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RESEARCH ARTICLE

Synergy Effects by Combination with Lactic Acid Bacteria and Frutooligosaccharides on the Cell Growth and Antimicrobial Activity

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Abstract The aim of this study was to identify probiotic possibility of lactic acid bacteria (LAB) from mustard leaf kimchi and investigate synergy effects of combination with LAB and fructooligosaccharide (FOS) on antimicrobial activity. *Lactobacillus acidophilus* GK20, *L. paracasei* GK74, and *L. plantarum* GK81 have high survival ratio under gastric and intestinal fluids and were resistant to ampicillin, streptomycin, or tetracycline. The pH of LAB gradually decreased but the titratable acidity increased owing to organic acids production. Especially, *L. acidophilus* GK20 and *L. paracasei* GK74 produced hydrogen peroxide and bacteriocin. When LAB was co-cultured with *Listeria monocytogenes* or *Staphylococcus aureus* in broth without FOS, the inhibition ratio of *L. monocytogenes* and *S. aureus* by *L. acidophilus* GK20 was 36 and 57%, but *L. paracasei* GK74 showed higher inhibitory effect against *L. monocytogenes* than *S. aureus*. Meanwhile, the cell growth, culture pH, titratable acidity, and bacteriocin activity of *L. acidophilus* GK20 and *L. paracasei* GK74 were significantly ($p<0.05$) increased in MRS with FOS than without prebiotic. In conclusion, *L. acidophilus* GK20 and *L. paracasei* GK74 could be potentially used as novel probiotic strains, moreover, the combination between *L. acidophilus* GK20 or *L. paracasei* GK74 and FOS has great potential as a synbiotic.

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Introduction

Probiotics, defined as a live microbial feed supplement, confer a health benefit on the host by improving its intestinal microbial balance (1). Although still far from fully understood, balanced colonic microflora, alleviation of lactose intolerance, and immunostimulation are major functional effects attributed to the consumption of probiotics (2). Also, action mechanisms of several probiotics have been proposed, including prevention of diarrhea and colitis, suppression of cancer, and cholesterol reduction (3). Especially, probiotic lactic acid bacteria (LAB) having the preservative action of starter strains in food and beverage systems produce antibacterial agents which show wide inhibitory spectrum against many harmful organism such as pathogenic *Escherichia*, *Salmonella*, *Clostridium,* and *Helicobacter* spp. (4).

It has been known that many LAB produce proteinaceous antimicrobial agents referred as bacteriocins, which generally act through inactivation of enzymes, depolarization of the target cell membrane, or inhibition of cell wall formation of pathogenic microflora including bacteria, mold, and yeast (5). Lactic acid is also produced in varying quantities as metabolic substances by probiotic bacteria, and it is thought that this may exert their antimicrobial effect by interfering with the maintenance of cytoplasmic membrane potential, inhibiting active transport, and hindering a variety of metabolic functions (6). Moreover, hydrogen peroxide can be inhibitory to some microorganisms by strong oxidizing effect on membrane lipids and cell proteins (7).

Meanwhile, prebiotics are nondigestible food ingredients which selectively stimulate the growth or activities of

beneficial microbes living in the gut to improve the host's intestinal microbial balance (8). Prebiotics must be able to withstand gastric acidity in the stomach, digestive processes by mammalian enzymes before they reach the colon, and absorption in the large intestine (9). They are obtained by extraction from natural sources (e.g., chicory, garlic, leek, and yacon) using water or solvent and enzymatic and chemical synthesis (trans-glycosylation reaction) from disaccharide substrates such as sucrose or lactose [e.g., fructooligosaccharides (FOS) and galacto-oligosaccharides (GOS)] (10).

These compounds play a role not only by facilitating host metabolism and mineral (Ca, Fe, and Mg) absorption, but also in stimulating immune system by IgA production and cytokine modulation and enhancing host defenses (11). In addition, recent studies suggest that oligosaccharides serve as substrate for proliferation of LAB and bifidobacteria and inhibit the growth of colonic cancer cells and putrefactive or pathogenic bacteria present in the colon through the production of short chain fatty acids (SCFA). The action mechanisms of these also include strengthening of the gut mucosal barrier, gut microflora modification, prevention of pathogen proliferation and adherence to intestinal mucosa, and transformation of bacterial enzyme activity (12).

Criteria for the preferable probiotic strains included acid and bile tolerance, antibiotics resistance, and antagonistic activity against pathogens. Based on this concept, the aim of the present study was to identify probiotic possibility of LAB from mustard leaf kimchi exhibiting resistance to reactive oxygen species and antioxidant activities (13) and to investigate synergy effects of combination with LAB identified as probiotic strain and prebiotic FOS on antimicrobial activity.

