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Original Article

Adhesion Activity of *Lactobacillus plantarum* PM 008 Isolated from Kimchi on the Intestine of Mice

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Lactic acid bacteria (LAB), including *L. plantarum* isolated from Kimchi, are beneficial and safe microorganisms that improve disturbances of the indigenous microflora and the host's immune system. The adhesion abilities of Kimchi-derived *L. plantarum* PM008 and yogurt-derived *L. casei* were measured *in vitro* and *in vivo*. When *L. plantarum* or *L. casei* was incubated with Caco-2 cells, these *Lactobacillus* strains were potently attached. When these strains were orally administered to mice, the LABs were attached on the large intestine of mice. The attachment of *L. plantarum* on murine intestine or Caco-2 intestinal epithelial cell lines was more potent than that of *L. casei*, although numbers of LAB between their feces were not different. Treatment with either *L. plantarum* or *L. casei* for 14 days suppressed fecal β-glucuronidase activity, although treatment for one day did not affect it. *L. plantarum* showed more potent inhibition than *L. casei*. In addition, *L. plantarum* and *L. casei* were stable to artificial gastric and intestinal juice. *L. plantarum* was more stable than *L. casei*. Based on these findings, the survival and adhesion effects of orally administered LAB strains in the intestine may increase numbers of LAB in intestine and express their biological activities.

Key Words: Lactic acid bacteria, *Lactobacillus plantarum*, *Lactobacillus casei*, Adhesion activity

INTRODUCTION

Normal intestinal microflora consist of an estimated 500 different bacterial species, and reach their highest concentrations in the terminal ileum and colon (1~3). Intestinal microflora consisted of beneficial and harmful bacteria, such as *Lactobacillus acidophilus*, *Bifidobacterium longum*, *Escherichia coli* and *Staphylococcus aureus*.

The harmful bacteria produce toxic compounds, such as gram-negative bacterial endotoxin, and harmful enzymes, such as β-glucuronidase and tryptophanase, which produce cytotoxic or carcinogenic agents (4~7). Cytotoxin and endotoxins may interact at the apical intestinal surface and induce responses in the intestinal epithelial cells, which produce proinflammatory cytokines and other mediators that induce inflammatory activation of the mucosal immune system, and cause colitis, diarrhea or constipation.

Lactic acid bacteria (LAB) are beneficial and safe microorganisms (8), that improve disturbances of the indigenous microflora (9), ameliorate the development of microflora (8), have antidiabetic and antihyperlipidemic effects (10, 11), inhibit carcinogenesis (12), have anticolitic effects (9, 13~15), and show non-specific activation of the host's immune system (12). The biological activities of the

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beneficial LAB have mainly been studied for many LAB derived from yogurts and healthy human intestinal microflora, including *Lactobacillus acidophilus*, *Lacobacillus casei*, *Bifidobacterium breve*, and *Bifidobacterium longum*. Although LAB derived from foods such as Kimchi has been taken in Asia countries, particularly Japan and Korea, their adhesion abilities on the intestine have not been thoroughly studied. Therefore, we investigated the adhesion ability of Kimchi-derived *Lactobacillus plantarum* and yogurt-derived *L. casei* on Caco-2 cells and mice.

MATERIALS AND METHODS

Materials and bacterial strains

p-Nitrophenyl-β-D-glucuronide and p-nitrophenyl-β-D-glucopyranoside were purchased from Sigma Co. (St. Louis, MO, USA). Ex Taq polymerase chain reaction (PCR) kit was purchased from TaKaRa Co. (Tokyo, Japan). All reagents used in slot hybridization analysis and fluorescence *in situ* hybridization were purchased from Roche Diagnostics (Indianapolis, IN, USA).

