Immunoenhancing Effects of a New Probiotic Strain, Lactobacillus fermentum PL9005

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ABSTRACT

The immunoenhancing effects of Lactobacillus fermentum PL9005 were assessed via mouse intragastric inoculation. The number of immunoglobulin A–positive cells in the small intestine, CD4⁺ T lymphocytes in the peripheral blood, and the lymphocyte proliferation response to mitogen stimulation (lipopolysaccharide) increased in mice fed L. fermentum PL9005. The lactic acid concentration also increased dose dependently in the small intestine of mice fed L. fermentum PL9005. No differences were found in body weight, food intake, and clinical signs between mice fed L. fermentum PL9005 and the control group. Results indicated that L. fermentum PL9005 is a probiotic with immunoenhancing properties.

Chemical agents have been widely studied and used to overcome immunodeficiencies that result from aging, stress, infectious diseases, and malnutrition. Unfortunately, the use of many of these agents is often associated with adverse effects. Natural food products with immunoenhancing properties and free of adverse effects would be beneficial to people with impaired immune function.

Lactic acid bacteria (LAB) such as lactobacilli and bifidobacteria are normal components of healthy human intestinal microflora and are commonly used for the fermentation of certain foods. Some LAB have health-promoting attributes, including antimicrobial properties (13, 17, 27, 30) and immunomodulation (11, 12, 21, 22). Because of interest from nutrition, health, and food scientists, new LAB with these health-promoting properties have been identified and successfully introduced into the food and pharmaceutical markets (19, 23, 26). However, much variation exists in the immunomodulating ability of LAB strains. For examples, Gill et al. (12) showed that ingestion of LAB enhanced the proliferation ability of spleen cells to be stimulated by concanavalin A (ConA) and lipopolysaccharide (LPS). However, Kirjavainen et al. (18) demonstrated that oral treatment with LAB inhibited lymphocyte proliferation, suggesting that these bacteria may have potential for use in the management of hypersensitivity responses.

In an attempt to find a new strain of LAB with strong probiotic effects, we screened LAB strains isolated from human infant feces for their antimicrobial effects against several bacteria and selected one strain showing the strongest effect. The strain was identified as Lactobacillus fermentum through the use of an API 50CH kit, colony staining, and 16S rRNA sequencing; the strain was designated L. fermentum PL9005. We examined the immunomodulatory effects of this new probiotic strain via intragastric inoculations in mice.

MATERIALS AND METHODS

Isolation of LAB. Human infant feces were collected using a cotton swab and then inoculated on deMan Rogosa Sharpe (MRS) agar (Difco, Becton Dickinson, Sparks, Md.) containing 0.002% bromphenol blue. Following an incubation period of 3 to 4 days at 25°C, bacteria were isolated from a single colony and tested for inhibitory effects on various other bacteria.

Disk filter assay of inhibitory effects of LAB on the growth of various other bacteria. The overnight culture of each LAB was extracted with an equal volume of ethyl acetate and concentrated in a vacuum evaporator. The concentrate was dispersed in water at 1:50. An extract aliquot of 50 μl was dried on an antibiotic disk filter (Whitman, Clifton, N.J.) before being placed with various bacteria, each at log phase, inoculated on brain heart infusion solid medium. Bacteria used for the test were Aeromonas hydrophila CCARM 6001 (Culture Collection of Antibiotic Resistance Microbes, Seoul, Korea), Bacillus subtilis ATCC 6633 (American Type Culture Collection, Rockville, Md.), Enterococcus faecalis ATCC 29212, Enterococcus faecium CCARM 10090, Escherichia coli O157 ATCC 43894, Listeria monocytogenes ATCC 19113, Shigella flexneri ATCC 9199, Shigella sonnei ATCC 9290, Salmonella enterica serotype Enteritidis CCARM 10290, Salmonella enterica serotype Typhimurium CCARM 11001, and Staphylococcus aureus ATCC 29213. After an overnight incubation, the diameter of the zone of growth inhibition was measured with a ruler.

