Food Sci. Biotechnol. 23(1): 141-150 (2014)
DOI 10.1007/s10068-014-0019-2

RESEARCH ARTICLE

Antimutagenicity Activity of the Putative Probiotic Strain *Lactobacillus paracasei* subsp. *tolerans* JG22 Isolated from Pepper Leaves Jangajji

Sung-Mee Lim

Received: 8 March 2013 / Revised: 7 June 2013 / Accepted: 31 July 2013 / Published Online: 28 February 2014

Abstract The aim of this study was to evaluate the probiotic nature, antimutagenicity, and mutagen-binding ability of the bacterial strain JG22 isolated from pepper-leaf *jangajji*. The strain, which was identified as *Lactobacillus paracasei* subsp. *tolerans* JG22, retained a high survival rate when exposed to pepsin-containing gastric juice and intestinal fluid supplemented with oxgall. This strain adhered to Caco-2 cells and exhibited resistance to ciprofloxacin, rifampicin, erythromycin, and cephalothin. Furthermore, against the 3 mutagens tested, live cells of strain JG22 displayed the greatest antimutagenicity against 2-nitroflourene (2-NF), with 51.37% inhibition, but exhibited relatively low antimutagenicity (11.07%) against 4-nitroquinoline-1-oxide (4-NQO). The antimutagenic effect of this strain was somewhat reduced after heat treatment for 30 min at 80°C. The most efficient mutagen-binding was observed with live cells of strain JG22, which bound 69.5% of the 2-NF. Thus, the putative probiotic strain JG22 could play a vital role in reducing the risk of cancer by absorbing mutagens and suppressing mutagenesis. Therefore, we consider the strain a good candidate for functional cultures and food system development.

Keywords: Ames test, antimutagenic activity, *Lactobacillus paracasei* subsp. *tolerans*, probiotic

Sung-Mee Lim (✉)
Department of Food Nutrition and Science, Tongmyong University, Busan 608-711, Korea
Tel: +82-51-629-1714; Fax: +82-51-629-1309
E-mail: limsm020@tu.ac.kr

Introduction

Mutagens are generated by various factors, both exogenous (e.g., natural radiation, radon, pesticides, and synthetic dyes) and endogenous (e.g., phagocytes generating H_2O_2, O_2^-, OCl^-, and chloramines during the consumption of living and dead bacterial cells, viral infections, food components, nitrates, and nitrosamine). The accumulation of mutagens and pro-mutagens in the body may induce DNA alteration or damage, thereby leading to genetic mutation and cancer initiation (1).

Some researchers have demonstrated *in vitro* the antimutagenic effects of probiotic lactic acid bacteria (LAB) and food products fermented by LAB starters against food-borne mutagens, including heterocyclic amines formed during cooking, aflatoxin B_1, benzo[a]pyrene, and 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide (AF2) (2-4). *Leuconostoc mesenteroides*, *Lactobacillus brevis, Lactobacillus fermentum*, *Lactobacillus plantarum*, and *Pediococcus acidilactici* isolated from kimchi prevented mutations mediated by 4-nitroquinoline-1-oxide (4-NQO), 2-amino-3,8-dimethylimidazo-[4,5-f]-quinoxaline (MeIQ), and 3-amino-1-methyl-5H-pyrido-[4,3-b]-indole (Trp-P-2). The antimutagenic activity of the strains was found in the cell wall fraction rather than in the cytosolic fraction, regardless of their viability (5). Lankaputhra and Shah (6) demonstrated the broad-spectrum antimutagenic activity of butyric acid and acetic acid produced by probiotics toward chemical mutagens or promutagens; they emphasized the importance of consuming live probiotic bacteria and of maintaining their viability in the intestines to inhibit mutagens. Thus, probiotics that can prevent the risk of DNA genotoxins are thought to be an important tool for reducing the incidence of gut and colon disorders.

Antimutagens that decrease the deleterious effects of mutagens are classified as either desmutagens or bioantimutagens. Desmutagens directly inhibit mutagens or their precursors by means of chemical or enzymatic inactivation. On the other hand, bioantimutagens inhibit the effects of mutagens mainly by acting on DNA replication and repair processes (7). The mechanisms underlying the antimutagenic action of fermented dairy products or probiotic LAB are not clearly understood. Possible mechanisms include detoxification by binding activated mutagens to the cell wall, production of a mutagen-inactivating factor or desmutagen, formation of a mutagen-antimutagen complex, inactivation of promutagens through cytochrome P450, and induction of DNA repair (4,8).

When selecting probiotic strains, several characteristics, such as tolerance to gastric juice and bile salts, resistance to antibiotics, and adhesion to the intestinal surface, must be considered. Probiotic strains that maintain or improve the intestinal microbial balance can protect the host against gastrointestinal disorders, including inflammatory diarrhea and intestinal injury by pathogen infection. In addition, the antimutagenic properties of probiotics have been widely studied (3,6,9). In this study, a strain isolated from pepper-leaf *jangajji*, a fermented food, was identified by morphological, physiological, and biochemical tests and 16S rRNA gene sequencing. In addition, tolerance to acid and bile, resistance to antibiotics, adhesion to Caco-2 cells, and antimutagenic activity against 3 mutagens [*N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG), 2-nitroflourene (2-NF), and 4-NQO] were assessed by comparing the strain from pepper-leaf *jangajji* with the probiotics *Lactobacillus brevis* MLK27 and *Pediococcus pentosaceus* MLK67 obtained from mustard-leaf *kimchi*.

