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Screening and Characterization of Probiotic Lactic Acid Bacteria Isolated from Korean Fermented Foods

Lim, Sung-Mee[1]* and Dong-Soon Im[2]

[1]*Department of Food Science and Technology, Tongmyong University, Busan 608-735, Korea*
[2]*College of Pharmacy and Research Institute for Drug Development, Pusan National University, Busan 609-735, Korea*

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To examine their potential as probiotics, acid and bile tolerance, antibiotics resistance, adhesion capacity to Caco-2 and HT-29, and antibacterial activity, of LAB isolated from Korean fermented foods such as *dongchimi*, *kimchi*, *Meju*, and *doenjang* were assayed against foodborne pathogenic bacteria. DC 55, DC 136, DC 222, KC 21, KC 24, KC 34, KC 43, KC 117, MJ 54, MJ 301, SP 33, and SP 170 strains were resistant to acid and bile conditions. In particular, DC 55, DC 136, KC 24, KC 43, and MJ 301 strains were highly resistant to higher than 20 μg/ml concentrations of vancomycin, streptomycin sulfate, or amoxicillin, whereas, DC 222, KC 21, KC 34, KC 117, MJ 54, and SP 33 strains were susceptible to lower than 2 μg/ml concentrations of those antibiotics. The adhesion to HT-29 and Caco-2 cells varied with the strains tested in a strain-dependent manner. The highest level of adhesion was observed with DC 55, KC 21, KC 24, and MJ 301 strains, having higher than 50% of adhesion to HT-29 or Caco-2 cells. In addition, *Staphylococcus aureus* was the most sensitive to KC 21, showing an inhibition of about 70%, and the antibacterial activity of KC 21 against *S. aureus* resulted most likely from both organic acids and bacteriocin. Based on its phenotypic characteristics and utilization of various sugars, the KC 21 strain was identified as *Lactobacillus plantarum*.

Keywords: Probiotics, acid and bile tolerance, antibiotics resistance, adhesion capacity, antibacterial activity

Lactic acid bacteria (LAB) are typically involved in a large number of spontaneous food fermentations such as cheese, yoghurt, butter, and *Kimchi*. Furthermore, they are closely associated with the human environment. LAB associated with fermented foods include species of the genera *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella* [60].

There has been much recent interest in the use of various strains of LAB as probiotics; that is, as live microbial feed supplements that beneficially affect the host by improving its intestinal microbial balance [17]. The probiotic microorganisms possess properties that make humans or animals healthier (e.g., protection against infections with pathogenic microorganisms, decrease of incidence and duration of antibiotic- and rotavirus-associated travelers' diarrhea, alleviation of symptoms of lactose intolerance, reduction of allergic reactions, anti-colon cancer and antimutagenic activities, antihypertensive or anticholesterol effects) [42]. Logan and Katzman [37] suggested that probiotics which have the potential to lower systemic inflammatory cytokines, decrease oxidative stress, improve nutritional status, and predominate beneficial bacteria in the small intestine may be an adjuvant to standard care in major depression disorder.

Because of recently growing demand for "healthy" foods and increasing consumer health consciousness and expenditure continuously, the food industry associated with probiotic products has a central role in facilitating consumer's health and represents a rapid growth within the global market [41, 27]. Probiotics microorganisms of the genera *Lactobacillus*, *Bifidobacterium*, and *Saccharomyces*, which are used not only in fermented dairy products and infant formula but also in pharmaceutical preparations have been recognized for their "gerally recognized as safe (GRAS)" status [53, 54].

The expected beneficial characteristics of probiotic strains fall into five basic categories: physiological, immunological, metabolic and genetic traits, and technological properties. Potential probiotic strains have to include characteristics such as nontoxicity, nonpathogenicity, ability to modulate

*Corresponding author
Phone: +82-51-629-1714; Fax: +82-51-629-1719;
E-mail: limsm020@tu.ac.kr

immune responses, and production of antimicrobial substances. In addition, they should be able to survive and proliferate in the target site, resist gastric acid and bile, adhere to the gastrointestinal tract, and antagonize pathogenic bacteria such as *Salmonella typhimurium*, *Listeria monocytogenes*, *Helicobacter pylori*, and *S. aureus* [42, 30].

The nutritious and therapeutic benefits of probiotic microorganisms have been most extensively investigated in dairy products such as milk, yogurt, and cheese [21, 31, 43, 66]; however, the potential probiotics properties of LAB isolated from traditional Korean fermentation foods have rarely been reported. In the present study, therefore, to determine the potential uses of LAB as probiotic strains, we assessed LAB isolated from various fermented foods such as *dongchimi*, *kimchi*, *meju*, and soybean paste (*doenjang*) for their properties including acid resistance, bile tolerance, adhesion to Caco-2 and HT-29 cells, antibiotics resistance, and *in vitro* antagonism against food-poisoning bacteria.