L. plantarum PM008 isolated from Kimchi and *L. casei* were donated from Pulmuone Co. (Seoul, Korea), and grown in MRS broth at 37℃ for 24 h. The cultured *Lactobacillus* strains were harvested by centrifugation at 3,000 × g for 10 min. Each pelleted bacterium was then washed two times in sterile PBS and resuspended 1×10^9 CFU/ml and 5×10^9 CFU/ml in PBS.

Survival Stability assays in gastric and intestinal juice

To evaluate the survival stabilities of orally administered *L. plantarum* and *L. casei* in intestine, they were stored in artificial gastric [pepsin (1,000 units/ml) containing MRS broth adjusted to pH 2.5 with 5 N HCl] and intestinal juices [GAM broth (pH 6.8) containing 0.1% mucin, 0.04% pancreatin, 0.2% bile, 0.85% NaCl, 0.04% trypsin] for 3 h. Numbers of surviving *Lactobacillus* strains were determined by plating serial dilutions (in PBS, pH 7.2) on MRS agar followed by incubation at 37℃ for 48 h.

Adhesion assay

Caco-2 cells, heterogeneous human epithelial colorectal adenocarcinoma cells, purchased from Korea Cell Line Bank (Seoul, Korea). The cells were cultured at 37℃ in a 5% CO_2-95% air atmosphere in RPMI 1640 (Sigma Co.) supplemented with 10% heat-inactivated (30 min, 56℃) fetal calf serum (FCS, Sigma Co.). To investigate the adhesion activity of *Lactobacillus* strains, postconfluent Caco-2 cells were washed twice with PBS. For each adhesion assay, 500 μL of the *Lactobacillus* suspension [bacteria (1×10^7 CFU) with broth culture supernatant] was mixed with Dulbecco's modified Eagle's medium (DMEM) (500 μL), and then added to each well of the tissue-culture plate (24 wells), which was then incubated at 37℃ for 2 h in 10% CO_2-90% air. After incubation, the cells were washed five times with sterile PBS, the cells were lysed with sterile H_2O, and appropriate dilutions were then plated on MRS agar to determine the number of viable cell-associated bacteria. Results were expressed as CFU/well of cell-associated bacteria.

Animals

Male ICR mice (20~25 g, 5 weeks old) were obtained from the Charles River Orient Experimental Animal Breeding Center (Seoul, Korea). All animals were housed in wire cages at 20~22℃, relative humidity of 50±10%, air ventilation frequency of 15~20 times/h and 12-h illumination (07:00~19:00; intensity, 150~300 Lux), fed standard laboratory chow (Charles River Orient Experimental Animal Breeding Center, Seoul, Korea), and allowed water *ad libitum*. All experiments were performed in accordance with the NIH and Kyung Hee University guides for Laboratory Animals Care and Use and approved by the Committee for the Care and Use of Laboratory Animals in the College of Pharmacy, Kyung Hee University.

Intestinal bacterial enzyme activity assay

Mice were randomly divided into 9 groups: normal; high dosage (1.0×10^9 CFU) of *L. plantarum* or *L. casei* for one day; high dosage (1.0×10^9 CFU) of *L. plantarum* or

L. casei for 14 days; low dosage (0.2 × 10^9 CFU) of *L. plantarum* or *L. casei* for one day; low dosage (0.2 × 10^9 CFU) of *L. plantarum* or *L. casei* for 14 days. Dosage was once a day with 200 μl of each LAB, and the normal group was treated with sterile PBS alone. Each group contains 12 mice, and half of each group was sacrificed 1st day after final treatment with LAB, and the rest were sacrificed 7th day after the final treatment.

Counting of bacteria in stool

Number of survival LAB were determined by plating serial dilutions (in PBS, pH 7.2) on MRS agar followed by incubation at 37℃ for 48 h.

Preparation of fecal bacterial suspension

The fresh mouse stools (0.5 g) from each group were separately collected in sterilized plastic cups, carefully suspended with 20-fold diluted anaerobic broth in a cooled tube and centrifuged at 250 × g for 5 min. The supernatant was re-centrifuged at 10,000 × g for 20 min. The resulting precipitates were used as the sources for the fecal enzyme assay. All procedures were performed at 4℃.