Materials and Methods

Isolation and identification of LAB isolates from pepper-leaf *jangajji* Pepper-leaf *jangajji* was prepared as follows in our laboratory. Soy sauce, sugar, and vinegar mixed at a ratio of 1:1:1 were added to fresh pepper leaves, which were then fermented for approximately 7 days at 4ºC. Samples were homogenized in sterile phosphate buffer solution (PBS, pH 7.0) for 2 min with a Waring blender (Waring Products Division, New Hartford, CT, USA) and suspended in PBS. Subsequently, 100 μL of the diluted solution was spread onto lactobacilli MRS agar (Difco Co., Sparks, MD, USA) plates with 1%(w/v) $CaCO_3$ for 24 h at 37ºC. Typical LAB colonies that formed a clear zone on MRS agar plates were incubated in PBS adjusted to pH 2.0 for 2 h at 37ºC. The bacteria that survived under acidic conditions were collected by centrifugation for 10 min at 7,000×*g*, washed twice with PBS (pH 7.0), and incubated on an MRS agar plate for 24 h at 37°C. Among the acid-tolerant strains, strain JG22 satisfied the criteria for probiotic and exhibited antimutagenic activity in preliminary experiments. The strain was aseptically selected, and a stock culture of the isolated strain was maintained in MRS broth with sterile glycerol (20%, v/v) at 80ºC in a deep freezer.

The selected strain, JG22, was phenotypically characterized by cell morphological, physiological, and biochemical tests including cell shape, Gram staining, spore formation, acid-fast staining, motility, pathogenicity, enzyme production, and optimal growth conditions. In addition, the sugar fermentation patterns and enzyme profiles of the strain were analyzed using an API 50CHL kit (BioMérieux Co., Marcy I'Etoile, France) and an API ZYM kit according to the manufacturer's instructions, respectively. Molecular identification was performed by 16S rRNA gene sequence analysis. The 16S rRNA gene was amplified by polymerase chain reaction (Bio-Rad Laboratories Ltd., Montreal, Quebec, Canada) using the universal primers, 518F (5'-CCAGCAGCCGCGGTAATACG-3') and 800R (5'-TACC AGGGTATCTAATCC-3') according to standard procedures. PCR was performed in 20 μL of reaction solution containing each primer (20 pmol), 10× reaction buffer, *Taq* DNA polymerase, each dNTP, and the isolated DNA. The following conditions were used for PCR: An initial denaturation step of 4 min at 94ºC; followed by 35 cycles each of denaturation for 0.5 min at 94ºC; annealing for 1 min at 50ºC; elongation for 1 min at 72ºC; and a final extension for 5 min at 72ºC. The PCR product was purified with the QIA-quick PCR Purification System (Qiagen, Valencia, CA, USA), verified by 16S rRNA gene sequencing, and compared to sequences in the National Center for Biotechnology Information (NCBI) database. Multiple alignments of the 16S rRNA gene were performed with the CLUSTAL W (version 1.81) program. The MEGA package (version 5.05) was used to reconstruct a phylogeny with 1,000 bootstrap replicates through the neighbor-joining method.

Potential evaluation as a probiotic strain

***Acid and bile tolerance*:** The tolerance of the tested strains to acidic conditions was estimated according to the method of Maragkoudakis *et al*. (10), with minor modifications. In brief, simulated gastric juice was prepared in PBS containing 125 mM NaCl, 7 mM KCl, 45 mM $NaHCO_3$, and 1 mg/mL pepsin (Sigma-Aldrich, St. Louis, MO, USA) and adjusted to pH 2.0 with 6 N HCl. Cells grown overnight in MRS at 37ºC were harvested by centrifugation at 7,000×*g* for 10 min, washed twice with PBS (pH 7.0), and resuspended in the same buffer to adjust the cell density to approximately 1.0×10^9 cells/mL. The cell suspensions (1 mL) were then added to simulated gastric

juice (1 mL), mixed by gentle vortexing, and incubated for 2 h at 37°C. Viable cell counts were determined by plating serial 10-fold dilutions on MRS agar and counting the colony numbers after aerobic incubation for 48 h at 37°C. Each experiment was carried out in duplicate and repeated 3 times. The tolerance of the strains to simulated intestinal fluid was determined by cultivating the cells in the presence of bile salt. In brief, cells grown overnight in MRS at 37°C were harvested by centrifugation (7,000×*g*, 10 min, 4°C) and washed 2 times with PBS (pH 7.0). After adjusting the number of cells to equal the number of viable cells remaining after incubation in gastric juice, the cells were resuspended in PBS containing 0.5%(w/v) oxgall (Sigma-Aldrich) and pancreatin (1 mg/mL) and maintained in an aerobic incubator at 37°C. After 3 h incubation, the cultures were serially diluted in sterile saline and plated on MRS agar to determine the viable cell counts (CFU/mL). The cell viability (%) was calculated as follows: (B/A) ×100, where A represents the initial cell count before incubation in simulated gastric juice and B describes the viable cells remaining after treatment. The bile salt hydrolase (BSH) activity of the tested strains was measured by determining the amount of amino acids liberated from glycine-conjugated bile salts, following the method described by Tankaka *et al*. (11).

***In vitro adhesion assay to Caco-2 cells*:** Adhesion of the selected strains to intestinal epithelial cells was investigated according to the method reported by Tuomola and Salminen (12), with minor modifications. Briefly, Caco-2 cells obtained from the Korean Cell Line Bank (KCLB) were routinely grown in Dulbecco's minimal essential medium (DMEM; Invitrogen-Gibco, Carlsbad, CA, USA) supplemented with 1%(v/v) non-essential amino acids, 100 U/mL penicillin, 50 μg/mL streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, and 20%(v/v) heat-inactivated (for 30 min at 56°C) fetal calf serum (FCS; Invitrogen-Gibco). Caco-2 cells were cultured for 15 days at 37°C in a humidified atmosphere containing 5% CO_2 to obtain confluence before the adhesion assay. During incubation, the culture medium was changed 3 times per week until the cells were completely differentiated. The cells grown overnight in MRS broth at 37°C were harvested by centrifugation (7,000×*g*, 10 min, 4°C), washed twice with PBS (pH 7.0), and then diluted appropriately in non-supplemented DMEM to achieve a cell concentration equal to that of the viable cell populations obtained after treatment with simulated intestinal juice. For the adhesion assay, monolayers of Caco-2 cells were prepared in 6-well tissue culture plates and seeded at a concentration of 5.0×10^5 cells/mL inside the wells. Subsequently, the bacterial DMEM suspensions were added to each well of the tissue culture plate containing Caco-2 monolayers, and the plates were incubated for 2 h at 37°C in a 5% CO_2/95% air atmosphere. The non-adhered bacterial suspensions were aspirated, and the bacterial cells that adhered to the Caco-2 monolayers were washed twice with PBS and detached using trypsin (0.1%)-EDTA (0.53 mM). The detached cells were diluted appropriately with PBS, plated on MRS agar, and incubated for 48 h at 37°C to count the number of bacteria adhered to Caco-2 cells. Each measurement was carried out in triplicate. The adhesion of the strains to Caco-2 cells was expressed as a percentage of the viable bacteria relative to the initial population in the DMEM suspension.