MATERIALS AND METHODS

Media and Reagents

Lactobacilli MRS broth and Brain Heart Infusion (BHI) broth were purchased from the Difco Co. (Franklin Lakes, NJ, U.S.A.). Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were from GIBCO (Invitrogen Ltd., Carlsbad, CA, U.S.A.). The API CHL 50 kit was obtained from the biomërieux Co. (Marcy I'Etoile, France), and other reagents and antibiotics were provided by Sigma-Aldrich (St. Louis, MO, U.S.A.).

Isolation and Identification of LAB from Korean Fermented Foods

The traditional Korean fermented food *doenjang* was purchased from the local Oriental grocery store in Busan and Gyeongnam. *Kimchi* and *dongchimi* were prepared with Chinese cabbage and radish in our laboratory and home. All samples were homogenized with sterile phosphate-buffered saline (PBS, pH 7.2) for 5 min with a blender. Appropriate dilutions were made with PBS, and a 0.1-ml aliquot was spread-plated onto Lactobacilli MRS agar plate containing 1% $CaCO_3$. Colonies forming a clear zone on the MRS agar plate were selected. Selected strains were characterized by physiological and biochemical tests according to the criteria of *Bergey's Manual of Systematic Bacteriology* [46]. Cell morphology was optical microscopically (DW-THN, Dongwon) determined. Carbohydrate fermentation patterns were determined using the API 50 CH system, according to the manufacturer's instructions.

Acid and Bile Tolerance of LAB

To investigate survival of LAB strains under acidic condition, each strain of LAB was harvested by centrifugation (7,000 $\times g$, for 10 min), washed twice in PBS, inoculated (1%) into MRS broth that had been acidified to pH 2.5 (using HCl) containing 1,000 unit of pepsin (Sigma, St. Louis, MO, U.S.A.) or non-acidified MRS broth, and incubated at 37°C for 2 h. To estimate bile tolerance of LAB, each strain was harvested by centrifugation (7,000 $\times g$, for 10 min), washed twice in PBS, inoculated (1%) into MRS broth containing 5% and 10% bovine bile, and incubated at 37°C for 24 h. Then, the number of viable LAB cells was determined by serial 10-fold dilution in PBS, and 0.1-ml aliquots were spread evenly on MRS agar. Plates were incubated aerobically at 37°C for 48 h, and the colony-forming units (CFU) were estimated.

Antibiotics Resistance Assay

The antibiotics used for antibiotic susceptibility assay were vancomycin, erythromycin, tetracycline, ampicillin, amoxicillin, and streptomycin sulfate. Antibiotics were dissolved in suitable solvents to make stock solutions, and then filter-sterilized through a 0.2 μm membrane filter (Millipore Corp., Billerica, MA, U.S.A.) and kept at -20°C. Antibiotic resistance testing by Minimal Inhibitory Concentration (MIC), which was defined as the smallest amount of antibiotic needed to totally inhibit the growth of the bacteria after incubation for 48 h, was determined by the disk diffusion method [5] with the following modifications. Each LAB tested was incubated overnight at 37°C in MRS broth and adjusted to approximately 1×10^8 CFU/ml, equivalent to an absorbance reading at 600 nm of 0.08–0.1. Twenty μl of culture was added into the test tube containing 20 ml of melted MRS agar, mixed gently, and the agar was poured into petri dishes. After solidification, sterilized paper disks (Φ 8 mm; Whatman, Florham Park, NJ, U.S.A.) were placed aseptically on the agar surface, and then serial 2-fold dilutions of the antibiotic solution (50 μl) were immediately applied to each disk. Agar plates with antibiotic disks were incubated for 48 h at 37°C. The MIC value (in μg/ml) was obtained by observing a distinct inhibition zone around the disks against the LAB.

Adhesion Assay of LAB

The adhesion properties of the LAB strains were examined using Caco-2 and HT-29 human colon adenocarcinoma epithelial cells, which were obtained from the Korean Cell Line Bank (KCLB). Caco-2 and HT-29 cells were grown in 75 cm^2 tissue culture flasks in DMEM supplemented with 10% (v/v) FBS, 2 mM glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin, and 50 mg/ml streptomycin at 37°C in a humidified 5% CO_2 incubator until approximately 90% confluent. Monolayers of Caco-2 and HT-29 cells were used at late post-confluence culture after 15 days, with a change of medium every 2 days. Just before use, monolayers were washed twice with PBS, and transferred to a 24-well multi-dish containing fresh tissue culture medium; the number of cells was within the range of 1×10^4 cells/well, and kept at 37°C in 5% CO_2-95% air atmosphere.