Materials and Methods

LAB and foodborne pathogenic bacteria culture conditions Lactic acid bacteria (LAB) such as *Lactobacillus acidophilus* GK20, *L. brevis* GK55, *L. paracasei* GK74, *L. plantarum* GK81, and *Leuconostoc mesenteroides* GK104 having antioxidant activities were isolated from the mustard leaf kimchi (13). The kimchi was prepared as follows; seasoning (red pepper powder, salted anchovy, minced garlic, ginger, and onion, and wheat flour paste) was added to mustard leaf dipped in 15%(w/v) brine for 4 h and then it was fermented at 4°C after keeping at room temperature for 12 h. These strains were incubated aerobically onto fresh Lactobacilli MRS broth (BD, Spark, MD, USA) for 48 h at 37°C and stored at −80°C in 40% glycerol. Besides, foodborne pathogenic bacteria (*Listeria monocytogenes* KCTC 3569 and *Staphylococcus aureus* ATCC 6538) used to examine antimicrobial activity were obtained from American Type Culture Collection (ATCC) and Korean Collection for Type Cultures (KCTC) and propagated in brain heart infusion (BHI, BD) at 37°C for 24 h under aerobic condition.

Simulated gastric and intestinal fluids tolerance The method to evaluate the acid-bile tolerance of test strains was according to Grimoud *et al*. (14) with some modifications. LAB cells (approximately 10^8 CFU/mL) grown up to log phase in MRS broth at 37°C for 24 h were harvested by centrifugation at 7,000×*g* for 10 min, washed twice in stile phosphate buffer solution (PBS, pH 7.2), inoculated in simulated gastric juice [NaCl 125 mM, KCl 7 mM, $NaHCO_3$ 45 mM, pepsin (Sigma-Aldrich, St. Louis, MO, USA) 1 mg/mL] at al final pH adjusted to 2.0, and maintained at 37°C for 2 h. After acid preincubation cells were centrifuged (7,000×*g* for 10 min), resuspended in simulated intestinal fluid (pancreatine 1 mg/mL and bovine bile 0.5%,w/v) at a final pH adjusted to 8.0, and maintained at 37°C for 3 h. And then the sample was removed from the tubes and subjected to serial dilutions in PBS (pH 7.0), and cell viability was assayed by pour plate method. The % of bacterial survival was calculated as follows: $(CFU_{assay}/CFU_{control})\times100$, where CFU_{assay} represented CFU after incubation in simulated gastric and intestinal fluids and $CFU_{control}$ described CFU after incubation in PBS as a control. Separate experiments were conducted in triplicate.

Resistance to antibiotics Antibiotic resistance of LAB was performed using the paper disc method (15). One-hundred mL of MRS broth were inoculated with 1 mL of an active culture of each LAB, which were incubated at 37°C for 24 h aerobically. Semisolid agar (0.8%), containing approximately 10^6 CFU/mL of LAB centrifuged at 7,000×*g* for 10 min and washed twice with PBS was overlaid on agar plates. After the medium solidified, antibiotic disks [ampicillin 25 μg, erythromycin 15 μg, kanamycin 30 μg, penicillin G 10 units, streptomycin 10 μg, tetracycline 30 μg, and vancomycin 30 μg (Oxoid, Basingstoke, Hampshire, England)] were immediately placed on the agar, and then agar plates with antibiotic disks were incubated at 37°C for 24 h. The diameter of the inhibition zone surrounding the wells was measured and scored as follows; as the diameter of the well is 8 mm, 8 mm equals no inhibition (-), diameter between 9 and 13 mm (weak, +), diameter between 13 and 18 mm (good, ++), and diameter larger than 18 mm (strong, +++). All experiments were performed in 3 independent experiments.

Determination of pH and titratable acidity LAB were cultivated at 37°C for 24 h under aerobic condition. Samples were taken at regular intervals during growth to

determine the LAB culture pH, which was measured by a pH meter (Hanna Instruments, Sarmeola di Rubano, PD, Italy). In addition, titratable acidity was determined during incubation period as described by Mante *et al*. (16) with some modifications. Briefly, for determination of titratable acidity, LAB culture (10 mL) was made up to 250 mL by addition of distilled water. Diluted sample (100 mL) was filtered and titrated with 0.1 N NaOH using 0.1% phenolphthalein as indicator. The titratable acidity value (%) was done in triplicate and calculated as follows: (0.009 × 0.1 N NaOH consumption × 0.1 N NaOH factor × dilution rate)/weight of sample × 100.

Detection of hydrogen peroxide production The screening of hydrogen peroxide production used was described previously by Otero and Nader-Macias (17), and studied by culturing microorganisms in 3,3'5,5'-tetramethyl-benzidine (TMB)-MRS agar plates. LAB were cultured on MRS agar containing 1 mM TMB and 2 U/mL peroxidase (Sigma-Aldrich) under anaerobic conditions at 37ºC. According to the color intensity after 48 h of incubation, hydrogen peroxide-producing colonies turned blue (strong, +++), brown (medium, ++), light brown (weak, +), or white (negative, -) in 20 min under exposure to air.

