* NK181 by the modified deferred method

Organisms	Culture medium[1)]	Inhibition zone diameter (mm)
Gram positive bacteria		
Lactobacillus delbrueckii ATCC 4797	MRS	15.0
Pediococcus acidilactici KCTC 1626	MRS	15.0
Leuconostoc mesenteroides KCCM 11324	MRS	0
Lactococcus lactis KCCM 40104	MRS	0
Bacillus cereus	TSB	>40.0
Bacillus pumilis	TSB	>40.0
Bacillus subtilis IFO 12113	TSB	0
Listeria monocytogenes ATCC 15313	TSB+0.6%YE	>40.0
Gram negative bacteria		
Aeromonas hydrophila	TSB	>40.0
Chryseomonas luteola SBA 9634	TSB	>40.0
Escherichia coli JM 109	TSB	0
Pseudomonas cepacia SBA 9611	TSB	>40.0
Pseudomonas cepacia SBB 9613	TSB	>40.0
Pseudomonas fluorescens SBB 9631	TSB	>40.0
Pseudomonas putida	TSB	18.0
Sphingomonas paucimobilis BNJ 9664	TSB	>40.0
Xanthomonas maltophila SBC 9611	TSB	>40.0

[1)]MRS, lactobacilli MRS (Difco); TSB, tryptic soy broth (Difco); YE, yeast extract.

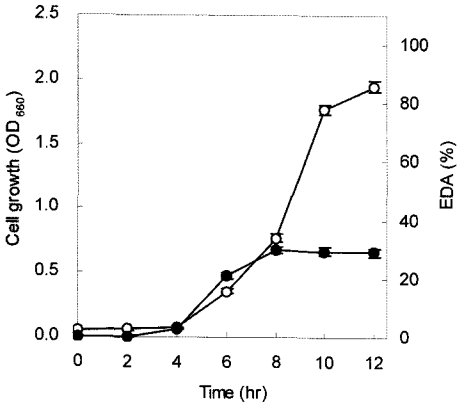

Fig. 3. Scavenging effect of *L. plantarum* NK181 on DPPH radicals. (●), EDA; (○), cell growth.

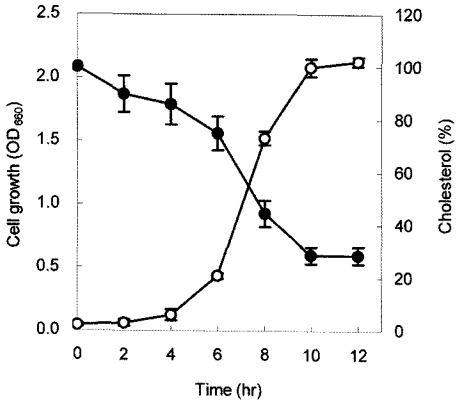

Fig. 4. Cholesterol-lowering activity of *L. plantarum* NK181. (●), cholesterol (%); (○), cell growth.

propionate), cholesterol assimilation by the bacteria, cholesterol binding to the bacteria cell wall, influence of bile acids to reducing cholesterol, disintegration in cell by cholesterol oxidase, inhibition of absorption cholesterol in the intestine and enzymatic deconjugation of bile acids (26).

In conclusion, these studies demonstrate that *L. plantarum* NK181 is a potentially beneficial probiotic strain due to its stability, antimicrobial activity, antioxidative activity, among others. Further investigation of *L. plantarum* NK181 for probiotic use involving *in vivo* and clinical studies is necessary.

Acknowledgments

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