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ORIGINAL ARTICLE

The effects of a vegetable-derived probiotic lactic acid bacterium on the immune response

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ABSTRACT

The objective of this study was to investigate the probiotic properties of the fermented vegetable derived lactic acid bacterium, *L. plantarum*. *L. plantarum* 10hk2 showed antibacterial activity against pathogenic bacteria and immunomodulating effects on murine macrophage cell lines. RAW 264.7 cells stimulated with viable cells of this probiotic strain increased the amounts of pro-inflammatory mediators such as IL-1β, IL-6 and TNF-α, as well as the anti-inflammatory mediator, IL-10. ICR mice fed with viable cells of *L. plantarum* 10hk2 had reduced numbers of enteric *Salmonella* and *Shigella* species in comparison to controls from 2 weeks after supplementation, and this effect was observed for up to 4 weeks. The findings of this study suggest that this specific lactic acid bacterial strain, which is derived from vegetable fermentation, holds great promise for use in probiotics and as a food additive since it can reduce the number of some pathogenic bacteria through production of lactic acids.

Key words Lactic acid bacteria, naturally fermented white cabbage, IL-6, IL-10.

It is well known that lactic acid bacteria are beneficial to host health because they inhibit the growth of harmful microorganisms. In particular, ingestion of certain members of the *Lactobacillus* genus is believed to have beneficial effects on the health of the host (1, 2). Probiotic strains may be able to maintain a metabolically active state during gastrointestinal passage. In other words, the viability of probiotics could be crucial for improvement of the balance of intestinal bacterial flora (3). Additionally viable bacteria have been reported to provide greater stimulation of intestinal immunity in hosts than nonviable bacteria (4). Furthermore, infectious diseases in general are still a major problem, intestinal infectious diseases caused by pathogenic or opportunistic microorganisms in particular posing a more serious problem because overuse of antibiotics has induced antibiotic-resistant bacteria. Among harmful and opportunistic bacteria, *Escherichia* and *Salmonella* together with *Shigella* species are the most serious pathogens (3, 5).

Macrophages are tissue-based phagocytes that play a central role in initiating the first line of defense in host immunity. Activated macrophages, which typically become activated in the presence of microbial components, phagocytose micro-organisms, secrete pro-inflammatory cytokines and NO, and hence may regulate the host's immunity (6, 7). Several studies have demonstrated that viable or heat-killed cells of *Lactobacilli* and *Bifidobacterium* species, as well as their cell components, are

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List of Abbreviations: CFU, colony forming units; C. *freundii*, *Citrobactor freundii*; DMEM, Dulbecco's modified Eagle's medium; *E. cloaceae*, *Enterobacter cloaceae*; *E. coli*, *Escherichia coli*; HKC, heat-killed cells; HPLC, high performance liquid chromatography; ICR, Institute of Cancer Research; IL-1β, interleukin-1β; IL-6, interleukin-6; *L. acidophilus*, *Lactobacillus acidophilus*; *L. casei*, *Lactobacillus casei*; *L. plantarum*, *Lactobacillus plantarum*; LPS, lipopolysaccharides; *L. rhamnosus*, *Lactobacillus rhamnosus*; mAbs, monoclonal antibodies; MRS, de Mann Rogosa Sharpe; NO, nitric oxide; *S. aureus*, *Staphylococcus aureus*; *S. dysenteriae*, *Shigella dysenteriae*; *S. flexneri*, *Shigella flexneri*; *S. paratyphi*, *Salmonella paratyphi*; *S. sonnei*, *Shigella sonnei*; *S. typhimurium*, *Salmonella typhimurium*; SRBC, sheep red blood cells; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α; VC, viable cells.

capable of stimulating production of NO, IL-6 and TNF-α in macrophage cell lines (8–10). Some investigators have found that the effects on induction of cytokines are greater in viable bacteria than in heat-killed cells (11, 12). In response to internal or external invasion, these cytokines contribute to the defense mechanisms of the host, while over-expression of these pro-inflammatory cytokines may induce immunopathological disorders (11). Therefore, a balance between pro- and anti-inflammatory cytokines is important for host immunity (9, 13).

Anti-inflammatory IL-10 is known to block induction of synthesis of TNF-α, IL-1β and IL-6 by mouse peritoneal macrophages (14, 15). IL-10 also stimulates B cells and augments antibody production in activated B cells (16, 17). Studies on the anti-inflammatory effects of lactic acid bacteria have focused solely on heat-killed forms of these bacterial cells (9, 13, 18). Interestingly, it has previously been demonstrated by others that, in RAW 264.7 cells, LPS of Gram-negative bacteria can induce not only production of pro-inflammatory cytokines, such as TNF-α and IL-6, but also production of anti-inflammatory molecules such as IL-10 (11, 12).