Preparation of bacteriocin and activity assay Preparation of LAB bacteriocin was performed as previously described (15). In detail, strains for testing were cultured in MRS broth at 37ºC for 24 h and the supernatant was collected from LAB cell culture by centrifugation at 7,000×*g* for 20 min, adjusted to pH 7.0 with 1 N NaOH, salted out with ammonium sulfate (60%,w/v) during overnight at 4ºC, desalted by cellulose dialysis membrane (Spectrum Labs., Gardena, CA, USA), and filter-sterilized through a syringe filter with a pore size of 0.45-μm membrane filter (Millipore Corp., Billerica, MA, USA) to prepare bacteriocin solution. And then the possible inhibitory action of hydrogen peroxide was eliminated by the addition of catalase (1 mg/mL) at 25ºC for 30 min. The proteinaceous nature of the solution was evaluated by treating this extract with a 1 mg/mL final concentration of the pepsin, trypsin, lipase, and α-amylase (Sigma-Aldrich). Any residual antimicrobial activity was determined by the agar well diffusion method. The *L. monocytogenes* KCTC 3569 or *S. aureus* ATCC 6538 (approximately 10^5 CFU/mL) cultured in BHI broth for 24 h at 37ºC was inoculated into 20 mL of semisolid BHI agar (BHI broth plus 0.8% agar) and after solidification of the medium, diameter wells (8 mm) were punched in the agar. And then 50 μL of serially diluted bacteriocin solution were filled into the each well separately and the plates were incubated overnight at 37ºC for 24 h under aerobic conditions. Bacteriocin activity was expressed as arbitray unit (AU/mL) which was calculated by multiplying 20 with the reciprocal of the greatest inhibitory dilution fold showing a clear inhibition zone against the indicator strain. All results are means of triplicate experiments.

Effects of FOS on the cell growth and antibacterial activity of LAB To investigate combined effects of probiotic strain and prebiotic FOS on the cell growth and antibacterial activity of LAB against the pathogenic bacteria, first of all, the FOS (Sigma-Aldrich) were added to a final concentration of 0, 0.5, and 1.0% in MRS broth, and then LAB (10^5 CFU/mL) were inoculated and incubated in an aerobic condition at 37ºC. During incubation time, a 1 mL LAB sample removed from each culture was serially diluted in sterile 0.1% PBS and plated on MRS agar by pour plate method as described above. Also, culture of LAB (10^5 CFU/mL) was carried out in MRS broth added FOS (0, 1, and 2%), and then the cells (10^5 CFU/mL) of *S. aureus* or *L. monocytogenes* harvested and washed with PBS were inoculated in the medium and incubated at 37ºC for 24 h. From the culture harvested at specified time, the viable cell counts of pathogen were examined using selective medium [mannitol salt egg yolk agar (BD) for *S. aureus* and Oxford agar (BD) for *L. monocytogenes*]. Inhibition (%) of pathogen was calculated as follows: $[(CFU_{control}-CFU_{assay})/CFU_{control}]\times100$. Besides, to examine effects of FOS on metabolic products of LAB during incubation, the culture pH and titratable acidity were measured, and bacteriocin activity was determined by the agar well diffusion assay using *L. monocytogenes* KCTC 3569 and *S. aureus* ATCC 6538 as indicator strain as described previously.

Data and statistical analysis The results of the experiment were presented as means±standard deviation (SD) (n=3) and analyzed with SPSS version 12.0 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was applied to analyze whether significant variation existed between the treatments using the Duncan's multiple range test at probability values of $p<0.05$.

Results and Discussion

Simulated gastric and intestinal fluids tolerance Five strains of *L. acidophilus* GK20, *L. brevis* GK55, *L. paracasei* GK74, *L. plantarum* GK81, and *L. mesenteroides* GK104 isolated from the mustard leaf kimchi were investigated for simulated gastric and intestinal fluid tolerance (Table 1). Among test strains, *L. acidophilus* GK20, *L. brevis* GK55, *L. paracasei* GK74, and *L. plantarum* GK81 revealed a significantly high viability in artificial gastric juice (pH 2.0) by maintaining viable cell

Table 1. Viable cell counts and survival ratio of LAB isolated from the mustard leaf kimchi after preincubation under simulated gastric and intestinal fluids

Strain	Viable cell counts[1)] (CFU/mL)		Survival ratio (%)
	Gastric juice	Intestinal fluid	
L. acidophilus GK20	$7.87\pm0.16\times10^7$	$8.62\pm0.59\times10^7$	43.10±2.95
L. brevis GK55	$6.91\pm0.56\times10^7$	$4.83\pm0.44\times10^4$	0
L. paracasei GK74	$8.07\pm0.20\times10^7$	$7.97\pm0.33\times10^7$	39.86±1.64
L. plantarum GK81	$7.61\pm0.48\times10^7$	$6.58\pm0.17\times10^7$	32.90±0.85
L. mesenteroides GK104	$5.14\pm0.34\times10^5$	$6.19\pm0.22\times10^3$	0

[1)]Initial LAB cells (2.0×10^8 CFU/mL) were incubated in simulated gastric juice at 37°C for 2 h, and then maintained in simulated intestinal fluid at 37°C for 3 h.