***Antibiotic resistance*:** The following 7 antibiotics were used for antibiotic resistance tests: Ampicillin, cephalothin, and vancomycin as inhibitors of cell wall synthesis; tetracycline and erythromycin as inhibitors of protein synthesis; and ciprofloxacin and rifampicin as inhibitors of nucleic acid synthesis. All antibiotic powders were obtained from Sigma-Aldrich, and stock solutions were kept at 20°C. Each of the antibiotics was diluted to an appropriate concentration (5 μg/mL of ciprofloxacin and rifampicin; 10 μg/mL of ampicillin and erythromycin; and 30 μg/mL of cephalothin, vancomycin, and tetracycline) and filter-sterilized before addition to MRS broth. Cells harvested by centrifugation (7,000×*g*, 10 min) were washed 2 times with PBS (pH 7.0) and resuspended in PBS containing each antibiotic, such that the cell counts equaled the number of cells adhered to Caco-2 cells. After the antibiotic-challenged cells were incubated for 2 h at 37°C, they were collected by centrifugation, plated on MRS agar, and incubated in an aerobic incubator for 48 h at 37°C to measure the viable cells.

Antimutagenic properties of the tested strains

***Ames test*:** MNNG, one of the most potent direct chemical mutagens, was obtained from Fluka (BioChemika, Germany); 2-NF and 4-NQO were purchased from Sigma-Aldrich. The antimutagenic activity of the tested strains was tested without exogenous metabolic activation using S9 mix because the mutagens are direct-acting genotoxins. A stock solution (1 mg/mL) of each mutagen was prepared in dimethyl sulfoxide (DMSO; Sigma-Aldrich) and stored at 20°C until the experiment. Working solutions of all mutagens were obtained at suitable dilutions in 0.05 M sodium phosphate buffer (pH 7.4) just before testing and sterilized through a 0.45-μm Millipore membrane filter (Billerica, MA, USA). The antimutagenicity of the tested strains against MNNG, 2-NF, and 4-NQO was determined by measuring the extent of reverse mutation of *Salmonella* Typhimurium TA98 (*his*) obtained from the Korean Culture Center of Microorganisms (KCCM 11862) according to the Ames test (13). *S.* Typhimurium TA 98 was freshly grown for 18 h at 37°C in Oxoid Nutrient Broth No. 2 (Oxoid, Basingstoke, Hampshire, UK) containing ampicillin

(8 mg/mL). To confirm the histidine requirement of *his* mutants, *S.* Typhimurium TA 98 was incubated for 48 h at 37°C on a minimal glucose agar plate, which contained per liter: Magnesium sulfate, 0.2 g; citric acid monohydrate, 2 g; potassium phosphate dibasic, 10 g; sodium ammonium phosphate, 3.5 g; glucose, 20 g; histidine, 0.05 g; biotin, 0.00074 g; ampicillin, 0.025 g; and agar, 15 g. LAB aerobically grown in MRS broth for 24 h at 37°C were separated from the supernatants by centrifugation (7,000×*g*, 10 min, 4°C), washed twice in PBS (pH 7.0), and resuspended in the same buffer. The cell suspensions were heated for 30 min at 80°C to obtain heat-killed cells. The live and heat-killed cell suspensions and the supernatants (100 μL) were added to sterile glass tubes with the cell cultures of *S.* Typhimurium TA 98 (100 μL), PBS (0.7 mL), and the mutagen diluted in buffer (100 μL). At this point, the final concentration of mutagen was 1 μg/plate for MNNQ, 1 μg/plate for 2-NF, and 0.5 μg/plate for 4-NQO. These concentrations, chosen according to the visual test of Maron and Ames (13), induced a significant frequency of *his*$^+$ revertants without causing detectable toxicity. The mixtures were vortexed, preincubated at 37°C with gentle agitation for 30 min in a shaking incubator, and combined with 2 mL of molten top agar (45°C) containing 0.05 mM L-histidine, 0.05 mM biotin, and 0.09 M NaCl. The solutions were then poured onto minimal glucose agar plates. After incubation for 48 h at 37°C to calculate revertants, the number of *his*$^+$ revertant colonies on the plates was counted and compared with the colony numbers of the control. All experiments were performed in triplicate, and the antimutagenic activity was expressed as a percentage of the mutagenic inhibition according to the following formula: Inhibition (%)=[(AB)/(AC)]×100, where A is the number of histidine revertants induced by the mutagen in the absence of the antimutagen, B is the number of revertants induced by the mutagen in the presence of the antimutagen, and C is the number of spontaneous *his*$^+$ revertants in the absence of the mutagen.

***Mutagen-binding*:** Overnight culture of the LAB were harvested by centrifugation (7,000×*g*, 10 min, 4°C) and washed twice with PBS (pH 7.0). The pellet was resuspended in PBS to yield an A_{600} of 1.30, the absorbance corresponding to a cell density of 1.0×10^{10} cells/mL. The bacterial pellet was then suspended in PBS containing 1 μg/mL of MNNG, 1 μg/mL of 2-NF, and 0.5 μg/mL of 4-NQO, concentrations chosen based on biologic responses in preliminary experiments to induce DNA damage without cytotoxic effects on cell viability. Cells were incubated with each mutagen under agitation (100 rpm) for 100 min at 37°C. The mutagen in PBS without a cell suspension was used as a control. After incubation, the supernatant containing unbound mutagen was collected by centrifugation (7,000×*g*, 10 min, 4°C) and filtered through a 0.45-μm membrane filter. The residual mutagen in the samples after bacterial incubation was quantified by HPLC (Shimadzu, Kyoto, Japan) by the method reported by Srinivasan *et al.* (14), with minor modifications. The mutagen was separated on a Zorbax XDB-C18 column (250 mm×4.6 mm; DuPont, France). The mobile phase consisted of solvent A (water with 0.1% trifluoroacetic acid) and solvent B (acetonitrile with 0.1% trifluoroacetic acid) with a gradient elution in which solvent B was increased from 1% to 15% over 30 min and then rapidly increased to 90% over 10 min. The flow rate was maintained at 1 mL 1/min, and the UV detection wavelength was set at 230 nm. The percentage of mutagen bound to the bacteria was calculated using the following formula: [1(mutagen peak area of sample/ mutagen peak area of control)]×100. In addition, some experiments involved cells previously incubated at 80°C for 30 min to determine the mutagen-binding of heat-killed cells. After heat treatment, the cells were vortexed for 5 min to break coagula before co-incubation. All the assays were performed in triplicate.