In this study, the biochemical characteristics of *L. plantarum* 10hk2 were examined with the aim of assessing its potential use as a probiotic. In addition, its probiotic effects when mice were fed with viable cells were assessed. Another purpose of the study was to determine the immune enhancing effects of viable cells in comparison to heat-killed cells of this strain on pro- and anti-inflammatory cytokine induction, using the macrophage cell line RAW 264.7.

MATERIALS AND METHODS

Isolation and identification of lactic acid bacteria

Vegetables, such as Chinese white cabbage, radishes and onions (90: 9: 1), were washed three times with tap water and the excess water decanted and fermented with salt and sugar (1%, w/w). Following 5 days of incubation, bacterial strains were isolated from the fermented vegetables at 37°C under culture conditions. Microscopic examination, Gram staining, assessment of colony morphology and catalase tests were carried out on the bacterial strains isolated. Firstly, colonies forming a clear zone on de Mann Rogosa Sharpe (Difco, Franklin Lakes, NJ, USA) agar plates containing 1% $CaCO_3$ were selected (19). The candidate bacterial strains were tested for production of lactic acid after incubation in lactobacilli MRS (Difco) broth at 37°C for 2 days. The strain that produced the largest amounts of lactic acid was selected for identification by 16S rDNA sequencing and verified using a BLASTX search (20). This bacterial strain was also subjected to an aid to identification by using an API CHL 50 kit (Biomérieux, Lyon, France) according to the instruction manual to determine sugar utilization (21).

Tolerance to low pH, bile salts and heat

To assess tolerance of the selected *L. plantarum* 10hk2, to low pH, lactobacilli MRS broth (Difco) was adjusted to pH 1.5 using hydrochloric acid containing 1000 units of pepsin (Sigma, St. Louis, MO, USA) (19). Lactic acid bacteria were inoculated into the pH adjusted broth and then incubated at 37°C for 2 hr. In order to assess tolerance to bile salts, the isolates were incubated in MRS broth (pH 7.0) supplemented with 0, 0.5 and 5% (w/v) oxgall (Difco), after which they were placed in acidic conditions for 2 hr. After 3 hr incubation at 37°C, the number of bacteria that had survived was quantified after serial 10-fold dilution in PBS (pH 7.0, Gibco BRL, Gaithersburg, MD, USA) (22). Finally their tolerance to heat was tested at 90°C for 10 min.

HPLC analysis of organic acids

The organic acids produced by the isolated *L. plantarum* 10hk2, as compared to a commercial strain, *L. plantarum* ATCC 14917, were determined. Chinese white cabbage waste obtained from the Kimchi factory was used as the sole nutritional carbon source. After 3 days of incubation at 37°C, cabbage fermented with either *L. plantarum* 10hk2 or *L. plantarum* ATCC 14917 was dried at 60°C and the lactic, malic, acetic and succinic acid content assessed by HPLC analysis. Organic acid analysis was conducted using a mobile phase column (300 × 7.8 mm, Biorad, Munich, Germany) with 20 mM sulfuric acid, and HPLC-grade reagents were used as standards (Sigma). Both solvents and standard solutions were filtered through a 0.45 μm membrane filter, and the concentration of each organic acid was determined.

Antibacterial effects of *L. plantarum* 10hk2

The antibacterial activity of *L. plantarum* was examined by co-culturing it with the following pathogenic strains: *E. coli* ATCC 53323, *S. aureus* ATCC 25923, *E. cloaceae* ATCC 13047, *C. freundii* ATCC 6750, *S. typhimurium* ATCC 14028, *S. paratyphi* ATCC 11511, *S. sonnei* ATCC 9290, *S. flexneri* ATCC 9199, and *S. dysenteriae* ATCC 9752. Each indicator bacterium was inoculated at 10^7 CFU/ml, and then co-incubated with *L. plantarum* at 37°C for 24 hr (6). Antibacterial activity was determined by measuring the viability of the pathogenic bacteria and each of the pathogenic bacteria was cultured alone as a control (19, 23). All tests were performed in triplicate and inhibition

was calculated as follows: (22).

$$\% \text{ inhibition} = \frac{\frac{\text{CFU}}{\text{ml}} \text{ in control} - \frac{\text{CFU}}{\text{ml}} \text{ in coculture}}{\frac{\text{CFU}}{\text{ml}} \text{ in control}} \times 100 \quad (1)$$

Murine cell line maintenance

The murine macrophage cell line, RAW 264.7, was maintained in DMEM supplemented with 10% (v/v) FBS and gentamicin (20 μg/ml, Sigma) at 37°C in a 5% CO_2 humidified incubator. The cells were adjusted to a density of 5.0×10^5 cells/ml for all experiments. The culture medium was refreshed every 2 days and the cell number assessed on a hemocytometer.