Prior to the adhesion assay, all the LAB cells were centrifuged for 10 min at 7,000 $\times g$ and washed twice with PBS after culture in MRS for 24 h at 37°C. The LAB cells were resuspened in 1 ml of DMEM, and 100-μl aliquots each of LAB susupension (about 1×10^7 CFU/ml) in triplicate were transferred to 24-well plates with monolayer of Caco-2 and HT-29 cells, respectively, and incubated for 2 h at 37°C in 5% CO_2. After incubation, the cells in each well were washed twice with PBS, fixed overnight with 2% formalin, stained with 2% eosin Y, and again washed twice with 1% acetic acid in 50% ethyl alcohol, and the OD was measured at 570 nm using an enzyme-linked immunosorbent assay (ELISA) reader (Spectrocount; Packard Instruments, Meriden, CT, U.S.A.). Individual histograms were made based on the percentage of the control level. Adhesion assays were conducted in triplicate.

Antibacterial Activity of LAB

Antibacterial activity was tested against foodborne pathogens such as *S. aureus* ATCC 6538, *S. typhimurium* KCTC 2514, *V. parahaemolyticus*

KCTC 2471, and *L. monocytogenes* KCTC 3569 by coculture experiments with the LAB. All foodborne pathogens were obtained from the ATCC (American Type Culture Collection) and KCTC (Korean Collection for Type Culture), and propagated in BHI broth at 37°C under aerobic conditions. All the LAB were grown overnight in MRS broth, and then the LAB as well as foodborne pathogens were centrifuged for 10 min at 7,000 ×*g* and washed twice with PBS (pH 7.2). One hundred µl of the LAB and foodborne pathogens was suspended with sterile PBS, and they were co-inoculated in BHI broth (100 ml) to a cell density of 1×10^6 CFU/ml and aerobically incubated at 37°C. Initially and then at predetermined intervals (after 12 h), 1 ml of cell culture was removed, serially diluted, and plated on TCBS (for *V. parahaemolyticus*), Oxford agar (for *L. monocytogenes*), *Staphylococcus* 110 medium (for *S. aureus*), or SS agar (for *S. typhimurium*) to determine viable cell counts. The agar plates were incubated at 37°C for 24 h, and the number of CFU was estimated. All tests were performed in triplicate, and inhibition was calculated according to the formula:

$$\frac{\text{(CFU/ml in control)}-\text{(CFU/ml in co-incubation culture)}}{\text{(CFU/ml in control)}} \times 100 = \%\ \text{Inhibition}$$

Preparation of Bacteriocin and Bacteriocin Activity Assay

Cell-free culture supernatant of LAB grown in MRS broth at 37°C for 12 h was obtained from a culture of LAB by centrifugation at 7,000 ×*g* for 10 min at 4°C, and the pH was adjusted to 6.5–7.0 using 1 M NaOH. Ammonium sulfate to 50% (w/v) saturation was added to the cell-free culture supernatant, the mixture was stirred overnight at 4°C, and the protein precipitate was obtained at 10,000 ×*g* for 20 min at 4°C. Then, the pellet was solubilized in 10 ml of sodium phosphate buffer (10 mM; pH 6.5), desalted by cellulose dialysis membrane (Spectrum Labs., Gardena, CA, U.S.A.), and filtered through a 0.22-µm membrane filter (Millipore Corp., Billerica, MA, U.S.A.). Bacteriocin activity was quantified by the microtiter plate assay [23]. Each well of the microtiter plate (BD Falcon, Franklin Lakes, NJ, U.S.A.) contained 200 µl of MRS broth, 50 µl of crude bacteriocin solution serially 2-fold diluted, and 100 µl of bacterial suspension of the indicator organisms, *S. aureus* ATCC 6538 (10^6 CFU/ml). After incubation at 37°C for 12 h, the growth inhibitory effect was determined by recording the bacterial growth by absorbance measurements at 660 nm using an enzyme-linked immunosorbent assay (ELISA) reader (Spectrocount; Packard Instruments, Meriden, CT, U.S.A.). One bacteriocin unit (BU) was arbitrarily defined as the reciprocal of the highest dilution fold that inhibited the growth of the indicator bacteria by 50% turbidity of the control culture without bacteriocin. The highest dilution was multiplied by 20 (1 ml: 50 µl) to obtain the activity units per milliliter (BU/ml). Bacteriocin solution (2 ml) was mixed with protease (50 mM Tris-HCl, pH 7.5) solution (2 ml) at a final concentration of 1 mg/ml, and the enzyme was inactivated by heating for 10 min at 80°C after incubation at 37°C for 1 h. Cells of *S. aureus* ATCC 6538 grown up to log-phase in BHI broth were harvested by centrifugation (7,000 ×*g*, for 10 min), washed twice in PBS, and resuspended in their appropriate broth to yield 1.0×10^5 CFU/ml. Bacteriocin untreated with protease or bacteriocin treated with protease was added to give a final concentration of 200 BU/ml. Bacteriocin-treated cells and cells without the treatment were incubated for 24 h at 37°C, and the number of viable cells was estimated by standard plate counting at indicated incubation periods.

Results and Discussion

Tolerance Under Acidic and Bile Conditions

Before reaching the gastrointestinal tract, probiotic bacteria must first survive transit through the stomach and have their health promoting effects as metabolically viable active cells when they arrive in the colon [14]. Therefore, the various LAB isolated from *dongchimi*, *kimchi*, *meju*, and *doenjang* were screened for their acid and bile resistance (Table 1).