Table 2. Antibiotic susceptibility profiles of LAB isolated from the mustard leaf kimchi

Antibiotics (concentration)	Inhibition zone[1)] (mm)				
	L. acidophilus GK20	*L. brevis* GK55	*L. paracasei* GK74	*L. plantarum* GK81	*L. mesenteroides* GK104
Ampicillin (25 μg)	-	++	+	-	-
Erythromycin (15 μg)	+	++	+	++	+++
Kanamycin (30 μg)	-	-	-	-	+
Penicillin G (10 units)	-	+	-	+	++
Streptomycin (10 μg)	-	++	+	-	+
Tetracycline (30 μg)	++	+	-	-	+
Vancomycin (30 μg)	+	++	+	+	++

[1)]Antibiotic susceptibility of LAB was performed using the paper disc method; Diameter of the inhibition zone surrounding the wells was scored as follows; (-), 8 mm; (+), 9-13 mm; (++), 13-18 mm; (+++), >18 mm.

counts of above 7 log scale, whereas *L. mensenteroides* GK104 showed acid susceptibility under the same condition. Especially, many cells of *L. acidophilus* GK20, *L. paracasei* GK74, and *L. plantarum* GK81 survived in artificial bile after incubation for 2 h in gastric fluid, which have comparatively high survival ratio under simulated gastric and intestinal fluids.

These results are in concordance with the previously reported some studies. Ha *et al*. (18) reported that *L. planatrum* CK102 survived at a level of 1.36×10^8 CFU/mL in pH 2.0 buffer for 6 h and showed exhibited excellent bile tolerance. Mechanisms underlying acid resistance of LAB include not only homeostasis of intracellular pH by the F_1F_0-ATPase proton pump but also alkalization of the external environment with amino acid decarboxylation and changes in the cell membrane and density (19). Although bile salts can enter the cell by directly traversing the outer membrane or by passage through porins such as OmpF, efflux system of some strains that have ability removing bile salts before significant damage occurs are responsible for resistance to bile salts (20). Therefore, these results indicated that the tolerance of LAB in simulated gastric and intestinal fluids is strongly LAB-dependent and suggested that *L. acidophilus* GK20, *L. paracasei* GK74, and *L. plantarum* GK81 could successfully transit the human stomach and may be capable of reaching the gastrointestinal tracts and expressing their probiotic functions.

Resistance to antibiotics The susceptibility to antibiotics of 5 LAB tested was summarized in Table 2. Among LAB having relatively high tolerance under simulated gastric and intestinal fluids, *L. acidophilus* GK20, *L. paracasei* GK74, and *L. plantarum* GK81 was highly resistant to ampicillin, kanamycin, penicillin G, streptomycin, or tetracycline. However, *L. brevis* GK55 and *L. mensenteroides* GK104 showed higher sensitivity to various antibiotics. It has been known that probiotics have a resistance to various antibiotics. Zhou *et al*. (21) showed that *L. acidophilus* and *L. plantarum* were susceptible to β-lactam antibiotics (penicillin, ampicillin, and cephalothin), Gram-positive spectrum antibiotics (erythromycin and novobiocin), and broad-spectrum antibiotics (chloramphenicol, rifampin, spectinomycin, and tetracycline). However, these results differ from those of Ruoff *et al*. (22) who reported that some LAB including strains of *L. plantarum* and *Leuconostoc* spp. were resistant to vancomycin, one of the last antibiotics broadly efficacious against clinical infections caused by multidrug-resistant pathogens. Action mechanisms of the several bactericidal antibiotics are known to inhibit macromolecular consolidation and the important enzymes for cell wall synthesis, to activate murein hydrolases,

Table 3. Profiles of the culture pH, titratable acidity, and antimicrobial substances production of LAB isolated from the mustard leaf kimchi during incubation

LAB[1)]	Incubation time (h)	pH	Titratable acidity	Hydrogen peroxide	Bacterocin activity (AU/mL)
L. acidophilus GK20	6	5.97±0.09	0.39±0.11	+++	ND[2)]
	12	4.34±0.16	0.67±0.14		80
	24	3.59±0.15	0.95±0.10		640
L. brevis GK55	6	6.02±0.07	0.14±0.05	-	ND
	12	5.06±0.18	0.31±0.07		ND
	24	4.58±0.06	0.65±0.12		ND
L. paracasei GK74	6	5.26±0.08	0.48±0.12	++	ND
	12	4.51±0.14	0.63±0.04		40
	24	3.60±0.10	1.12±0.06		2,560
L. plantarum GK81	6	5.19±0.09	0.43±0.13	+	ND
	12	4.41±0.08	0.77±0.07		ND
	24	3.51±0.20	0.99±0.16		ND
L. mesenteroides GK104	6	5.89±0.18	0.20±0.09	-	ND
	12	4.98±0.22	0.36±0.05		ND
	24	4.62±0.17	0.69±0.11		ND

[1)]Initial pH of MRS broth inoculated LAB before incubation was approximately 6.81±0.05.
[2)]Not detected

which active in bacterial cell wall degradation, and to block cell division and growth (23). However, resistance mechanism to antibiotics of intrinsically avirulent organisms such as lactobacilli or enterococci is manifested by changes in antibiotic permeability, alteration of target molecules, enzymatic degradation of the antibiotics, and efflux of antimicrobials from the cytosol, thus these strains are protected from the toxic effects of antibiotics (24).