NO determination

The RAW 264.7 cells were treated with VC or HKC at 5.0×10^6, 1.0×10^5 or 5.0×10^4 CFU/ml, respectively. After 48 hr, the culture supernatants were collected and analyzed for NO production (16, 24). Nitrite produced from NO in RAW 264.7 cells was measured indirectly by determining the concentration of nitrite in the cell culture supernatants, as nitrite produced from NO in the presence of H_2O and O_2 accumulates in the culture medium and reflects the amount of NO produced. The concentration of nitrite in the culture supernatant was determined using Griess reagent (1% sulfanilamide in 50% H_3PO_4 [w/v], 0.1% *N*-1-napthylethylenediamine dihydrochloride [1:1]) (25). Color development was measured by absorbance at 540 nm using a 96 well-plate reader. A positive control of LPS (2 μg/ml, from *E. coli*, Sigma) was also used.

Induction of cytokine release

To measure the amount of various cytokines released from RAW 264.7 cells upon stimulation, the cells were stimulated as described before, except that the culture time was changed to 24 hr. The amounts of cytokines released were compared against the amounts of cytokines observed in RAW 264.7 cells in DMEM alone as a negative control, and in cells stimulated with LPS (2 μg/ml) as a positive control. All culture supernatants were collected and stored at −20°C until analysis (26). The induction of various cytokines was measured using a commercial ELISA kit (BD OptEIA Set; BD Biosciences, San Diego, CA, USA) for IL-1β, IL-6, TNF-α and IL-10 according to the manufacturer's recommendations, and absorbance was measured at 490 nm using a 96-well plate reader (Molecular Devices, Sunnyvale, CA, USA) (10, 24).

Animals and experimental diets

ICR mice (male, 5 weeks old) weighing around 30 g were purchased from the Institute of Laboratory Animal Resources (Seoul National University). A total of 75 mice were randomly allocated into three groups, a control, an *L. plantarum* 10hk2 and an *L. plantarum* ATCC 14917 fed group. After one week of acclimatization, the mice were freely offered water and a diet (AIN93G, Han Sam R&D, Seoul, Korea) supplemented with lyophilized viable cells of *L. plantarum* 10hk2 or *L. plantarum* ATCC (0.1%, 1.0×10^8 CFU/g) for 4 weeks. Control mice received normal AIN93G diets without the lactic acid bacteria. All housing and handling was approved by the Animal Care and Ethics Committee of Seoul National University and was in compliance with the guidelines of the Committee for Institutional Animal Care and Use for Scientific Purposes.

Microflora of feces

Each fecal specimen was transferred to a tube containing PBS buffer (1×, pH 7.0), serially diluted, and then the enteric bacteria were plated on to MacConkey agar and *Salmonella* and *Shigella* media. Lactobacilli MRS broth was used for lactic acid bacteria, and tryptic soy broth, MacConkey agar and *Salmonella* and *Shigella* media were used for enteric bacteria. All culture media were obtained from Difco. All plates were simultaneously incubated at 37°C for 48 hr under anaerobic (GasPaks, BBL, Sparks, MD, USA) and facultative anaerobic conditions.

Flow cytometric analysis

At the end of the feeding period, the ICR mice (8 weeks) were intraperitoneally injected with SRBC. The SRBC were suspended in PBS as a 2% suspension based on the packed cell volume, 1 ml of the 2% suspension containing approximately 5×10^6 red blood cells. After 2 weeks, the mice were killed by cervical dislocation and their spleens aseptically removed and pooled. The splenocytes were then harvested using the Histopaque 1077 (Sigma, USA). Surface marker expression was measured by staining the cells with specific mAbs. A total of 1×10^6 cells was incubated with the FITC-conjugated rat anti-mouse CD19 mAb and CD3 mAb (BD Biosciences) according to the manufacturer's recommendations. Analysis was carried out on a FACSCalibur flow cytometer (Becton Dickinson Instruments, Waltham, MA, USA).