Assay of β-glucuronidase and β-glucosidase activities

The reaction mixture (2.0 ml), consisting of 0.04 ml of 2 mM p-nitrophenyl-β-D-glucuronide (or p-nitrophenyl-β-D-glucopyranoside), 0.76 ml of 0.1 M phosphate buffer (pH 7.0), and 0.2 ml of fecal suspension, was incubated for 30 min at 37℃, with the reaction stopped by the addition 1 ml of 0.5 M NaOH. The mixture was then centrifuged at 3,000 × g for 10 min and the absorbance measured at 405 nm.

Quantification of LAB in mouse intestine

Species-specific probes for *L. plantarum* and *L. casei* used the following three oligonucleotide probes: LPANG (5'-TATCATTGCCATGGTGA-3'), *L. plantarum* species-specific probe; and 5'-GAGATTCAACATGGAACG-3', *L. casei* species-specific probe; and UP041 (5'-CTGCTGCC-TCCCGATGGAGT-3'), a universal oligonucleotide complementary to virtually all 16S rRNA (16, 17). These oligonucleotide probes were marked with digoxigenin at 3'-ends for immunoblot and fluorescent *in situ* hybridization (FISH). Total bacterial genomic DNAs were isolated and purified from the intestine using the Qiagen Blood and tissue DNA purification kit (Qiagen GmbH, Hilden, Germany). The rDNA was amplified by using the TaKaRa Ex Taq polymerase kit (Takara Co., Tokyo, Japan), and the following primers were selected for the PCR reactions: 27F, 5'-AGAGTTTGATCCTGGCTCAG-3'; and 1492R, 5'-GGCTACCTTGTTACGACTT-3'. The PCR cycle was 4 min at 94℃; 30 sec at 92℃, 30 s at 52℃ and 1.5 min at 72℃, repeated 20 cycles; and 10 min at 72℃. The PCR products were purified using QIAquick PCR purification kit (Qiagen GmbH). To calibrate the data, PCR amplification using *L. plantarum* or *L. casei* genomic DNA as a template was also performed by following the same method.

Quantification of LAB attached in intestine by slot blot hybridization

Sections of 3.5 cm colon from each mouse were sampled and homogenized with 500 μl of sterile PBS on ice. The sludge was centrifuged at 800 rpm at 4℃ for 5 min, and then the supernatant was collected. The supernatant was centrifuged at 15,000 × g at 4℃ for 2 min to collect the pellet. Total bacterial genomic DNA was isolated and purified from the pellet using the Quiagen Blood and tissue DNA purification kit (Qiagen GmbH), according to the manufaturer's manual and amplified by PCR. PCR products (2 μg) were denatured with 0.3 M NaOH for 10 min at 98℃, and then loaded onto the positively charged nylon membrane using Minifold-II slot blotting system (Whatman Inc. Florham Park, NJ, USA). The membrane was fixed twice with UV-crosslinker. The membrane was hybridized in DIG-easy hybridization solution containing 100 pmol of LPANG for 2 h at 40℃, and then washed twice in the washing buffer (0.5 × SSC buffer containing 0.1% SDS) at 49℃. The hybrids were detected by using a CSPD-DIG detection kit (Roche), according to the manufacturer's manual. The chemiluminescent signals were detected by image analyzer, LAS-4000 mini (Fujifilm Co., Tokyo, Japan). After the detection, the membrane was stripped with a stripping solution (0.2 M NaOH containing 0.1% SDS) and then rehybridized with the universal DIG-labeled probe, UP041, for control detection. The hybridization and washing temperatures for UP041 were 48 and 58℃, respectively,

and the other protocols were the same as those described above for LPANG. The signal intensity was converted to the mass unit (μg) by the calibration curve, and the percentage of *L. plantarum* or *L. casei* in the total microbes of the stool was calculated.