DC 39, DC 55, DC 136, DC 222, KC 21, KC 24, KC 34, KC 43, KC 59, KC 117, MJ 07, MJ 54, MJ 301, SP 33, and SP 170 strains were resistant at acidic condition, showing more than 7 log cycle per milliliter after incubation for 2 h at pH 2.5, whereas KC 62, KC 109, and MJ 150 were the most acid-sensitive. Additionally, most of the LAB were stable in 5% bovine bile for 24 h. Among the acid-resistant LAB, DC 136, KC 117, and MJ 301 strains were particularly the most bile-resistant, whereas DC 39, KC 59, and MJ 07 strains were the least bile-resistant, at 10% bovine bile. Accordingly, DC 55, DC 136, DC 222, KC 21, KC 24, KC 34, KC 43, KC 117, MJ 54, MJ 301, SP 33, and SP 170 strains could most likely survive in the stomach and the small intestine, and colonize in the large intestine.

Table 1. Acidic pH and bovine bile tolerance of various LAB isolated from Korean fermented foods.

Strains	Source	Condition		Conc. of bovine bile (%)		
		Control	pH 2.5	Control	5	10
DC 39	*Dongchimi*	8.76	8.30	8.79	7.59	5.01
DC 55	*Dongchimi*	8.41	8.14	8.71	8.04	7.77
DC 89	*Dongchimi*	8.68	3.75	8.60	7.83	4.61
DC 136	*Dongchimi*	8.91	8.63	8.45	8.34	7.96
DC 222	*Dongchimi*	8.85	8.70	8.73	7.94	7.01
KC 21	*Kimchi*	8.04	7.95	8.48	8.36	7.29
KC 24	*Kimchi*	8.67	7.77	8.41	7.79	7.71
KC 34	*Kimchi*	8.57	7.80	8.66	8.51	7.58
KC 43	*Kimchi*	8.91	7.75	8.80	8.30	7.78
KC 59	*Kimchi*	8.41	7.90	8.69	6.63	4.56
KC 62	*Kimchi*	8.85	2.85	8.53	7.64	4.46
KC 96	*Kimchi*	8.70	2.97	8.62	7.15	3.01
KC 109	*Kimchi*	8.56	2.71	8.28	7.38	4.03
KC 117	*Kimchi*	8.80	8.90	8.89	8.04	8.55
MJ 07	*Meju*	8.78	7.70	8.04	6.70	2.80
MJ 54	*Meju*	8.40	8.90	8.59	8.71	7.76
MJ 61	*Meju*	8.88	3.83	8.79	6.48	2.63
MJ 150	*Meju*	8.60	1.66	8.38	6.61	3.74
MJ 301	*Meju*	8.80	7.32	8.34	8.46	7.85
SP 33	*Doenjang*	8.68	7.81	8.46	8.39	7.49
SP 68	*Doenjang*	8.74	5.56	8.28	7.86	6.44
SP 170	*Doenjang*	8.53	7.73	8.40	8.23	7.68

The spore-forming LAB including all *Sporolactobacillus* strains tested, except *Sp. racemicus* IAM 12395, were resistant to pH 3.0 [24]. Masco *et al.* [39] suggested that most *Bifidobacterium* isolated from human origin and probiotic product displayed a considerable loss of viability when exposed to an acidic pepsin-containing solution (pH 2.0), whereas cultures of *B. animalis* ssp. *lactis* were capable of surviving gastric transit. Ding and Shah [13] indicated that, because microencapsulated probiotic bacteria (*L. rhamnosus*, *B. longum*, *L. salivarius*, *L. plantarum*, *L. acidophilus*, and *L. paracasei*) survived better ($P<0.05$) than free probiotic bacteria in MRS containing HCl or oxgall, microencapsulation improved the survival of probiotic bacteria when exposed to acidic condition and bile salts. *L. acidophilus* ATCC 4356 showed similar growth in the presence or absence of bile salt, whereas the growth of *L. casei* ASCC 290 was inhibited in the presence of oxgall compared with the control [36]. Sanchez *et al.* [56] indicated that low-pH adaptation and the acid tolerance response of *B. longum* biotype longum involve changes in the glycolytic flux and the ability to regulate the internal pH. These changes were accompanied by a higher ammonium content in the cytoplasm, most likely due to amino acid deamination and a decrease of bile salt hydrolase activity. Furthermore, the bile-adapted *B. animalis* strain is able to tolerate bile by increasing intracellular ATP reserve and inducing proton pumping by the F_1F_0-ATPase [55].

Resistance of LAB to Antibiotics

Antibiotics are a major tool utilized by the medical and pharmacological industries to fight pathogenic bacteria; however, antibiotic resistance can cause significant danger and suffering for many people with common pathogen infections, and is a growing problem that complicates the treatment of important nosocomial and community-acquired infections [40, 44]. In order to be used as a probiotic, LAB that exhibit profitable effects on the health of the host must show an ability to resist various antibiotics. Therefore, the antibiotics resistance of acid- and bile-resistant LAB was assessed using a modified MIC protocol (Table 2).