Statistical analysis

All statistical analyses were performed using SPSS software (version 13.0) and mean values were expressed with

Table 1. Tolerance of *L. plantarum* strains to acidity, bile salts and heat

	Condition†		Concentration of bile acids‡			Heat	
Strains	Control	pH 1.5	Control	0.5%	5%	Control	90°C
L. plantarum 10hk2	7.12 ± 0.40	8.00 ± 0.78 (112%)	7.52 ± 0.28	7.38 ± 0.83 (98%)	6.53 ± 0.44 (87%)	8.11 ± 0.32	7.51 ± 0.79 (93%)
L. plantarum ATCC 14917	7.72 ± 0.83	6.81 ± 0.09 (88%)	7.72 ± 0.43	7.20 ± 0.59 (93%)	5.76 ± 0.60 (75%)	8.17 ± 0.37	4.72 ± 0.90 (58%)

All values represent the average log number ± SD of three independent experiments and values in parentheses represent the percentage of surviving bacteria after treatment when the control before treatment is assumed to be 100%.
†, acid tolerance in response to 2 hr incubation at pH 1.5; ‡, bile acid tolerance of the probiotic strains after 2 hr incubation at pH 1.5, estimated after 3 hr incubation in a medium containing the indicated oxgall concentrations; , each probiotic strain was treated at 90°C for 10 min.

SD from three independent experiments. The values were evaluated with one-way analyses of variances and compared between groups using Tukey and Dunnett's multiple tests at $P \leq 0.05$.

RESULTS

Identification and biochemical characterization of lactic acid bacteria

Based on 16S rDNA gene sequencing and utilization of sugars, the 10hk2 strain was identified as *L. plantarum*. To examine the potential of using the selected lactic acid bacterial strains as probiotics, acidity tolerance was assessed at pH 1.5. As shown in Table 1, the viability of *L. plantarum* 10hk2 was unaffected by low pH conditions. The bacteria were generally tolerant of bile salts at 0.5% concentration after passage at pH 1.5, and had a survival rate of approximately 87% with a 5% bile salt concentration at the same pH. The tolerance of *L. plantarum* ATCC 14917 to acidic conditions was slightly less than that of *L. plantarum* 10hk2. Finally, the tolerance of the *L. plantarum* species to heat treatment was estimated. At 90°C the survival rate of *L. plantarum* 10hk2 was over 93%, compared to only 58% for *L. plantarum* ATCC14917.

HPLC analysis of organic acids

The compositions of non-volatile organic acids produced by these two *L. plantarum* species were analyzed by HPLC after fermentation using a white cabbage waste. *L. plantarum* 10hk2 produced a significant amount of lactic acid (52.26%) with minor amounts of acetic, succinic and malic acids, whereas only approximately 10% of the organic acids produced by *L. plantarum* ATCC 14917 was lactic acid and slightly larger amounts of malic and smaller amounts of acetic acid were produced. Their pH in solution was also different from each other (Table 2).

Antibacterial effects

A comparison between the isolated *L. plantarum* 10hk2 and *L. plantarum* ATCC 14917 of antibacterial effects against pathogenic bacteria is shown in Figure 1. The two strains both showed excellent antibacterial effects against *E. coli*, *S. aureus*, *E. cloaceae*, *C. freundii*, and *S. typhimuriun*. However, the sensitivities of *S. paratyphi* and *S. flexneri* to these probiotic strains seemed to be different from each other, *L. plantarum* 10hk2 showing approximately 90% inhibition against these pathogenic bacteria.

Table 2. Composition of organic acids in 10% solution† of *L. plantarum* strains with white cabbage fermentation‡

Strains	Lactic	Malic	Acetic	Succinic	pH in 10% solution
L. plantarum 10hk2	52.26%	0.11%	0.65%	0.36%	pH 4.18
L. plantarum ATCC 14917	11.79%	2.75%	0.03%	0.13%	pH 3.44

Content of the organic acids is expressed as a percentage (gram per 100 gram of dried white cabbage fermentation) and was determined by HPLC.
†, the dispersed solution (10% in water, w/v) was measured for pH; ‡, Chinese white cabbage waste from the Kimchi factory was used as sole nutritional source and 5% of each starter (*L. plantarum*) was inoculated. After 3 days of incubation at 37°C, both cabbage fermentations were dried at 60°C to approximately 5% moisture.