Analysis of LAB attached in intestine by FISH

The colon (3.5 cm) from each mouse was sampled and homogenized with 500 μl of sterile PBS on ice. The homogenized sludge was centrifuged at 1,000 × g at 4℃ for 5 min, and the supernatant was collected. A 200 μl volume of supernatant was fixed with 600 μl of 4% paraformaldehyde in PBS at 4℃ for 4 h. To remove the paraformaldehyde, the cell suspension was pelleted by centrifugation at 15,000 × g at 4℃ for 2 min, and resuspended in ice-cold PBS. These washing steps were repeated 3 times. The fixed pellet was finally resuspended in 200 μl of a 1:1 mixture of EtOH and PBS, and then stored at -20℃ before use. The procedure was used to identify *L. plantarum* by FISH method using DIG-labeled oligonucleotide (18). To view the cell smears hybridized with fluorescein-labeled anti-DIG Fab fragment, the slide was mounted with Vectashield (Vecta Laboratories, Inc., Burlingame, CA, USA), and the hybridized cells were counted visually with Axio Observer.D1 epifluorescence microscope (× 1,000, Carl Zeiss MicroImaging GmbH, Göttingen, Germany).

Statistical analysis

The data were calculated with mean values, and standard deviations (mean ± SD) were determined from triplicate trials. Statistical significance of the results was evaluated by one way ANOVA (analysis of variance) test ($p < 0.05$).

RESULTS AND DISCUSSION

We investigated the adhesion ability of *L. plantarum* isolated from Kimchi to evaluate whether the LAB can attach on the intestine (Fig. 1). When *L. plantarum* or *L.*

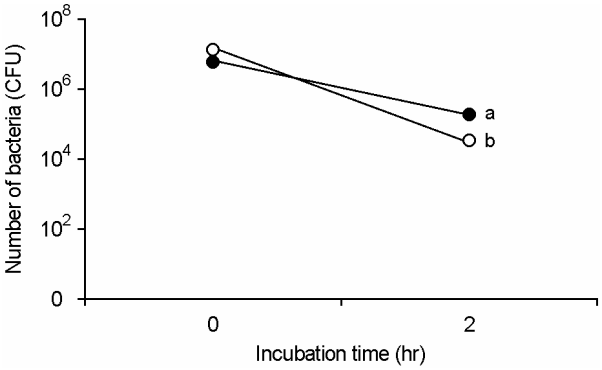

Figure 1. Adhesion ability of lactic acid bacteria to Caco-2 cells. Lactic acid bacteria (closed circle, 1 × 10^7 *L. plantarum* per well; open circle, 1.0 × 10^7 *L. casei* per well) were incubated with Caco-2 cells for 2 h, washed with PBS and then inoculated in MRS agar plate.

Table 1. Colony forming unit (CFU) of fecal bacteria grown in MRS and BL agar plates in mice orally treated with lactic acid bacteria

	Dosage	Treated period (day)	MRS		BL	
			Before	After	Before	After
L. plantarum	0.2 × 10^9 CFU	1	0.4 × 10^9	0.1 × 10^9	1.5 × 10^8	1.3 × 10^8
	1 × 10^9 CFU	1	1.6 × 10^9	1.5 × 10^9	1.1 × 10^8	1.0 × 10^8
L. casei	0.2 × 10^9 CFU	1	2.1 × 10^9	1.3 × 10^9	2.8 × 10^8	0.5 × 10^8
	1 × 10^9 CFU	1	4.1 × 10^9	3.8 × 10^9	3.8 × 10^8	3.7 × 10^8
L. plantarum	0.2 × 10^9 CFU	14	2.4 × 10^8	3.1 × 10^8	0.8 × 10^8	1.1 × 10^8
	1 × 10^9 CFU	14	2.3 × 10^8	3.5 × 10^8	0.3 × 10^8	1.3 × 10^8
L. casei	0.2 × 10^9 CFU	14	1.4 × 10^8	2.9 × 10^8	0.5 × 10^8	0.2 × 10^8
	1 × 10^9 CFU	14	2.2 × 10^8	3.1 × 10^8	0.3 × 10^8	3.2 × 10^8

Lactic acid bacteria (*L. plantarum* or *L. casei*) were orally administered once a day for one or 14 days. Numbers of intestinal bacteria were counted by plating serial dilutions (in diluted anaerobic broth, pH 7.2) on MRS and BL agars followed by anaerobic incubation at 37℃ for 48 h. All values are mean (n=3).