DC 55, DC 136, KC 24, KC 43, and MJ 301 strains were highly resistant at higher than 20 μg/ml concentrations of vancomycin, streptomycin sulfate, or amoxicillin, whereas DC 222, KC 21, KC 34, KC 117, MJ 54, and SP 33 strains were susceptible at lower than 2 μg/ml concentrations of those antibiotics. Although DC 55, DC 136, KC 43, and MJ 301 showed high MICs for vancomycin or streptomycin sulfate, they exhibited low MICs for erythromycin, tetracycline, or ampicillin. Consequently, antibiotics resistance against LAB appeared to be species-specific.

In particular, the antibiotic resistance and susceptibility of LAB in the present study was similar to the results of Zhou *et al.* [65], who showed that *Lactobacillus* sp. and *Bifidobacterium* sp. were sensitive to erythromycin, tetracycline, and ampicillin, whereas they were resistant to streptomycin. Many studies on the antibiotic sensitivity and resistance of LAB, which were isolated from various products and human or animal gastrointestinal tracts, have been reported; the lactobacilli strains isolated from infant feces were resistant to kanamycin and streptomycin, but affected by amoxicillin, chloramphenicol, erythromycin, penicillin G, and tetracycline [2]; all *Oenococcus oeni* isolated from wine showed susceptibility to erythromycin, tetracycline, rifampicin, and chloramphenicol, whereas they exhibited resistance to aminoglycosides, vancomycin, sulfamethoxazole and trimethoprim, which could represent intrinsic resistance [52]; the susceptibility and resistance of various LAB to many antibiotics were variable, depending on the species [10, 12, 40].

Garofalo *et al.* [18] detected antibiotic resistance genes, *tet*(M), *tet*(O), *tet*(K), *erm*(A), *erm*(B), *erm*(C), *van*A, *van*B, *aac* (6')-Ie *aph* (2")-Ia, *mec*A, and *bla*Z, encoding

Table 2. MICs of selected antibiotics against acid- and bile-resistant strains.

Isolate No. of LAB	MIC against antibiotics (μg/ml)[a]					
	Vancomycin	Amoxicillin	Streptomycin sulfate	Erythromycin	Tetracycline	Ampicillin
DC 55	12.5	0.78	40.00	2.50	5.00	2.50
DC 136	3.13	0.10	20.00	0.63	0.16	0.63
DC 222	0.39	0.02	0.02	0.31	2.50	0.08
KC 21	0.78	0.20	0.63	0.08	5.00	1.25
KC 24	6.25	0.10	160.00	5.00	0.63	5.00
KC 34	0.10	3.13	0.16	0.63	0.08	0.08
KC 43	25.00	20.00	0.04	0.31	0.04	0.31
KC 117	1.56	1.56	<0.01	0.16	0.02	0.08
MJ 54	0.20	0.20	1.25	<0.01	0.63	1.25
MJ 301	160.00	<0.01	2.50	5.00	0.08	2.50
SP 33	0.20	0.05	1.25	0.16	0.63	0.31
SP 170	10.00	2.50	5.00	0.04	5.00	0.63

[a]The MIC value (in μg/ml) was defined as the smallest amounts of antibiotic that produced a distinct inhibition zone around the disks against the LAB.

resistance to some antibiotics that are widely used in clinical practice, and Kastener *et al.* [26] identified the lincosamide resistance gene *lnu*(A) in *L. reuteri* SD 2112. Irreversible loss of antibiotic resistance by elimination of plasmids from a strain indicates that the resistance is plasmid-linked. The transfer of this plasmid to a sensitive strain that lacked the resistance-associated plasmid makes the strain antibiotic resistant [54]. Ammor *et al.* [1] reported that the genes encoding resistance to tetracycline [*tet*(W), *tet*(O), and *tet*(O/W)], erythromycin and clindamycin [*erm*(B)], and streptomycin [*aph*(E) and *sat*(3)] are located on the bacterial chromosome, except for *tet*(M), which was identified on plasmids in *L. lactis*. O'Connor *et al.* [50] determined the complete nucleotide sequence of plasmid pEOC01 (11,661 bp) from *P. acidilactici* NCIMB 6990 that contains an *erm*B gene encoding erythromycin and clindamycin resistance, and a streptomycin resistance gene, *aad*E. Moreover, Danielsen [11] and Gevers *et al.* [19] studied results on molecular characteristics of the antibiotic resistance plasmid from LAB.