Statistical analysis Results on the antimutagenic activity by Ames test are expressed as the mean and standard deviation (SD) of the data obtained from three separate experiments and analyzed by one-way analysis of variance (ANOVA) using SPSS software (version 12.0; Chicago, IL, USA) program. Comparison of means was undertaken by Duncan's multiple range test at 5% significance level ($p<0.05$).

Results and Discussion

Identification of LAB isolated from pepper-leaf *jangajji* Sixteen LAB strains were isolated from pepper-leaf *jangajji* and screened for potential probiotic activity and antimutagenic activity in a preliminary study. Strain JG22, which showed the highest activity among the LAB tested, was selected for further study. Physiological and biochemical tests and 16S rRNA gene sequencing were conducted to determine the genus and species of strain JG22. As shown in Table 1, the selected strain was found to be a gram-positive, rod-shaped, non-spore-forming, non-motile, catalase-positive, oxidase-negative, and urease-negative facultative anaerobe that could produce gas and L-lactic acid from carbohydrate. It was able to grow at 10°C-45°C and in 1%-10% NaCl, and it did not induce hemolysis. In addition, this strain did not produce indole and ammonia from tryptophan and arginine, respectively, and it was not able to hydrolyze starch and reduce nitrate. Additionally, strong leucine and valine arylamidase and acid phosphatase; moderate esterase, cystine arylamidase, and α- and β-galactosidase; and weak alkaline phosphatase, esterase-

Table 1. Morphological and biochemical properties and carbohydrate fermentation ability of JG 22 strain isolated from pepper-leaf *jangajji*

Morphological and biochemical test	Result	Enzyme profile	Result[1)]	Carbohydrate	Result	Carbohydrate	Result
Cell shape	Rod	Alkaline phosphatase	1	Glycerol	-	Salicine	+
Gram staining	+	Esterase	3	Erythritol	-	Cellobiose	+
Spores staining	-	Esterase lipase	1	D-Arabinose	-	Maltose	-
Motility	-	Lipase	-	L-Arabinose	-	Lactose	+
Nitrate reduction	-	Leucine arylamidase	5	Ribose	+	Melibiose	-
Gas from glucose	+	Valine arylamidase	4	D-Xylose	-	Saccharose	+
H_2S production	-	Cystine arylamidase	2	L-Xylose	-	Trehalose	+
Indole production	-	Trypsin	-	Adonitol	-	Inuline	-
Lactic acid	L	α-Chymotrypsin	-	β-Methyl-xyloside	-	Melezitose	+
Methyl red	+	Acid phosphatase	4	Galactose	+	D-raffinose	-
Voges-Proskauer	-	Naphthol-AS-BI-phosphohydrolase	1	D-Glucose	+	Amidon	-
Horse blood hemolysis	-	α-Galactosidase	2	D-Fructose	+	Glycogene	-
Catalase	+	β-Galactosidase	2	D-Mannose	+	Xylitol	-
Oxidase	-	β-Glucuronidase	-	L-Sorbose	-	β-Gentiobiose	-
Urease	-	α-Glucosidase	-	Rhamnose	-	D-Turanose	+
Arginine hydrolysis	-	β-Glucosidase	-	Dulcitol	-	D-Lyxose	-
Lysine hydrolysis	+	*N*-acetyl-β-glucosaminidase	-	Inositol	-	D-Tagatose	+
Ornithine hydrolysis	+	α-Mannosidase	-	Mannitol	+	D-Fucose	-
Starch hydrolysis	-	α-Fucosidase	-	Sorbitol	+	L-Fucose	-
Growth in aerobic condition	+			α-Methyl-D-mannoside	-	D-Arabitol	-
Growth in anaerobic condition	+			α-Methyl-D-glucoside	-	L-Arabitol	-
Growth at 10°C-45°C	+			*N*-Acetyl glucosamine	+	Gluconate	+
Growth in 1%-10% NaCl	+			Amygdaline	+	2-Ceto-gluconate	-
				Arbutine	+	5-Ceto-gluconate	-
				Esculine	-		

[1)]4-5, strong; 2-3, moderate; 1, low enzyme activity

lipases, and naphthol-AS-BI-phosphohydrolase activities were detected in the culture supernatant of strain JG22. However, the activities of lipase, trypsin, α-chymotrypsin, β-glucuronidase, α- and β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase enzymes were not detected.

The JG22 strain identified in this study did not produce the carcinogenic enzyme β-glucuronidase, implicated in the bioavailability of some toxicants and in the formation of carcinogens, mutagens, and various tumor promoters (9). On the other hand, strain JG22 produced β-galactosidase, which is beneficial for lactose intolerance.

According to the sugar fermentation pattern analysis performed using API 50 CHL test strips, the selected strain belongs to the species *Lactobacillus paracasei* subsp. *paracasei.* However, because the carbohydrate fermentation profiles could not clearly define an isolated strain that exhibited a similarity of 95%, we examined the genotypic characteristics of the strain for accurate identification. When we compared the 16S rRNA gene sequences of strain JG22 with those of other strains in the NCBI database, we found that strain JG22 was closely related to *Lactobacillus paracasei* subsp. *tolerans* JCM 1171 (99.24%; Fig. 1). Thus, we definitively identified the strain isolated from pepper-leaf *jangajji* as *L. paracasei* subsp. *tolerans* JG22 from morphological analysis, and biochemical profiles, and genotypic data.