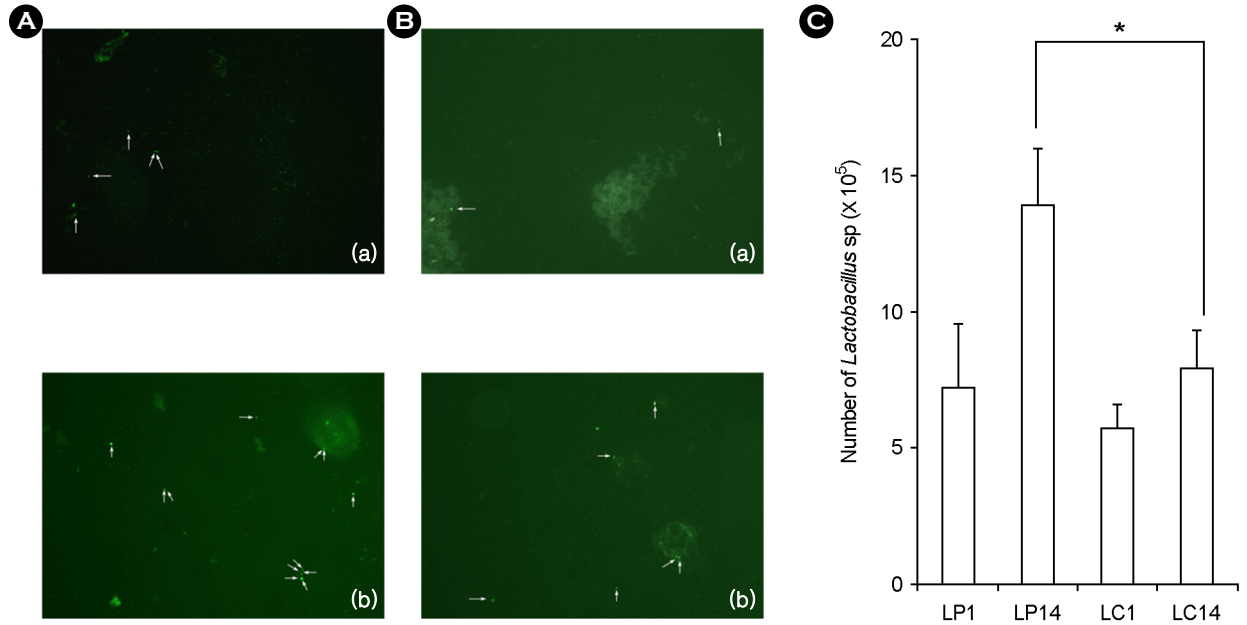

Figure 2. Number of intestinal bacteria attached in colon membrane in *Lactobacillus plantarum* (A) or *Lactobacillus casei* (B)-treated mice by a fluorescent hybridization method. Lactic acid bacteria were orally administered once a day for one (a) or 14 days (b): LP1, treated with 1.0×10^9 *L. plantarum* per mouse for one day; LP14, 1.0×10^9 treated with *L. plantarum* per mouse for 14 days; LC1, 1.0×10^9 *L. casei* per mouse for one day; LC14, 1.0×10^9 treated with *L. casei* per mouse for 14 days). All values are mean ± SD. (n=6). *significantly different compared to control group.

casei were incubated with Caco-2 cells, these *Lactobacillus* strains potently attached on Caco-2 cells. The adhesion ability of *L. plantarum* was superior to that of *L. casei* isolated from yogurt. To investigate whether these *Lactobacillus* strains are able to attach and proliferate in the intestine, we orally administered them to mice and counted numbers of LAB in the stools of mice using LAB selection media (MRS and BL agars) (Table 1). Treatment with both strains increased numbers of LAB on MRS and BL. Treatment with *L. plantarum* and *L. casei* were increased LAB numbers, but their increases were not dependent on treatment periods.