Adhesion of LAB to HT-29 and Caco-2 Cells

LAB can exert beneficial effects and seem to be important for modifying the host's immune system, for reducing the colonization of pathogenic microorganism, and for enhancing the healing of damaged mucosa during bacterial adhesion to the epithelium [8, 15, 57]. Adherence to human intestinal cell lines such as HT-29 and Caco-2 cells and the ability to colonize the gastrointestinal tract should be estimated during preselection and assessment of probiotic strains [14]. The capacity of isolated LAB to adhere to HT-29 and Caco-2 is shown in Fig. 1. The adhesion to HT-29 and Caco-2 cells varied among the bacteria tested in a strain-dependent manner. The highest level of adhesion was observed with DC 55, KC 21, KC 24, and MJ 301 strains, having more than 50% of adhesion to HT-29 or Caco-2 cells. In contract, the adhesion capacity of DC 136, DC 222, KC 34, and KC 117 strains to the intestinal cell lines was low.

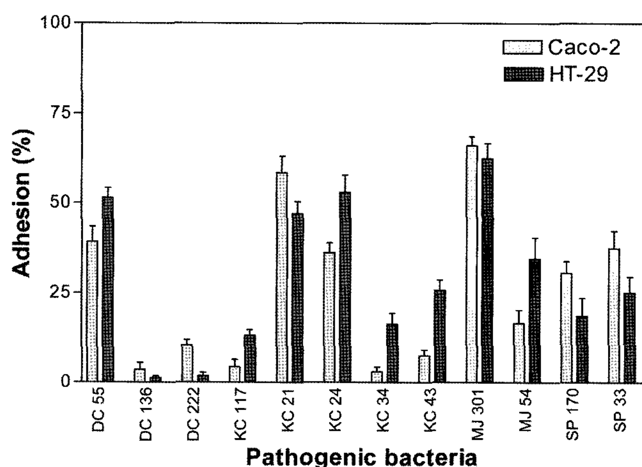

Fig. 1. Adhesion of LAB isolated from Korean fermented foods to Caco-2 and HT-29.

Each LAB suspended (about 1×10^7 CFU/ml) in DMEM was transferred in triplicate to 24-well plates with Caco-2 and HT-29 cells, respectively, and incubated for 2 h at 37°C in 5% CO_2. After incubation, the cells were washed, fixed, stained, and measured at 570 nm using an ELISA reader.

Tuomola and Salminen [62] reported that the four most adhesive strains were *L. casei*, *L. acidophilus* 1, *L. rhamnosus* LC-705, and *Lactobacillus* GG, whereas *L. casei* var. *rhamnosus* was the least adhesive strain to Caco-2 cultures, indicating that the adhesion property was strain-specific among *Lactobacillus* spp. Adhesion of *S. typhimurium* is significantly inhibited by probiotic *L. johnsonii* LJ1 and *L. casei* Shirota [63], and *L. rhamnosus* strain GG may reduce the accumulation of aflatoxins in the intestine *via* increased excretion of an aflatoxin-bacteria complex [25]. Adhesion of *L. acidophilus* LA1 onto Caco-2 cells requires a proteinaceous adhesion promoting factor present in the bacterial broth culture supernatant [6]. Moreover, adhesion of *L. acidophilus* 1 and *L. rhamnosus* strain GG is reduced by boiling, autoclaving, or by pepsin and trypsin treatments, but increased by ethanol and propanol treatments, suggesting that bacteria protein structures are essential for their adhesion [64]. Lakhtin *et al.* [32] reported that lactobacilli and bifidobacteria have cell-surface adhesion factors such as lectin/adhesion proteins of S-layers, secreted lectin-like bacteriocins, and lectin-like complexes. Granato *et al.* [20] suggested that the mechanism of adhesion of *L. johnsonii* La1 to Caco-2 cells involves lipoteichoic acid, a nonproteinaceous component of the bacterial surface. In addition, the adhesion mechanisms of probiotic LAB involved in their interaction with intestinal epithelial cells include passive forces, electrostatic and hydrophobic, interactions, and steric forces [58]. Moreover, Baccigalupi *et al.* [4] indicated that small (less than 3 kDa) surface-associated factors are involved in mediating adhesion of *L. fermentum* to Caco-2.

Antibacterial Activity of LAB

Another essential condition for LAB with probiotic activity is the productive capacity of inhibitory substances that antagonize pathogenic strains [47]. We selected 4 strains that showed the acid and bile resistance and high adhesion property to HT-29 and Caco-2, and examined the antibacterial activity by co-incubation of foodborne pathogenic bacteria with the LAB (Fig. 2). *S. aureus* was the most sensitive to the presence of KC 21, showing an inhibition of about 70%, but the inhibition of *L. monocytogenes*, *V. parahaemolyticus*, and *S. typhimurium* showed the highest ratio by the K 24 strain. However, the inhibitory effect of MJ 301 strain on pathogenic bacteria was somewhat low.