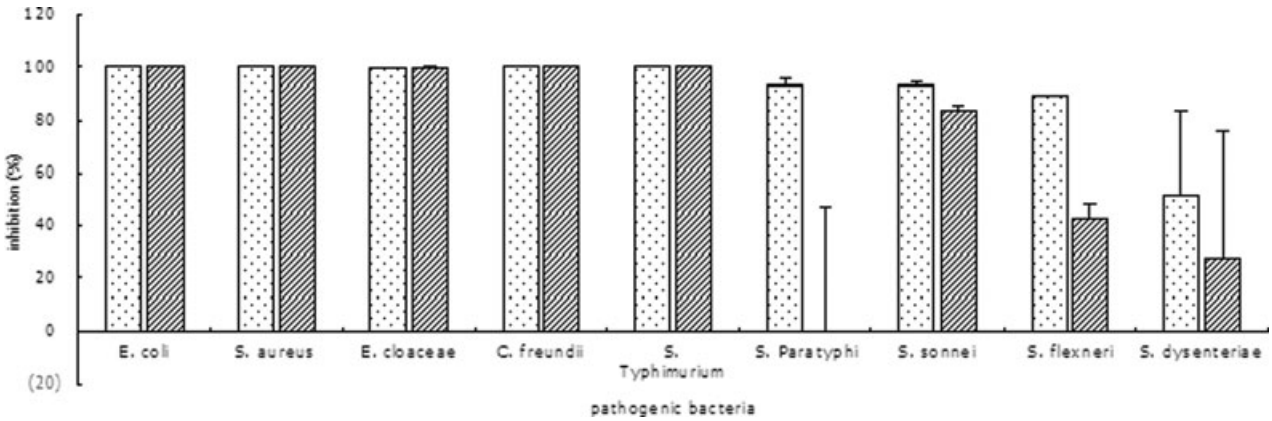

Fig. 1. Antibacterial effects of *L. plantarum* against pathogenic bacteria. The pathogenic strains used as indicators have been described in the text. Each indicator bacterium was inoculated at a concentration of 10^7 CFU/ml, and then co-incubated with *L. plantarum* at 37°C for 24 hr. Antibacterial activity was determined by measuring the viability of the pathogenic bacteria and each of the pathogenic bacteria was cultured separately for the controls. (▨), *L. plantarum* 10hk2; (■), *L. plantarum* ATCC 14917.

NO production and cytokine induction

RAW 264.7 cells in DMEM (control cells) produced small amounts of NO in the culture supernatant, whereas cells stimulated with LPS (positive control) generated 12.4 ± 0.7 μM. When cells were stimulated with VC at a cell density of 5.0×10^6 CFU/ml, NO production increased to the same amount as was found with LPS. However NO production induced by HK stimulated cells at the same density was much less than that induced by VC (Fig. 2). Dose-dependent release of various cytokines from RAW 264.7 cells by VC and HK is shown in Figure 3. IL-1β concentrations in RAW 264.7 cells cultured with LPS (2 μg/ml) were 20.0 ± 3.0 pg/ml, and release of IL-1β after stimulation by VC at a density of 5×10^6 CFU/ml was greater than after LPS stimulation. The most noticeable effects of VC were on secretion of TNF-α, treatment at a density of 5×10^6 CFU/ml generating much more TNF-α (4401 ± 169 pg/ml) than was observed in the negative control cells. In addition, release of IL-6 was increased only when the cells were stimulated with VC at a density of 5×10^6 CFU/ml, which was at least three times greater than the production induced by HK treatment. Interestingly, the anti-inflammatory mediator IL-10 was also detected only with VC treatment, and in amounts equal to those found with the positive control, LPS.

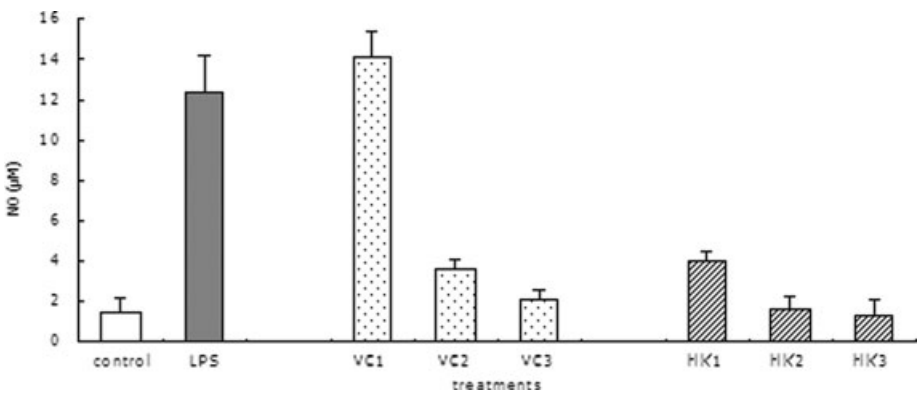

Fig. 2. Nitric oxide production by RAW 264.7 cells at various concentrations of viable and heat-killed cells of *L. plantarum* 10hk2. RAW 264.7 cells at a density of 5.0×10^5 cells/ml were stimulated with VC or HKC for 48 hr. The culture supernatants were collected and analyzed for NO production. Results were expressed as μM and presented as means ± SD of three independent experiments. 1, 5×10^6 CFU/ml; 2, 5×10^5 CFU/ml; 3, 5×10^4 CFU/ml; HKC, heat killed cells; VC, viable cells; (■), positive control, LPS (2 μg/ml); (▨), VC; (■), HKC.