To measure the attachment ability of orally administered LAB in intestine *in vivo*, we orally administered *L. plantarum* or *L. casei* to mice and measured numbers of LAB attached to intestines using the slot blot hybridization method (Fig. 2). Numbers of LAB attached in intestines were found to be dependent on the administration period. To confirm numbers of LAB directly attached to the intestines, we measured LAB numbers by fluorescent *in situ* hybridization (Fig. 3). Both strains were detected in the

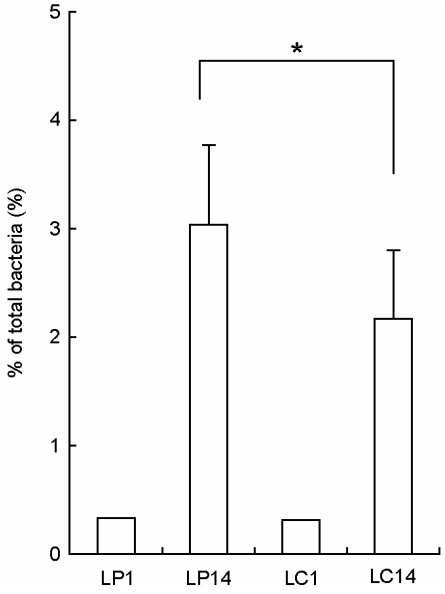

Figure 3. Number of intestinal bacteria attached in colon membrane in *Lactobacillus plantarum* (LP) or *Lactobacillus casei* (LC)-treated mice by a slot blot hybridization method. Lactic acid bacteria were orally administered once a day for one or 14 days: LP1, treated with 1.0×10^9 *L. plantarum* per mouse for one day; LP14, 1.0×10^9 treated with *L. plantarum* per mouse for 14 days; LC1, 1.0×10^9 *L. casei* per mouse for one day; LC14, 1.0×10^9 treated with *L. casei* per mouse for 14 days). All values are mean ± SD. (n=6). *significantly different compared to control group.