Fermentation of vegetables occurs because of the growth of LAB, which impart unique metabolic characteristics and influence the flavor, shelf life, and texture of fermented products. LAB involved in the fermentation of vegetables play different roles in food processing: (1) preserving food through the formation of inhibitory metabolites, such as organic acid, ethanol, bacteriocin, hydrogen peroxide, and diacetyl; (2) improving food safety through the inhibition of pathogens or removal of toxic compounds; (3) increasing the nutritional value; and (4) influencing the organoleptic quality of food (15). LAB isolated from fermented foods, such as vegetables pickled in soy sauce (*jangajji*), usually belong to the *Leuconostoc*, *Lactobacillus*, and *Pediococcus* genera. Species of *Lactobacillus curvatus*, *Leuconostoc mesenteroides* subsp. *mesenteroides*, *Weissella confusa*, and *Weissella cibaria* were identified from raw or spontaneously fermented peppers. Specific LAB strains

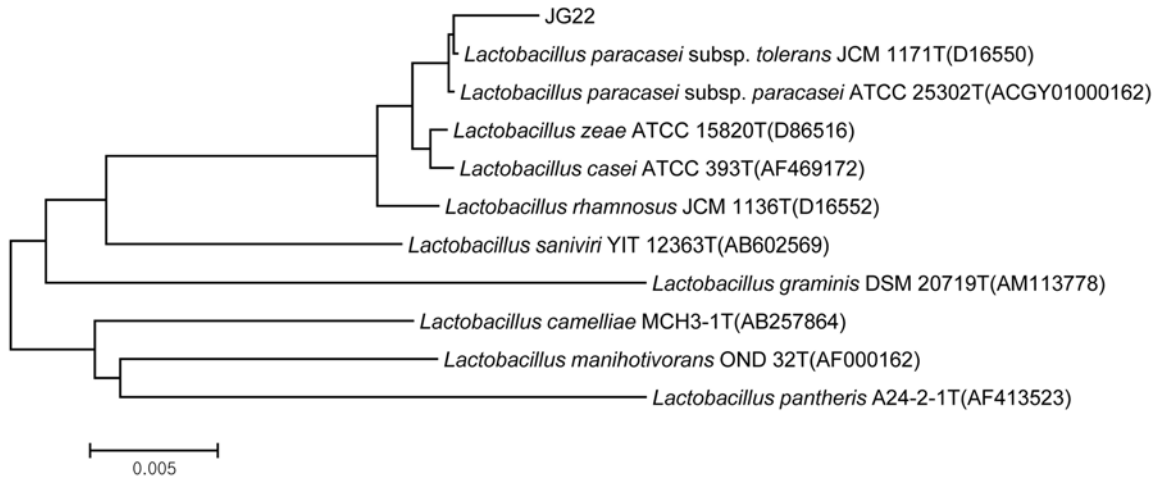

Fig. 1. Phylogenetic tree of *L. paracasei* subsp. *tolerans* JG22 and related species constructed on the basis of 16S rRNA sequences using the neighbor-joining method. The numbers next to the branches indicate percentage values for 1,000 bootstrap replicates. Genbank accession numbers for the sequences are shown in parentheses. The scale bar represents 0.005 substitutions per site.

collected from fermented vegetable pickles have increasingly been considered for incorporation into probiotic cultures that contain high levels of beneficial substances and health-promoting features (16).

***In vitro* evaluation for probiotic potential of *L. paracasei* subsp. *tolerans* JG22** To function in the intestinal tract, probiotic microorganisms must first pass through the stomach, which secretes gastric acid and digestive enzymes. Viability after exposure to bile salts, adhesion to and colonization of epithelial cell lines, and resistance to various antibiotics, as well as the ability to improve intestinal microbial balance and gut functionality, are generally included among the criteria used to select a probiotic strain (10). To evaluate the suitability of *L. paracasei* subsp. *tolerans* JG22 as a probiotic strain, we assessed its survival in a harsh environment, its adhesion to Caco-2 cells, and its resistance to antibiotics (Table 2). Strain JG22 was compared with *L. brevis* MLK27 and *P. pentosaceus* MLK67 strains, which showed probiotic activity, such as antibacterial activity and cholesterol assimilation ability, respectively, in previous studies (17,18). When exposed to pepsin-containing simulated gastric juice (pH 2.0) for 2 h, the cell counts of *L. paracasei* subsp. *tolerans* JG22 were more than 7 log cycles from the initial concentration (2.0×10^9 CFU/mL). The viable cell count of *P. pentosaceus* MLK67 was similar to that of strain JG22, but *L. brevis* MLK27 exhibited higher viability than the other 2 strains under the same conditions. Maragkoudakis *et al*. (10) observed a wide variation in the survival of LAB subjected to pepsin solution at pH 2.0 *Lactobacillus rhamnosus* ACA-DC 112 and *L. paracasei* subsp. *paracasei* ACA-DC 130 strains showed <2.0 log cycles reduction after 3 h of exposure to pepsin, but *Lactobacillus acidophilus* ACA-DC 295, *L. paracasei* subsp. *paracasei* ACA-DC 116, and *L. paracasei* subsp. *tolerans* ACA-DC 4037 strains showed loss of viability of >3 log cycles. Because some strains possess numerous acid-shock proteins that promote survival, they can survive exposure to extreme acidic environments (19). Furthermore, they have mechanisms for acid tolerance, such as operation of F_1F_0-ATPase proton pumps and specific signal transduction systems, neutralization by alkali compounds, alteration of the bacterial membrane, formation of biofilms, and transition of metabolic pathways (20).