Changes of the culture pH, titratable acidity, and antimicrobial substances production of the LAB during incubation The changes of the pH values, titratable acidity, and production of antimicrobial substances such as hydrogen peroxide and bacteriocin in MRS broth during incubation at 37°C for 24 h are shown in Table 3. The initial pH of MRS broth inoculated LAB before incubation was approximately 6.81±0.05, and then the culture pH gradually decreased during incubation owing to organic acids production. After incubation for 24 h, titratable acidity produced from LAB was 0.95% (*L. acidophilus* GK20), 0.65% (*L. brevis* GK55), 1.12% (*L. paracasei* GK74), 0.99% (*L. plantarum* GK81), and 0.69% (*L. mesenteroides* GK104), respectively. In addition, *L. acidophilus* GK20, *L. paracasei* GK74, and *L. plantarum* G81 produced hydrogen peroxide having antimicrobial effects, i.e., *L. acidophilus* GK20 and *L. paracasei* GK74 were hydrogen peroxide-hyperproducing and *L. plantarum* GK81 was less efficient, whereas, *L. brevis* GK55 and *L. mesenteroides* GK104 did not produce any hydrogen peroxide. Particularly, bacteriocin, which specific inhibitory activity against closely related species and foodborne pathogens was produced by *L. acidophilus* GK20 and *L. paracasei* GK74, but not shown by *L. brevis* GK55, *L. plantarum* GK81, and *L. mesenteroides* GK104. The supernatant of 2 strains did have an effect on their inhibitory activity against the indicator organisms despite the addition of pepsin, lipase, and α-amylase, however, the bacteriocin activity of *L. paracasei* GK74 was disappeared by trypsin (data not shown). *L. acidophilus* GK20 produced highest bacteriocin levels (640 AU/mL) against *L. monocytogenes* KCTC 3569 after incubation for 24 h at 37°C, but its activity was decreased to half after 36 h of incubation (data not shown). And the bacteriocin activity of *L. paracasei* GK74 was first detected to 40 AU/mL after incubation for 12 h, arrived at maximum activity (2,560 AU/mL) after 24 h, and drastically decreased due to cell lysis by autolysin afterward.

Xanthopoulos *et al*. (25) indicated that *L. paracasei* ssp. *paracasei* and *L. acidophilus* strains isolated from infant faeces had weak antibacterial activity on Gram-negative bacteria such as *E. coli* ATCC 25922 and *Yersinia enterocolitica* ATCC 1501, which differs somewhat from these results in this study. In other words, the bacteriocin produced from *L. acidophilus* GK20 and *L. paracasei* GK81 presented a high inhibitory effect against Gram-positive bacteria, whereas was unable to inhibit Gram-negative bacteria such as *E. coli*, *Salmonella* Typhimurium, and *Vibrio parahaemolyticus* (data not shown), therefore, the antagonistic activity and spectrum of 2 strains was strain-dependent. This findings is consistent with the