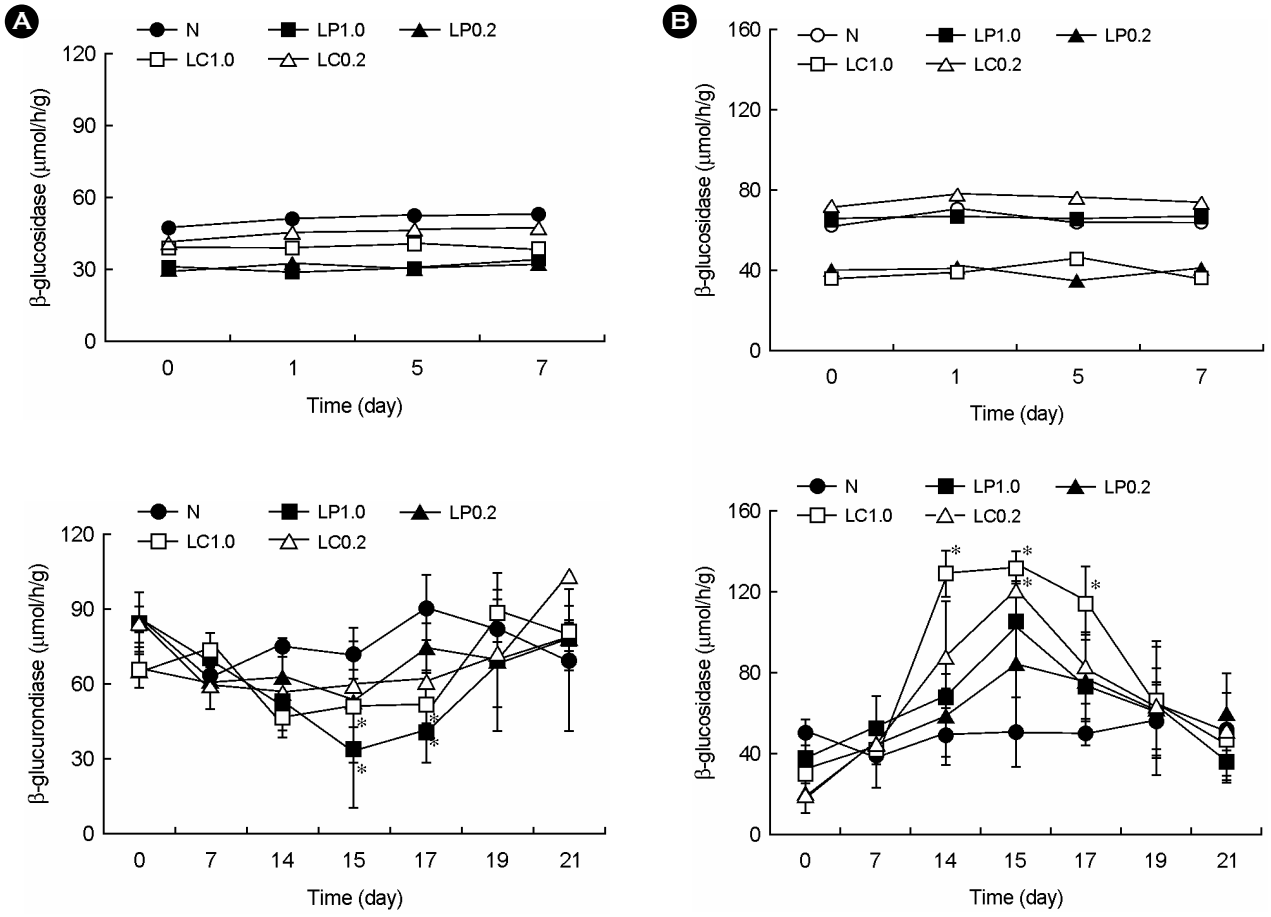

Figure 4. Effect of lactic acid bacteria on fecal bacterial β-glucosidase (A) and β-glucuronidase activities (B) in mice. Lactic acid bacteria (LP0.2, 0.2 × 10^9 *L. plantarum* per mouse; LP1.0, 1.0 × 10^9 *L. plantarum* per mouse; LC0.2, 0.2 × 10^9 *L. casei* per mouse; LC1.0, 1 × 10^9 *L. casei* per mouse) were orally administered once a day for one (upper) or 14 days (bottom). All values are mean ± SD. (n=6). *significantly different compared to control group.

intestines and the attached numbers were dependent on the administration period. When *L. plantarum* was orally administered to mice for one and 14 days, the numbers of *L. plantarum* attached to large intestine were 7.0 × 10^6 and 1.4 × 10^7, respectively. *L. plantarum* attached more efficiently to the intestine than *L. casei*.

To understand the reason why *L. plantarum* more plentifully attached on the intestine than *L. casei*, we measured the survival ability of *L. plantarum* and *L. casei* in gastric and intestinal juices. When these *Lactobacillus* strains were stored in artificial gastric and intestinal juices (Fig. 4). *L. plantarum* was more stable to gastric and intestinal juices than *L. casei*.