After acidic preincubation, the cells of *L. paracasei* subsp. *tolerans* JG22 exhibited a significantly high survival rate (74.34±13.24%) in simulated intestinal juice consisting of 0.5% oxgall, a concentration higher than that usually recommended for simulating the small intestine environment (0.3%) (21). Moreover, *L. brevis* MLK27 and *P. pentosaceus* MLK67 were relatively resistant to 0.5% oxgall, although the survival of these strains was lower than that of strain JG22. Burns *et al.* (22) showed that *Lactobacillus casei, L. paracasei, L. plantarum, and L. acidophilus* (with the exception of *L. acidophilus* DRU) displayed much higher tolerance to bile salts than *L. delbrueckii* subsp. *bulgaricus*, *L. delbrueckii* subsp. *lactis*, and *L. helveticus* strains. Bile salts that enhance the emulsification and absorption of lipid nutrients are synthesized from cholesterol in the liver, secreted from the gall bladder into the duodenum by formation of *N*-acyl compounds conjugated with glycine or taurine, and reabsorbed in the distal small and large intestine following enzymatic deconjugation by resident bacterial flora (21). The ability of probiotic strains to hydrolyze bile salts is often included among the criteria for potential probiotic selection, and this ability may play a

Table 2. Tolerance to simulated gastric and intestinal juices, adhesion to Caco-2 cells, and resistance to antibiotics of LAB obtained from pepper-leaf *jangajji* and mustard leaf kimchi

Treatment condition		Strain	Initial cell counts before treatment (CFU/mL)	Viable/Adhered cell counts after treatment (CFU/mL)	Survival/Adhesion (%)
Tolerance in pepsin at pH 2.0		JG22	$2.00\pm0.15\times10^{9}$[1)]	$8.28\pm2.01\times10^{7}$	4.10±0.70
		MLK27	$2.03\pm0.25\times10^{9}$	$1.69\pm0.75\times10^{8}$	8.72±4.85
		MLK67	$2.35\pm0.36\times10^{9}$	$9.70\pm0.98\times10^{7}$	4.23±0.95
Tolerance in 0.5% oxgall		JG22	$8.28\pm2.01\times10^{7}$	$5.98\pm0.43\times10^{7}$	74.34±13.24
		MLK27	$1.69\pm0.75\times10^{8}$	$2.35\pm0.85\times10^{7}$	13.90±3.55
		MLK67	$9.70\pm0.98\times10^{7}$	$2.77\pm1.00\times10^{7}$	28.07±7.64
Adhesion to Caco-2 cells		JG22	$5.98\pm0.43\times10^{7}$	$9.42\pm1.50\times10^{6}$	15.69±1.38
		MLK27	$9.35\pm0.85\times10^{6}$	$2.55\pm0.83\times10^{6}$	27.27±7.00
		MLK67	$2.77\pm1.00\times10^{7}$	$3.15\pm2.56\times10^{6}$	11.37±0.02
Resistance to antibiotics	Ciprofloxacin (5 μg/mL)	JG22	$9.42\pm1.50\times10^{6}$	$6.23\pm0.56\times10^{6}$	66.62±4.79
		MLK27	$2.05\pm0.83\times10^{7}$	$1.05\pm0.23\times10^{7}$	54.87±13.29
		MLK67	$6.15\pm2.56\times10^{4}$	$3.34\pm1.47\times10^{4}$	53.85±1.60
	Rifampicin (5 μg/mL)	JG22	$9.42\pm1.50\times10^{6}$	$3.66\pm1.20\times10^{6}$	38.11±6.77
		MLK27	$2.05\pm0.83\times10^{7}$	$4.33\pm0.50\times10^{4}$	0.23±0.08
		MLK67	$6.15\pm2.56\times10^{4}$	$5.30\pm0.29\times10^{4}$	96.56±38.49
	Ampicillin (10 μg/mL)	JG22	$9.42\pm1.50\times10^{6}$	$8.52\pm2.04\times10^{5}$	8.96±0.75
		MLK27	$2.05\pm0.83\times10^{7}$	$7.25\pm1.68\times10^{5}$	3.75±0.79
		MLK67	$6.15\pm2.56\times10^{4}$	$5.05\pm2.54\times10^{4}$	79.66±9.05
	Erythromycin (10 μg/mL)	JG22	$9.42\pm1.50\times10^{6}$	$4.56\pm1.08\times10^{6}$	47.99±4.02
		MLK27	$2.05\pm0.83\times10^{7}$	$3.21\pm0.25\times10^{5}$	1.74±0.66
		MLK67	$6.15\pm2.56\times10^{4}$	$6.06\pm1.33\times10^{3}$	10.48±2.35
	Cephalothin (30 μg/mL)	JG22	$9.42\pm1.50\times10^{6}$	$7.03\pm1.02\times10^{6}$	74.65±1.12
		MLK27	$2.05\pm0.83\times10^{7}$	$2.71\pm1.61\times10^{5}$	1.26±0.30
		MLK67	$6.15\pm2.56\times10^{4}$	$8.20\pm1.98\times10^{3}$	14.11±3.00
	Vancomycin (30 μg/mL)	JG22	$9.42\pm1.50\times10^{6}$	$9.09\pm1.66\times10^{4}$	0.96±0.02
		MLK27	$2.05\pm0.83\times10^{7}$	$5.51\pm2.32\times10^{5}$	2.69±0.12
		MLK67	$6.15\pm2.56\times10^{4}$	$1.10\pm0.53\times10^{2}$	0.17±0.02
	Tetracycline (30 μg/mL)	JG22	$9.42\pm1.50\times10^{6}$	$1.73\pm0.30\times10^{5}$	1.83±0.03
		MLK27	$2.05\pm0.83\times10^{7}$	$3.27\pm1.03\times10^{6}$	19.79±14.44
		MLK67	$6.15\pm2.56\times10^{4}$	$7.99\pm0.95\times10^{1}$	0.14±0.05

[1)]Experiments were performed in triplicate and results were expressed as means±SD.

role in maintaining the equilibrium of gut microflora. Some probiotics, such as *L. acidophilus*, produce BSH, which catalyzes the hydrolysis of glycine- and/or taurine-conjugated bile salts into amino acid residues and free bile salts (23). Deconjugated bile salts lower the cholesterol levels in blood serum by interfering with the enterohepatic absorption of cholesterol at pH 5.5 or less. Strain JG 22 showed high BSH activity (1.27±0.58 U/mL) toward glycine-conjugated bile; therefore, this strain may be able to survive in and colonize the intestinal tract and reduce serum cholesterol levels. The BSH activity of strain JG22 was slightly higher than that of *L. brevis* MLK27 (0.92±0.24 U/mL) and *P. pentosaceus* MLK67 (0.89±0.20 U/mL), which showed the strong BSH activity towards glycine-conjugated bile in our previous study (18). These findings are in agreement with Maragkoudakis *et al*. (10), who reported that *L. paracasei* subsp. *tolerans* ACA-DC177, ACA-DC196, and ACA-DC4037 strains exhibited BSH activity on MRS agar, enriched with 0.5%(w/v) bile acid. In addition, *L. brevis* BCCM 7944 and BCCM 11998 strains expressed both taurodeoxycolic acid hydrolase and taurocholic acid hydrolase activities (24). *P. pentosaceus* VJ13, VJ41, VJ56, and VJ49 strains produced BSH enzyme (25).