Identification of LAB. The LAB strain with the highest level of activity was selected and identified according to Bergey’s Manual of Systematic Bacteriology (9) using an API 50CH kit (bioMérieux Vitex, Hazelwood, Mo.) and staining. This bacterium was identified as L. fermentum and was submitted to the Korea Culture Collection of Microbes (KCCM; Seoul, Korea) as L. fer-
mentum PL9005 (KCCM 10250). DNA sequencing of the full 16S rRNA gene of *L. fermentum* PL9005 was performed in an automatic DNA sequencer (ABI Prism, Perkin-Elmer, Foster City, Calif.), submitted to the GenBank database, and assigned the accession number AF477498.

Animals. Five-week-old specific-pathogen-free BALB/c female mice (Biogenomics, Seoul, Korea) were housed in individual cages at 22 ± 2°C under a light-dark cycle of 14 and 10 h. Food and water were provided ad libitum and were removed from the cages 5 h prior to inoculation of *L. fermentum* PL9005. Body weights were measured weekly, and food intake was measured daily. Mice were checked twice daily for clinical signs such as diarrhea, ruffled fur, and lethargy. All animal experiments were performed in accordance with the laboratory animal guidelines of Seoul National University.

Bacterial preparation and experimental design. Five mice were randomly allocated into each of three groups. *L. fermentum* PL9005 was lyophilized (10^{11} CFU/g) and stored at 4°C. The bacteria were diluted with sterile phosphate-buffered saline (PBS; pH 7.2) to make solutions of 10^7 and 10^9 CFU/0.5 ml immediately prior to inoculation. The bacterial viability was checked by plating on MRS agar and always was over 90%. Each mouse was fed daily with either 10^7 or 10^9 CFU/0.5 ml of the inoculum via the intragastric route for 28 days, and the control mice were fed 0.5 ml of PBS.

Blood collection. At the end of the experiment, all mice were anesthetized with ether, and blood samples were collected by cardiac puncture using a heparin-treated syringe. After the blood samples were centrifuged at 2,700 × g for 30 min, buffy coats were carefully collected into separate microtubes. 0.87% Tris-NH_4Cl was added, and the solution was incubated for 10 min at room temperature to lyse erythrocytes. Cells were washed twice with PBS and pooled from two or three mice of each group, and flow cytometry analyses were performed to assay the expression of CD4^+ and CD8^+ T lymphocytes.

Spleen cell preparation. Spleens were removed aseptically from mice and placed individually into petri dishes containing 3 ml of complete RPMI 1640 medium. Single cell suspensions were prepared by chopping the spleens into small pieces with sterile scissors and then forcing the spleen tissue up and down through a 3-ml syringe as previously described (12). The suspension was transferred to a 15-ml conical tube containing 3 ml of complete RPMI 1640 and centrifuged at 1,700 rpm for 10 min. The cells were resuspended in 0.87% Tris-NH_4Cl and incubated for 10 min at room temperature to lyse erythrocytes. After washing twice in complete RPMI 1640, the suspensions were adjusted to a final concentration of 2 × 10^6 cells per ml in complete RPMI 1640.

Assay of cells positive for IgA in the small intestine. After blood collection, the mice were killed by cervical dislocation and necropsied. Small intestine samples (from 2 cm distal to the stomach to 0.5 cm proximal to the cecum) were fixed in 10% neutral formalin for 24 h. Five portions were removed at regular intervals from each small intestine. Each portion was dehydrated in an alcohol-xylene series and embedded in paraffin for immunohistochemistry. From each paraffin block, 2-μm sections were prepared on poly-L-lysine-coated slides and dried overnight at 55°C. Following deparaffinization and rehydration with the xylene-alcohol series, the sections were incubated in 3% H_2O_2 in methanol for 10 min to block any endogenous peroxidase activity. The sections were then incubated with normal goat serum for 1 h at 4°C to block any nonspecific reactions. The sections were incubated overnight at 4°C with horseradish peroxidase (HRP)–conjugated goat anti-mouse immunoglobulin (Ig) A (Zymed, South San Francisco, Calif.) diluted 1:100 with 0.05% Tween 20 in PBS (T-PBS). After the sections were washed three times with T-PBS for 5 min each, peroxidase activity was assayed using 3,3′-diaminobenzidine (Vector Laboratories, Burlingame, Calif.) as a chromogen, and sections were counterstained with Mayer hematoxylin for 15 s and mounted. The number of positive cells in 10 villi in the small intestine (2 villi per portion) was counted as previously described by Matar et al. (22).