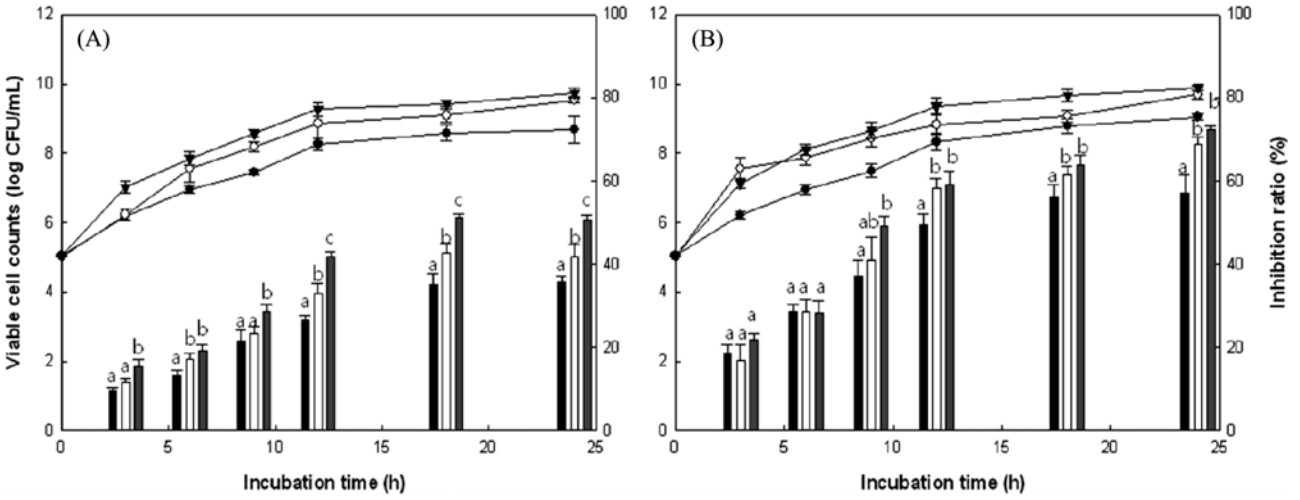

Fig. 1. Effects of FOS on the cell growth and antagonistic activity of *L. acidophilus* GK20 against pathogenic bacteria in co-culture with *L. monocytogenes* KCTC 3569 (A) or *S. aureus* ATCC 6538 (B). Line graph: viable cell counts of *L. acidophilus* GK20 in MRS broth containing FOS (-●- 0%, -○- 1%, -▼- 2%) at 37°C for 24 h; bar graph: inhibition ratio of pathogen when mixed culture with *L. acidophilus* GK20 (10^5 CFU/mL) and pathogenic bacteria (10^5 CFU/mL) in medium containing FOS (-■- 0%, -□- 0.5%, -▨- 1%). Different letters in the same incubation time are significantly different estimated by Duncan's multiple range test at $p<0.05$.

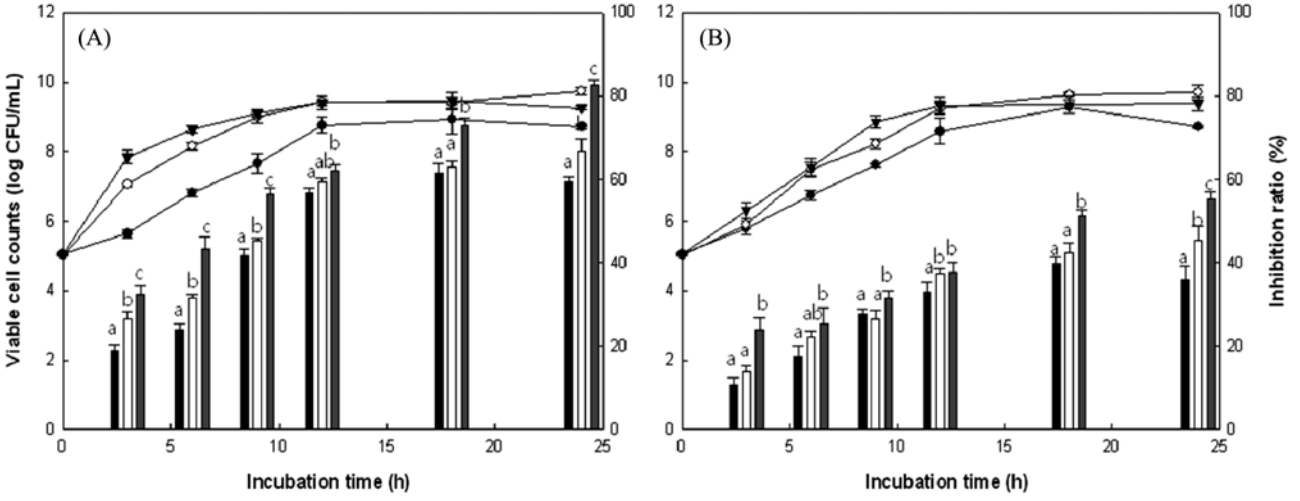

Fig. 2. Effects of FOS on the cell growth and antagonistic activity of *L. paracasei* GK74 against pathogenic bacteria in co-culture with *L. monocytogenes* KCTC 3569 (A) or *S. aureus* ATCC 6538 (B). Line graph: viable cell counts of *L. paracasei* GK74 in MRS broth containing FOS (-●- 0%, -○- 1%, -▼- 2%) at 37°C for 24 h; bar graph: inhibition ratio of pathogen when mixed culture with *L. paracasei* GK74 (10^5 CFU/mL) and pathogenic bacteria (10^5 CFU/mL) in medium containing FOS (-■- 0%, -□- 0.5%, -▨- 1%). Different letters in the same incubation time are significantly different estimated by Duncan's multiple range test at $p<0.05$.

results of Pinto *et al*. (26) that the inhibitory activity of *L. plantarum* from traditional African fermented milk products, as well as human intestinal isolates was not due to the production of a heat-stable bacteriocin, but observed could be explained by the production of organic acids and hydrogen peroxide. However, *L. plantarum* ST8KF isolated from kefir, produced a 3.5 kDa bacteriocin active against lactobacilli and *L. innocua* (27).