For *L. paracasei* subsp. *tolerans* JG22 cells that survived in intestinal fluid, the adherence rate to Caco-2 cells was 15.69%±1.38%, which was somewhat lower than that of *L. brevis* MLK27 cells. However, the adherence rate of strain

Table 3. Inhibition of MNNG, NP, and 4-NQO-induced mutagenesis in *S.* Typhimurium TA98 by live and heat-killed cells of LAB

Strain		MNNG (1 μg/plate)		2-NF (1 μg/plate)		4-NQO (0.5 μg/plate)	
		Number of revertants (plate±SD)	Inhibition (%)	Number of revertants (plate±SD)	Inhibition (%)	Number of revertants (plate±SD)	Inhibition (%)
	Positive control	130±20[1)]		308±28		194±17	
	Spontaneous revertant	27±5		29±4		30±5	
JG22	Cell-free supernatant	132±23	ND	297±25	3.87±0.75	201±33	ND
	Live cell	98±11*[2)]	30.47±4.58	165±21*	51.37±1.90	176±18	11.07±1.40
	Heat-killed cell	113±13	16.07±4.47	199±35*	39.43±5.91	185±29	5.87±7.76
MLK27	Cell-free supernatant	129±22	ND	299±31	3.31±1.37	201±25	ND
	Live cell	133±22	ND	212±23*	34.48±1.18	177±21	10.37±2.77
	Heat-killed cell	131±25	ND	267±28	14.77±1.28	195±20	ND
MLK67	Cell-free supernatant	135±27	ND	302±30	ND	201±22	ND
	Live cell	140±28	ND	311±33	ND	199±25	ND
	Heat-killed cell	131±19	ND	312±29	ND	192±28	ND

[1)]Results are presented as means±SD for three plates.
[2)]*Statistically significant difference compared to the positive control value, ANOVA test ($p<0.05$); ND, not detected

JG22 was slightly higher than that of probiotic strains such as *L. casei*, *L. acidophilus* 1, *L. rhamnosus* LC-705, and *Lactobacillus* GG ATCC 53103 (12). Adhesion and colonization at the intestinal surface may be important prerequisites for probiotic strains to have a beneficial effect in the large intestine. Probiotics that adhere to epithelial cells may prevent damage to the mucosa by competitive exclusion or displacement of pathogenic bacteria (9). Granato *et al.* (26) noted that adhesion was mediated by other, more specific, mechanisms involving cell-surface proteins and carbohydrates on the cell wall of LAB. The cell surface hydrophobicity of strain JG 22 was 30.52±6.84% (data not shown), suggesting that the adhesion ability of this strain is derived from its hydrophobicity. Furthermore, the hydrophobicity and adherence of *L. brevis* MLK27 showed some correlation. However, the adhesion ability of *P. pentosaceus* MLK67, which showed very low hydrophobicity, may involve another mechanism (17).

L. paracasei subsp. *tolerans* JG22 was sensitive to ampicillin, vancomycin, and tetracycline but was resistant to ciprofloxacin, rifampicin, erythromycin, and cephalothin. In particular, strain JG22 showed greater resistance to ciprofloxacin, erythromycin, and cephalothin than the MLK27 and MLK67 strains. Thus, the antibiotic resistance of the LAB tested varied depending on the type of antibiotic and the strain. The results for the antibiotic resistance of strain JG22 closely corresponded with those of Kirtzalidou *et al.* (27), who found that the *L. paracasei* C4, C41, C65, C68, C69, C70, and C71 strains isolated from infant faeces were susceptible to ampicillin, amoxicillin, clavulanic acid, tetracycline, erythromycin, cephalothin, chloramphenicol, and rifampicin. Antibiotic resistance, which can readily spread to other bacterial genera, occurs via 3 major mechanisms: (i) blocking of the interaction between the drug and the target molecule through mutation of key binding elements, such as ribosomal RNA, or by reprogramming of biosynthetic pathways; (ii) efflux of the antibiotic from the cytosol by cell membrane-associated pumping proteins; and (iii) synthesis of specific enzymes that degrade or modify the compound (28).

Antimutagenic properties of live and heat-killed *L. paracasei* subsp. *tolerans* JG22 Table 3 shows the number of *S.* Typhimurium TA 98 revertants exposed to MNNG, 2-NF, and 4-NQO in the presence of live and heat-killed cells of *L. paracasei* subsp. *tolerans* JG22. To examine the cell components that may contribute to the antimutagenic activity of strain JG22, the antimutagenic activity of the cell-free supernatants and the cell pellets separated from the strain cultures was also determined using the Ames test.