Next, we measured the effect of these *Lactobacillus* strains by their attachment on the intestine of mice. We orally administered these *Lactobacillus* strains to mice and monitored the activities of the fecal enzymes, β-glucuronidase and β-glucosidase (Fig. 5). Treatment with either *Lactobacillus* strain for 14 days suppressed fecal β-glucuronidase activity, but increased fecal β-glucosidase activity: these enzyme activities were gradually affected from 7th day after treatment with either LAB. *L. plantarum* more potently suppressed β-glucuronidase activity than *L. casei*, which more potently increased β-glucosidase activity. However, treatment with either LAB for one day did not affect fecal enzyme activities.

LAB is recognized beneficial microorganisms and have been demonstrated to exert preventive and therapeutic effects on diarrhea, constipation, and inflammatory diseases. Fermented foods, such as, yogurt, cheese, and Kimchi, are

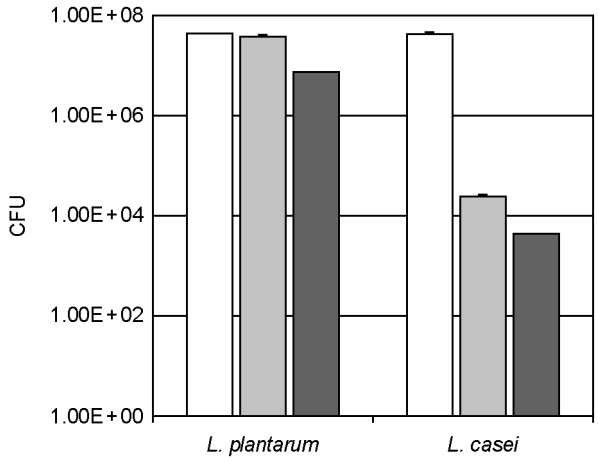

Figure 5. Stability of lactic acid bacteria against artificial gastric juice and intestinal juice. Lactic acid bacteria (*L. plantarum* or *L. casei*) were incubated in saline (white bar), artificial gastric [gray bar, pepsin (1,000 units)-contained MRS broth adjusted at pH 2.5 with 5 N HCl] or intestinal juice [black bar, GAM broth containing 0.1% mucin, 0.04% pancreatin, 0.2% bile, 0.85% NaCl, and 0.04% trypsin (pH 6.8)] for 3 h at 37℃ and number of survival LAB were determined by plating serial dilutions (in PBS, pH 7.2) on MRS agar followed by incubation at 37℃ for 48 h. All values are mean ± SD. (n=6). *significantly different compared to saline control group.

considered to be excellent sources of microorganisms and a variety of LAB strains have been detected in these foods (19), and been shown to regulate host defense mechanisms against pathogens (20, 21). Of them, the Kimchi-derived *L. plantarum* may have beneficial effects in terms of improving disturbances of indigenous bacteria in the gut and yogurt-derived *L. casei*, *L. acidophilus*, and *B. longum* have been reported to have similar effects. Nevertheless, the attachment ability of LAB isolated from Kimchi has not been thoroughly studied.

In the present study, the number of LAB detected in intestinal epithelia increased with administration period, and *L. plantarum* was more effective in this respect than *L. casei*. Furthermore, *L. plantarum* was found to be resistant to gastric and intestinal juices more than *L. casei*. These results were supported that *L. plantarum* was highly tolerant for acid and alkaline stress because the arginine dihydrolase pathway and glutamate dicarboxylation (22~24). In addition, *L. plantarum* more potently attached to Caco-2 cells, which is intestinal cell lines, than *L. casei*. These results also were supported by previous reports that highest attachment ability was observed with *L. plantarum* (25). When these strains were orally administered to mice for one and 14 days, they both increased LAB numbers in intestine and inhibited intestinal bacteria-producing β-glucuronidase activity which is known to cause colon cancer and liver injury (7, 26).

Based on these findings, the survival and attachment of orally administered *Lactobacillus* strains in the intestine increases numbers of parental *Lactobacillus* strains residing in intestine, inhibit harmful enzyme production of intestinal microflora, and thus, ameliorate intestinal diseases, such as constipation and diarrhea.

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