Effects of FOS on the cell growth and antagonistic activity of the LAB On the basis of these results, *L. acidophilus* GK20 and *L. paracasei* GK74 having possibility as probiotic strains were selected for evaluation effects of FOS on the cell growth and antimicrobial activity of LAB. Effects of FOS on the cell growth in MRS broth inoculated with each of the *L. acidophilus* GK20 and *L. paracasei* GK74 during incubation and antagonistic activity by LAB in co-culture with *L. monocytogenes* KCTC 3569 or *S. aureus* ATCC 6538 are shown in Fig. 1 and 2. When *L. acidophilus* GK20 was co-cultured with *L. monocytogenes* KCTC 3569 or *S. aureus* ATCC 6538 in MRS broth without FOS, the number of *L. acidophilus* GK20 gradually increased throughout incubation and showed viable cell counts of above 10^8 CFU/mL after 24 h,

and the inhibition ratios of *L. monocytogenes* and *S. aureus* by mixed culture were 36 and 57% due to the antimicrobial substance of LAB, respectively. As the number of *L. acidophilus* GK20 increased during incubation, the inhibition ratio against foodborne pathogen was proportionally increased. And the cell growth and antimicrobial activity of *L. paracasei* GK74 in the same incubation condition was similar to those of *L. acidophilus* GK20, but *L. paracasei* GK74 showed higher inhibitory effect against *L. monocytogenes* than *S. aureus*. Meanwhile, the cell growth of *L. acidophilus* GK20 and *L. paracasei* GK74 were sharply increased by addition of FOS in MRS broth after 24 h incubation. Namely, in MRS broth with 1.0% FOS, the viable cell counts of *L. acidophilus* GK20 and *L. paracasei* GK74 were higher about 1 log scale than in medium without FOS. And after 24 h in medium containing 1.0% FOS, the inhibitory activities of *L. acidophilus* GK20 against *L. monocytogenes* and *S. aureus* were 51 and 73%, respectively, and those of *L. paracasei* GK74 were detected to 83 and 57%. The inhibitory activities of 2 strains were significantly ($p<0.05$) increased in broth with FOS than in medium without prebiotic, furthermore, effects of FOS on antagonistic activity of LAB were dose-dependent manner.

Some studies have reported that the addition of prebiotic lactulose in skim milk significantly stimulated the counts of probiotic bifidobacteria (28), and GOS increased the growth parameters (turbidity and generation time) of the bifidobacteria and lactobacilli (14). And FOS and GOS promoted the growth of the beneficial strains but not of pathogenic microorganisms (29). A previous study proved that the inhibitory effect against pathogenic bacteria is usually considered to be achieved by reduction in pH results from acid production, secretion of hydrogen peroxide, and release of natural antibiotics (bacteriocin) from beneficial microflora selectively stimulated by various prebiotics (30). Rousseau *et al*. (29) suggested that the growth of vaginal lactobacilli in the absence of carbon sources was very low, but the strains were able to grow on FOS as indicated by the turbidity, pH, generation time, and lactate levels obtained after 48 h incubation. Consequently, the endogenous human vaginal lactobacilli strains and oligosaccharides are good candidate for incorporation in a formula to prevent vaginal infections.

Effects of FOS on the culture pH, titratable acidity, and bacteriocin production of the LAB To investigate the influence of FOS on the culture pH, titratable acidity, and bacteriocin production of *L. acidophilus* GK20 and *L. paracasei* GK74, the selected 2 strains were conducted in MRS broth supplemented with FOS. As shown in Table 4, after 24 h incubation in medium without FOS, the culture pHs of *L. acidophilus* GK20 and *L. paracasei* GK74 were 3.61±0.12 and 3.54±0.07, respectively. And the titratable acidity was higher *L. paracasei* GK74 (1.14±0.06) than *L. acidophilus* GK20 (0.99±0.09). The culture pH and titratable acidity of 2 strains were significantly ($p<0.05$) stimulated by the addition of FOS at 0.5 and 1.0%. Further, the bacteriocin activity of *L. acidophilus* GK20 against *L. monocytogenes* was first recorded after incubation at 37°C for 12 h in the absence of FOS, and it maximally increased to 640 AU/mL after 24 h. And that of the strain against *S. aureus* was escalated as incubation time goes by and their maximum activity was 1,280 AU/mL after 24 h incubation. Meanwhile, the bacteriocin activity (2,560 AU/mL) of *L. paracasei* GK74 against *L. monocytogenes* was maximal at 24 h incubation, but the activity (640 AU/mL) of the strain against *S.aureus* showed lower level than against *Listeria*. FOS enhanced the bacteriocin activity of *L. acidophilus* GK20 and *L. paracasei* GK74, i.e., the bacteriocin activity against pathogenic bacteria was increased more than 2 times in medium supplemented with FOS than the control. In particular, the maximum bacteriocin levels of *L. acidophilus* GK20 and *L. paracasei* GK74 against *L. monocytogenes* were 1,280 and 2,560 AU/mL in the presence of 1.0% FOS after 21 h incubation, respectively, but their activity was rapidly decreased owing to degradation by specific proteolytic enzymes thereafter.