The viable cells of strain JG22 significantly ($p<0.05$) reduced the number of *S.* Typhimurium TA98 revertants when MNNG or 2-NF was used as the mutagen. Live bacterial cells showed the greatest antimutagenicity against 2-NF, with 51.37% inhibition, but relatively low antimutagenicity (11.07%) against 4-NQO. The antimutagenicity of live cells of strain MLK27 against 2-NF and 4-NQO was 34.48±1.18% and 10.37±2.77%, respectively, which was lower than that of strain JG22. When the JG22 and MLK27 strains were heat treated (for 30 min at 80°C) before exposure to each mutagen, the antimutagenic effect was somewhat reduced. No antimutagenic activity was observed when MNNG and 4-NQO were mixed with the cell-free supernatants recovered from the stationary growth

Table 4. Mutagen-binding ability by live and heat-killed cells of LAB against MNNG, 2-NF, and 4-NQO

Strain	Cell type	Mutagen-binding (%)		
		MNNG (1 μg)	2-NF (1 μg)	4-NQO (0.5 μg)
JG22	Live cell	48.82±2.90[1)]	69.50±1.98	17.02±2.05
	Heat-killed cell	28.29±3.42	40.97±3.87	9.45±1.52
MLK27	Live cell	ND	36.17±5.06	12.91±2.08
	Heat-killed cell	ND	20.88±7.11	ND

[1)]Results are presented as means±SD for three plates; ND, not detected

phase of strain JG22 or MLK27. The *L. brevis* MLK27 strain did not show antimutagenic activity against MNNG, and the *P. pentosaceus* MLK67 strain did not show any antimutagenic activity against the 3 mutagens tested.

Generally, the antimutagenicity of probiotic LAB, bifidobacteria, and propionibacteria depends on desmutagenicity (8). Lankaputhra and Shah (6) reported that the live cells of *L. acidophilus* 2400 reduced the mutagenicity of MNNG, 2-NF, and 4-NQO by more than 50%. Additionally, Nadathur *et al*. (29) demonstrated that a strain of *L. acidophilus* obtained from the extracts of fermented milk inhibited MNNG by 59%-95%. Furthermore, Cassand *et al*. (30) noted that cultured milk containing *L. acidophilus* and bifidobacteria reduced the mutagenic activity of 4-NQO and 2-NF by 20%-60%. Thus, the antimutagenic activity of bacteria is strain-specific.

The mutagenic activity of MNNG and 2-NF was strongly suppressed by live cells of *L. paracasei* subsp. *tolerans* JG22, suggesting that this probiotic strain plays an important role in preventing mutagen formation in the intestinal tract. Strain JG22 could be used to treat obstructing colon cancer. The antimutagenic activity resided in the cell pellet, and not the cell-free supernatant. Therefore, the strain tested may inhibit mutagens through chemical-biological interaction, including simple absorption or binding to cell wall components. Because the antimutagenicity of heat-killed *L. paracasei* subsp. *tolerans* JG22 was lower than that of live cells, the antimutagenic effect of the strain likely involves thermolabile components in the cell wall or enzymes present in the cytoplasmic membrane.

In studies involving *L. acidophilus*, Pool-Zobel *et al.* (3) observed anti-MNNG activity in the peptidoglycan fraction, freeze-dried whole cells, and the acetone extract containing soluble metabolites, but not in heat-inactivated cells, the cell wall skeleton, and the cytoplasmic fraction. In contrast, the antimutagenic activity of *Propionibacterium* spp. against 4-NQO was related to genotoxin link by extracellular sulfhydryl compounds (4). Lankaputhra and Shah (6) determined that organic acids produced by live bacterial cells of probiotic bacteria showed higher broad-spectrum antimutagenic activity against several mutagens and promutagens than heat-killed cells.

Mutagen-binding ability of live and heat-killed *L. paracasei* subsp. *tolerans* JG22 Information related to the mutagen-binding ability of live and heat-killed *L. paracasei* subsp. *tolerans* JG22 and *L. brevis* MLK27 is presented in Table 4. These strains possessed variable mutagen-binding ability against MNNG, 2-NF, and 4-NQO. The most efficient binding was observed with live cells of *L. paracasei* subsp. *tolerans* JG22, which bound 69.50% of 2-NF. This strain showed weaker binding (17.02±2.05%) to 4-NQO than to MNNG and 2-NF. The mutagen-binding ability was significantly reduced when *L. paracasei* subsp. *tolerans* JG22 was heat-treated for 30 min at 80°C. The mutagen-binding of live cells of *L. brevis* MLK27 for 2-NF and 4-NQO was significantly lower than that of strain JG22, and no binding ability was observed in the presence of MNNG. The extent of mutagen-binding by *L. paracasei* subsp. *tolerans* JG22 differed depending on the type of mutagen and the cell activity, which is consistent with the findings of Lankaputhra and Shah (6). Thus, *L. paracasei* subsp. *tolerans* JG22 may play a vital role in reducing the risk of cancer by absorbing mutagens and thereby suppressing mutagenesis.

Zhang and Ohta (31) found that peptidoglycan complex and polysaccharides isolated from the cell wall of *Streptococcus cremoris* Z-25 and *L. acidophilus* IFO 13951 strongly bound some mutagenic amino acid pyrolysates, which are produced during high-temperature cooking. In addition, Sreekumar and Hosono (32) studied the antimutagenicity and binding properties of heterocyclic amine-binding receptors obtained from the cell walls of *L. gasseri* and *Bifidobacterium longum*; they found that the degree of heterocyclic amine binding positively correlated with carbohydrate content. The binding ability of *L. gasseri* strains is dependent on many factors, such as bacterial species, cell wall structures, pH, and the chemical complexity of the mutagen (33). Lankaputhra and Shah (6) demonstrated that live cells bound or inhibited the mutagens permanently and that mutagen-binding by viable cells was higher than binding by heat-treated cells because cell components, such as carbohydrates and proteins, that comprise binding receptors for the absorption of mutagens are vulnerable to heat.

In conclusion, *L. paracasei* subsp. *tolerans* JG22 exhibited

high tolerance to acidic conditions (pH 2.0) and bile salts (0.5%), strong resistance to various antibiotics, and good adhesion to epithelial cells. Therefore, this strain could maintain activity in the simulated gut environment. Furthermore, because *L. paracasei* subsp. *tolerans* JG22 had strong antimutagenic activity when co-incubated with MNNG and 2-NF mutagens, this bacterium, identified as a putative probiotic, is a good candidate for functional cultures and food system development to help prevent colon mutagenesis or tumorigenesis.

In the future, we will conduct additional experiments to evaluate the protective effect of *L. paracasei* subsp. *tolerans* JG22 in *in vivo* conditions and to validate its use in functional foods and pharmaceuticals.

Acknowledgments This study was supported by a Research Grant of Tongmyong University.

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