Lee *et al.* [33] showed that when LAB (K1, D2, F35-2) isolated from feces of newborn baby or *dongchimi* were cocultured with *L. monocytogenes*, *S. typhimurium*, or *E. coli* O157:H7 in YS medium, the foodborne microorganisms

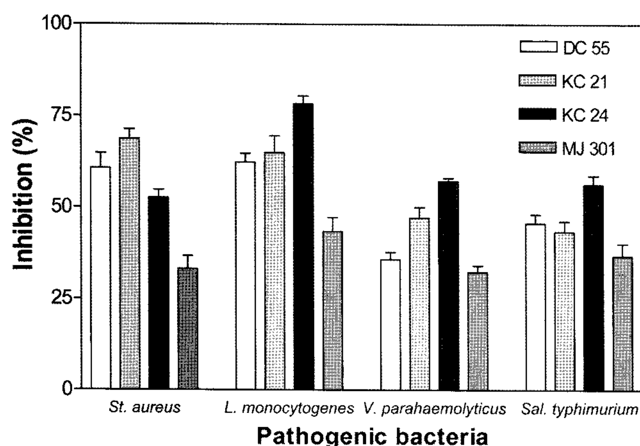

Fig. 2. The antibacterial effect of pathogenic bacteria by co-incubation with LAB.

After co-incubation with LAB for 12 h, cell cultures of pathogenic bacteria (1 ml) were plated on TCBS (*V. parahaemolyticus*), Oxford agar (*L. monocytogenes*), *Staphylococcus* 110 medium (*S. aureus*), or SS agar (*S. typhimurium*) to determine viable cell counts. The agar plates were incubated at 37°C for 24 h and the number of cell was estimated.

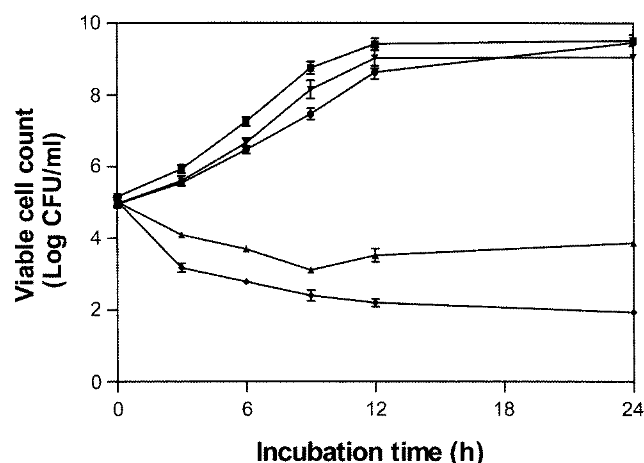

Fig. 3. Viable cell counts of *S. aureus* ATCC 6538 during the growth in BHI broth in the presence of the crude bacteriocin (200 BU/ml) or protease-treated bacteriocin produced by *L. plantarum* KC 21.

■, Control; ▲, KC 24 bacteriocin; ▼, KC 24 bacteriocin treated with protease; ◆, KC 21 bacteriocin; ●, KC 21 bacteriocin treated with protease.

were completely inhibited within 72 h of incubation. *L. acidophilus* KFRI 233, a strain isolated from human, exhibited an antagonistic effect on *Clostridium perfringens* [29].

To investigate bacteriocin production by the four strains, we prepared crude bacteriocin of the strains and examined their antibacterial activity using the microtiter plate assay. Among the strains, KC 21 and KC 24 produced bacteriocin-like substance, but DC 55 and MJ 301 did not suggesting that the antibacterial activities of the KC 21 and KC 24 strains were due to both organic acids and bacteriocin-like inhibitory substances. In particular, viable cell counts of *S. aureus* decreased by about 1 log unit from the initial cell counts within 24 h after exposure to 200 BU/ml of the bacteriocin produced by KC 24, whereas the initial cell counts of *S. aureus* were reduced by about 3 log units by the addition of the bacteriocin of KC 21 (200 BU/ml) after 24 h. Because the bacteriocin activity of the two strains was completely inhibited by protease, bacteriocin-like inhibitory substances seem to be proteinaceous in nature (Fig. 3).

Simon *et al.* [59] and Hernandez *et al.* [22] reported sakacin G, a 37-amino-acid-residue-long class IIa bacteriocin produced by *L. sake* 2512, and plantaricin TF711, a bacteriocin-like substance produced by *L. plantarum* TF711. Specifically, antimicrobial activity of crude bacteriocin produced by *L. plantarum* 27 was retained after autoclaving, DNase or catalase treatment, and exposure to pHs ranging from 4.0 to 9.0 [28]. The antimicrobial activities of many LAB are mainly due to the production of hydrogen peroxide, which exerts bacteriocidal effects on most pathogens and organic acids (lactic or acetic acids) [35]. In addition, other antimicrobial components, diacetyl, reuterin, pyroglutamic acid, and especially bacteriocin(s), are ribosomally synthesized, and extracellularly released proteinaceous antimicrobial compounds with antibacterial activities against bacteria are generally closely related to the producer strains [48, 51]. The bacteriocidal action of bacteriocin appears to be due to the formation of pores in the bacterial membrane, the induction of ATP efflux from inside the cells, the inhibition of cell wall formation, the dissipation of proton motive force of target organisms, and the blockade of the incorporation of DNA, RNA, and protein precursors [34, 38, 45].