Cebeci and Gurakan (31) indicated that potential probiotic *L. plantarum* strains have the ability to decrease pH of the medium as a result of acidic end products of FOS fermentation. The results are in agreement with the reports of Ignatova *et al*. (32), which showed that the added oligosaccharides induced LAB to form end-products of a typical mixed acid fermentation. Audisio *et al*. (33) reported that *E. faecium* CRL1385 isolated from the crop grew well in the presence of FOS, but its ability to produce bacteriocin, active against poultry pathogens such as *Enterococcus hirae*, *Salmonella pullorum,* and *L. monocytogenes* was not significantly modified. Chen *et al*. (34) suggested that the highest cell number and bacteriocin production of *L. lactis* ssp. *lactis* C101910 and *Lactococcus* sp. GM005 were observed with FOS or trehalose.

In conclusion, based on these results, because *L. acidophilus* GK20 and *L. paracasei* GK74 isolated from mustard leaf kimchi have the antioxidant activity (13), tolerance in the human gastric and intestinal tract, resistance against various antibiotics, and antimicrobial activity against foodborne pathogenic bacteria, these strains could be potentially used as novel probiotic strains. Moreover, since FOS stimulated the proliferation and metabolites production of the probiotic lactobacilli, which had a synergistic effect on enhancing the antagonistic activity against pathogenic bacteria in the presence of FOS, the combination between *L. acidophilus* GK20 or *L. paracasei*

Table 4. Changes of the culture pH, titratable acidity, and antimicrobial substances production of the LAB in MRS broth supplemented with FOS

Strain	Incubation time (h)	Concentration of FOS (%)											
		0	0.5	1.0	0	0.5	1.0	0	0.5	1.0	0	0.5	1.0
		pH			Titratable acidity			Bacteriocin activity against *L. monocytogenes* (AU/mL)			Bacteriocin activity against *S. aureus* (AU/mL)		
L. acidophilus GK20	0	6.88±0.06[a1)]	6.84±0.08[a]	6.88±0.10[a]	ND	ND	ND	ND	ND	ND	ND	ND	ND
	9	5.44±0.08[a]	5.30±0.07[a]	5.12±0.07[b]	0.45±0.08[a]	0.51±0.12[a]	0.55±0.08[a]	ND	ND	20	ND	ND	80
	12	4.30±0.11[a]	4.07±0.05[b]	3.92±0.11[b]	0.69±0.11[a]	0.73±0.09[a]	0.89±0.07[a]	80	80	160	160	160	320
	15	4.15±0.07[a]	3.97±0.10[b]	3.88±0.08[b]	0.75±0.15[a]	0.90±0.07[ab]	0.96±0.10[b]	80	160	320	320	320	640
	18	3.97±0.05[a]	3.81±0.05[b]	3.64±0.05[c]	0.81±0.06[a]	0.99±0.10[b]	1.04±0.05[b]	160	320	640	320	640	640
	21	3.85±0.11[a]	3.67±0.06[b]	3.49±0.09[c]	0.91±0.04[a]	1.05±0.03[b]	1.17±0.09[c]	320	640	1280	640	640	1280
	24	3.61±0.12[a]	3.50±0.09[ab]	3.31±0.09[b]	0.99±0.09[a]	1.19±0.05[b]	1.24±0.06[b]	640	640	640	1280	1280	2560
L. paracasei GK74	0	6.91±0.04[a]	6.90±0.08[a]	6.87±0.08[a]	ND	ND	ND	ND	ND	ND	ND	ND	ND
	9	4.88±0.05[a]	4.79±0.05[a]	4.61±0.11[a]	0.58±0.04[a]	0.61±0.09[a]	0.67±0.12[a]	ND	ND	ND	ND	ND	ND
	12	4.47±0.13[a]	4.13±0.12[b]	4.04±0.20[b]	0.66±0.15[a]	0.75±0.07[a]	0.84±0.06[a]	40	80	160	ND	20	20
	15	4.26±0.09[a]	4.03±0.10[b]	3.96±0.13[b]	0.68±0.05[a]	0.88±0.11[b]	0.91±0.09[b]	320	320	640	80	80	160
	18	4.06±0.13[a]	3.81±0.08[b]	3.75±0.06[b]	0.72±0.11[a]	1.01±0.05[b]	1.06±0.04[b]	640	1280	1280	320	320	640
	21	3.75±0.10[a]	3.66±0.09[ab]	3.51±0.12[b]	1.00±0.08[a]	1.08±0.04[ab]	1.20±0.08[b]	1280	1280	2560	640	640	640
	24	3.54±0.07[a]	3.34±0.15[b]	3.14±0.15[c]	1.14±0.06[a]	1.21±0.09[a]	1.66± 0.11[b]	2560	2560	1280	640	1280	1280

[1)]Values were mean±SD of triplicate determinations; ND, not detected; Significance of the differences ($p<0.05$) between means were estimated by Duncan's multiple range test.

GK74 and FOS has great potential as a synbiotic that can be utilized in the manufacturing of fermentation foods and dietary or medical supplements. Though these results showed the properties of probiotics combined with prebiotic FOS *in vitro*, in the future we will study on synbiotic characteristics using *in vivo* models to further evaluate for their technological aptitudes.

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