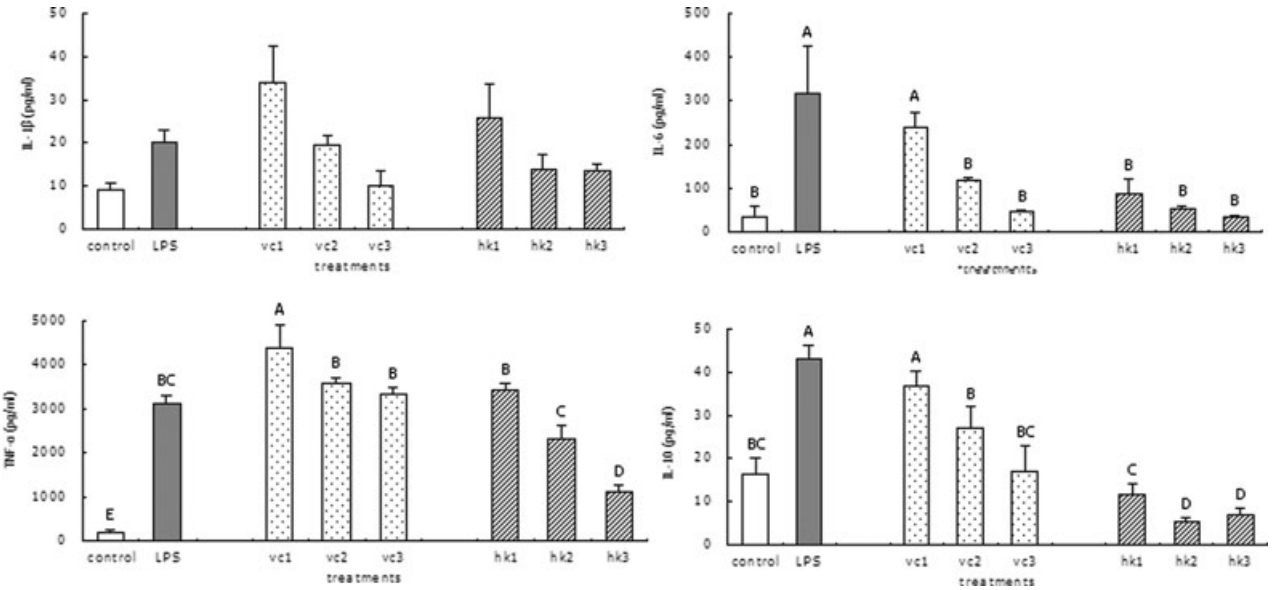

Fig. 3. Comparison of the effects of viable and heat-killed cells of *L. plantarum* on pro- and anti-inflammatory cytokine production by RAW 264.7 cells. The RAW 264.7 cells were stimulated with VC or HKC for 24 hr. The culture supernatants were collected and analyzed for cytokine induction. Data are presented as the means ± SD of three independent experiments and expressed in pg/ml units. Means with different superscripts (A, B, C and D) are significantly different at the level $P \leq 0.05$; 1, 5×10^6 CFU/ml; 2, 5×10^5 CFU/ml; 3, 5×10^4 CFU/ml; HKC, heat killed cells; VC, viable cells; (■), positive control, LPS (2 μg/ml); (▨), VC; (■), HKC.

Effects of *L. plantarum* 10hk2 supplementation on fecal microflora

Table 3 shows the fecal microflora in mice fed *L. plantarum* 10hk2 in comparison to the control group and mice fed *L. plantarum* ATCC 14917 for 4 weeks. Lactic acid bacteria co-existed with other harmless bacteria in the intestines of the ICR control mice at a concentration of 7.11 ± 0.10 log/ml. *Salmonella* and *Shigella* species constituted approximately 5% of the enteric bacteria. The *L. plantarum* 10hk2 fed groups showed reduced ratios of enteric bacteria, including *Salmonella* and *Shigella* species, which were reduced from 18% and 5% to 3% and 0.5%, respectively, after 4 weeks of supplementation. This tendency was also observed in the *L. plantarum* ATCC 14917 fed group, but this commercial species showed slightly fewer lactic acid bacteria after 2 weeks, and more enteric bacteria, excluding lactic acid bacteria and *Salmonella* and *Shigella* species, after 4 weeks of supplementation.