Spent culture supernatant of human *L. acidophilus* strain LB decreased the *in vitro* viability of *S. aureus*, *L. monocytogenes*, *S. typhimurium*, *Shigella flexneri*, *E. coli*, *Klebsiella pneumoniae*, *B. cereus*, *Pseudomonas aeruginosa*, and *Enterobacter* spp. [9]. The cell-free supernatant of *L. casei* subsp. *rhamnosus* strain inhibited human pathogenic bacteria, enterotoxigenic *E. coli*, enteropathogenic *E. coli*, *E. faecalis*, and *Cl. difficile* [16].

Identification of Probiotic KC 21 Strain

In conclusion, although the KC 21 strain was susceptible to antibiotics, the strain showing acid and bile- tolerance, adhesion properties to human intestinal cell line, and strong bacteriocin activity against pathogenic bacteria are considered to be the strain to have potential as probiotic bacteria. Table 3 shows the physiological and biochemical characterizations and carbohydrate fermentation patterns of probiotic KC 21 strain. The KC 21 strain was a Gram-positive, facultatively anaerobic, catalase-, urease-, or oxidase-negative, nonmotile, and nonsporing bacteria. The strain showed rod-shaped morphology and produced no gas from glucose. The strain had the ability to grow at 10–45°C, pH 5.0–9.0, and less than 5% NaCl. Furthermore, the KC 21 fermented ribose,

Table 3. Phenotypic characteristics of and the fermentation of various sugars by *L. plantarum* KC 21.

Contents	Results	Sugar	Results	Sugar	Results
Cell shape	Rod	Glycerol	–	Salicine	+
Gram staining	+	Erythritol	–	Cellobiose	+
Spores staining	–	D-Arabinose	–	Maltose	+
Acid-fast staining	–	L-Arabinose	+	Lactose	+
Motility	–	Ribose	+	Melibiose	+
Gas from glucose	–	D-Xylose	–	Saccharose	+
H_2S production	–	L-Xylose	–	Trehalose	+
Lactic acid	L	Adonitol	–	Inulin	–
Methyl red	+	β-Methyl-xyloside	–	Melezitose	+
Voges-Proskauer	–	Galactose	+	D-Raffinose	–
Horse blood hemolysis	–	D-Glucose	+	Amidon	–
Sheep blood hemolysis	–	D-Fructose	+	Glycogen	–
Catalase	–	D-Mannose	+	Xylitol	–
Oxidase	–	L-Sorbose	–	β-Gentiobiose	+
Urease	–	Rhamnose	–	D-Turanose	+
Arginine hydrolysis	+	Dulcitol	–	D-Lyxose	–
Lysine	+	Inositol	–	D-Tagatose	–
Ornithine	–	Mannitol	+	D-Fucose	–
Growth in aerobic condition	+	Sorbitol	+	L-Fucose	–
anaerobic	+	α-Methyl-D-mannoside	+	D-Arabitol	–
Growth at 10–45°C	+	α-Methyl-D-glucoside	–	L-Arabitol	–
Growth at pH 5.0–9.0	+	N-Acetyl glucosamine	+	Gluconate	–
10.0	–	Amygdaline	+	2-Ceto-gluconate	–
Growth in 1–5% NaCl	+	Arbutine	–	5-Ceto-gluconate	–
10% NaCl	–	Esculine	+		

+, Positive reaction; –, negative reaction; L, configuration of lactic acid produced from glucose.

mannose, mannitol, sorbitol, esculine, cellobiose, lactose, and saccharose, but did not ferment glycerol, xylose, rhamnose, and gluconate. Therefore, the KC 21 strain was identified as *L. plantarum* KC 21 with a confidence level of 99.9% and T=0.76 value using the API 50CHL kit.

L. plantarum 37, 80, NCIMB 1193, and HU were found to be the most promising strains as probiotics that have tolerance to acid and bile salts, ability to ferment fructooligosaccharides, and β-galactosidase activity, and susceptibility to antibiotics [3]. Bujalance *et al.* [7] showed that a probiotic strain of *L. plantarum* may exert immunomodulatory effects despite limited colonization ability, and may improve the immune function damaged by immunosuppressive agents. Nguyen *et al.* [49] reported that *L. plantarum* PH04, which has bile/acid tolerance and bile salt hydrolase activity, might be effective as a probiotic with cholersterol-lowering activities. Recently, Sul *et al.* [61] suggested that the multiplex PCR method would be an efficient tool for the simple, rapid, and reliable identification of LAB used as probiotic strains. To reveal the the potential of LAB of the *L. plantarum* KC 21 as a probiotic, our future studies will be focused on the antioxidant effect, cholesterol-lowering effect, immuno-modulatory effect, anticancer property, and characterization and purification of bacteriocin produced by this strain.

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