Splenocyte population in ICR mice as determined by flow cytometric analysis

Spleen cells isolated from mice fed with a normal diet or *L. plantarum* 10hk2 diets for 4 weeks were collected and surface markers of the lymphoid cells, namely B and T lymphocytes, were assessed. Flow cytometric analysis demonstrated that the spleen was made up of 12.2% $CD19^+$ splenocytes (B lymphocytes) and 18.4% $CD3^+$ splenocytes (T lymphocytes) in normal mice at the age of 10 weeks (Table 4). The percentage of $CD19^+$ and $CD3^+$ splenocytes in the control mice were similar after stimulation with the external antigen, SRBC. However, the percentage of B lymphocytes was slightly greater in the micefed *L. plantarum*, this tendency being a little stronger in the micefed *L. plantarum* 10hk2.

DISCUSSION

Based on its tolerance to acid, heat and freezing, the *L. plantarum* 10hk2 isolated in this study holds promise as a potentially beneficial probiotic strain. The rate of recovery of this strain after freeze drying was quite different from that of *L. plantarum* ATCC 14917. After freeze drying under the same culture and freezing conditions (including similar initial cell numbers of 1.1 and 1.5×10^9 CFU/g, respectively, data not shown), the numbers of viable *L. plantarum* 10hk2 and *L. plantarum* ATCC 14917 were 3.0×10^{12} CFU/g and 1.7×10^{11} CFU/g, respectively. The viability of lactic acid bacteria after lyophilization seems to be species-specific. Many factors which influence the rate of survival after freeze-drying, such as bacterial species, cell density, use of a lyoprotectant, drying medium and

Table 3. Comparison of fecal lactic acid and enteric bacteria, and *Salmonella* and *Shigella* species population of mice

		Periods			
		1st week	2nd week	3rd week	4th week
Control	LAB	7.69 ± 0.25 (80 ± 8%)	7.81 ± 0.14 (82 ± 6%)	7.79 ± 0.05 (78 ± 2%)	7.89 ± 0.07 (78 ± 2%)
	S & S†	ND	6.47 ± 0.23 (6 ± 4%)	6.65 ± 0.06 (6 ± 1%)	6.66 ± 0.17 (5 ± 2%)
	Enteric‡	7.07 ± 0.10 (20 ± 8%)	7.03 ± 0.03 (14 ± 2%)	7.10 ± 0.14 (16 ± 3%)	7.25 ± 0.07 (18 ± 1%)
L. plantarum 10hk2	LAB	7.56 ± 0.11 (70 ± 10%)	7.87 ± 0.05 (93 ± 1%)	7.79 ± 0.02 (92 ± 1%)	8.43 ± 0.10 (96 ± 1%)
	S & S	ND	5.93 ± 0.07 (2 ± 1%)	6.00 ± 0.15 (2%)	6.16 ± 0.10 (0.5%)
	Enteric	7.18 ± 0.17 (30 ± 10%)	6.68 ± 0.10 (6 ± 1%)	6.62 ± 0.08 (6 ± 1%)	6.90 ± 0.05 (3%)
L. plantarum ATCC 14917	LAB	7.64 ± 0.14 (68 ± 11%)	7.82 ± 0.06 (83 ± 7%)	7.92 ± 0.04 (90 ± 1%)	8.10 ± 0.11 (90 ± 3%)
	S & S	ND	6.23 ± 0.07 (2 ± 1%)	6.16 ± 0.15 (2%)	6.32 ± 0.05 (2%)
	Enteric	7.31 ± 0.12 (32 ± 11%)	7.02 ± 0.22 (14 ± 6%)	6.87 ± 0.07 (8 ± 1%)	7.04 ± 0.09 (8 ± 2%)

All values represent the average log number ± SD and values in parentheses represent the average percentage ± SD of the total bacteria.
†, intestinal *Salmonella* and *Shigella* species were counted using MacConkey agar and *Salmonella* and *Shigella* media; ‡, the other enteric bacteria apart from LAB and *Salmonella* and *Shigella* species were counted; LAB, lactobacilli; ND, not detected; S & S, *Salmonella* and *Shigella* species.

freezing rate, have been reported (27, 28). In conclusion, *L. plantarum* 10hk2 was found to be a less stress sensitive species than *L. plantarum* ATCC 14917.

In our study, each mouse consumed 4 to 5 grams of the experimental diet per day on average (data not shown), accordingly the amount of lactic acid bacteria consumed was calculated to be at least 1.0×10^8 CFU/g/day. Supplementation of probiotics results in an excessive number of lactic acid bacteria relative to the number of pathogenic bacteria in the intestinal microflora; thus, this change in balance of intestinal flora may result in effective antibacterial activity that could inhibit the *in vivo* growth of enteric bacteria. The relative proportions of enteric and *Salmonella* and *Shigella* species had decreased at the end of two and three weeks of supplementation from 14% to 6% and 6% to less than 1%, respectively. For probiotic applications in chickens, dried bacterial cells of the *Lactobacillus* strains were mixed with commercial feed to achieve a desired bacterial count of 10^6 CFU/g of the feed. This treatment resulted in stable lactobacilli counts and reduced the numbers of *Enterobacteriaceae* in the intestine (29). This probiotic strain is believed to pass safely from the stomach to the intestine, and could therefore inhibit the growth of enteric bacteria such as *E. coli*, *Salmonella* and *Shigella* species. The present results could be explained by the ability of *L. plantarum* 10hk2 to produce huge amounts of lactic acid compared to the commercial strain, *L. plantarum* ATCC 14917. *In vitro,* the antibacterial activity of the cultural supernatant of *L. plantarum* 10hk2 against *S. paratyphi* and *S. flexneri* was found to be stronger than that of *L. plantarum* ATCC 14917. One plausible reason for this having occurred is the huge amounts of lactic acid that are produced by *L. plantarum* 10hk2. Some previous studies have also indicated that the inhibitory action of lactic acid bacteria is due to their production of lactic acid (30, 31). In addition, the dry weight of the feces was measured and found to be significantly less in the *L. plantarum* 10hk2 group (over 30% less) relative to the control group (data not shown). This observation suggests that

Table 4. Effect of 4 weeks of supplementation with viable *L. plantarum* on splenocyte populations of 10 week old mice

	$CD19^+$ cells (%)		$CD3^+$ cells (%)	
Group	PBS	SRBC†	PBS	SRBC
Control	12.2	11.5	18.4	17.8
L. plantarum 10hk2	10.1	14.9	18.7	20.1
L. plantarum ATCC 14917	9.7	12.2	17.5	17.2

†, ICR mice (8 weeks) were intraperitoneally injected with SRBC at the end of a 4 weeks experimental period. After 2 weeks, the spleens were aseptically removed, pooled and stained with mAb.

improved dietary digestion is achieved by adding lactic acid bacteria to the feed.

An increase in the percent of $CD19^+$ cells in the spleens of the healthier mice which had been injected with an external antigen (SRBC) and fed with *L. plantarum* 10hk2 may be explained by the reduction in the number of harmful enteric bacteria and altered balance of the intestinal microflora. In other words, the ability of the viable *L. plantarum* 10hk2 to induce pro-inflammatory mediators such as NO, IL-6 and TNF-α and anti-inflammatory IL-10 may have resulted in induction of immune responses, resulting in healthier hosts. This finding is in accordance with the fact that the *Lactobacillus* strains were demonstrated to have strain-specific effects on B- and T cell proliferation. Also *L. acidophilus* treatment has been found to enhance B cell response with external LPS stimulation (32). Furthermore, TNF-α production is enhanced by exposure to lactic acid bacteria (12, 16), and therefore the effectiveness of B cells would, at least in part, have been enhanced. In a more recent review, a response regulator of B lymphocytes, CD19 deficiency, was found to inhibit infiltration of neutrophils and macrophages, and to decrease expression of cytokines such as IL-6 and TGF-β in mice. CD19, a regulator of B cell response, has also been demonstrated to regulate IL-6 expression in wound healing tissues in mice (33).

In this study, we compared the ability of viable and heat-killed cells of *L. plantarum* 10hk2 to induce production of pro- and anti-inflammatory responses in RAW 264.7 cells. The ability of this strain to induce release of these cytokines was reduced after viable cells had been subjected to heat treatment. These results are consistent with a recent study which examined *Lactococcus* strains (18), and several other previous studies (8–12). In regards to the release of anti-inflammatory cytokines in J774.1 cells, viable cells of *L. casei* have been found to stimulate greater amounts of TGF-β than do heat-killed bacteria (12). However, this study has demonstrated that viable cells of *L. plantarum* 10hk2 are able to simultaneously induce both pro- and anti-inflammatory mediators. A previous study found that, in human peripheral blood mononuclear cells stimulated with live *L. rhamnosus* and *L. acidophilus,* production of both pro- and anti-inflammatory cytokines, such as IL-6, TNF-α and IL-10, was induced in amounts even greater than those produced by LPS (11).

In conclusion, it was found in this study that *L. plantarum* 10hk2 supplementation enhances the health of mice by reducing the number of harmful enteric bacteria in the intestines, these changes being due to production of huge amounts of lactic acids and to the immune-enhancing effects of *L. plantarum* 10hk2. The present study also demonstrates that viable cells of this strain can induce both pro- and anti-inflammatory mediators, which helps to maintain a balance between these two adverse types of mediator. Based on these results, it appears that this probiotic strain may be a good candidate for a food additive to keep organisms healthier.

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