Analysis of CD4^+ and CD8^+ T lymphocytes in blood. The prepared blood cells were added to 96-well V-bottom tissue culture plates in triplicate and incubated with 1:100 diluents (vol/vol in PBS) of rat anti-mouse CD4 and CD8 antibodies (Research Diagnostics, Flanders, N.J.) for 30 min at 4°C. After washing with a primary wash buffer (10 ml of 20% NaNO_3, 100 ml of acid-citrate-dextrose [0.73% citric acid, 2.20% sodium citrate, and 2.45% dextrose, pH 7.2], 40 ml of EDTA, 20 ml of phenol red, 20 ml of horse serum, and 900 ml of PBS), the cells were incubated with 1:400 diluent (vol/vol in PBS) of fluorescein-conjugated anti-rat IgG antibodies (Rockland, Gilbertsville, Pa.) for 30 min at 4°C. The cells then were washed twice and fixed with 2% formalin buffer, and the expression of CD4^+ and CD8^+ T lymphocytes was analyzed with a FACSCalibur flow cytometer (Becton Dickinson Instruments, Cambridge, Mass.).

Proliferation assay. Proliferation responses of spleen cells to mitogens were determined using a commercial cell proliferation enzyme-linked immunosorbent assay (ELISA) kit (Roche Diagnostics, Mannheim, Germany) as previously described (8). A 50-μl solution of 10^6 cells in complete RPMI 1640 medium was added to the wells of a 96-well tissue culture plate and cultured in the presence or absence of T- and B-cell mitogens. Fifty microliters of ConA (2.5 μg/ml; Sigma, St. Louis, Mo.) and LPS (5 μg/ml, derived from *E. coli*; Sigma) was added to the wells. Control wells received 50 μl of complete medium. The cells were cultured for 72 h at 37°C in a humidified incubator supplemented with 5% CO_2. Cell proliferation over the final 18 h of culture was determined by measuring the incorporation of 5-bromo-2-deoxyuridine (BrdU) using peroxidase-conjugated anti-BrdU antibodies and a peroxidase substrate system. The absorbance of each well was read at 450 nm with an ELISA reader (Sunrise, TECAN Austria GmbH, Salzburg, Austria), and the results were expressed as the absorbance of mitogen-treated wells divided by the absorbance of control wells.

Assay of lactic acid in the small intestine. A part of the small intestine of each mouse was homogenized in PBS (0.01 g ml⁻¹) with an Ultra Turrax T8 (Ika, Staufen, Germany). The homogenate was centrifuged at 1,700 × g for 10 min, and the supernatant was collected. The amount of lactic acid in the supernatant was assayed with a lactate kit (Sigma) according to the manufacturer’s protocol.

Statistical analysis. Significant differences between the experimental and control groups were identified using the Duncan multiple range test (version 8.1, SAS Institute, Inc., Cary, N.C.) and t tests. Differences were considered significant at *P* < 0.05.

RESULTS

Inhibitory activity against various bacteria and identification of *L. fermentum* PL9005. The extract of the overnight culture had strong inhibitory effects against various bacteria. The diameters of the growth inhibition zones
FIGURE 1. Immunohistochemical assay of IgA-positive cells in the mouse small intestine was conducted to determine the number of positive cells in 10 villi in the small intestine (each two of five portions) (A). Mean values significantly different from those of control mice ($P < 0.05$) are indicated (*). Immunohistochemical assay was also conducted using HRP-conjugated goat anti-mouse IgA in the small intestine of a mouse fed $10^9$ CFU $L$. fermentum PL9005 (B) and a control mouse $L$. fermentum (C). Mayer hematoxylin stain was used as background; $\times 320$ (B) and $\times 300$ (C).

on inoculated plates of each bacterium were 21.5 mm for $A$. hydrophila, 24 mm for $B$. subtilis, 25 mm for $E$. faecalis, 19 mm for $E$. faecium, 17 mm for $E$. coli O157, 25 mm for $L$. monocytogenes, 17 mm for Salmonella Enteritidis, 16 mm for Salmonella Typhimurium, 24.5 mm for $S$. flexneri, 16 mm for $S$. sonnei, and 23 mm for $S$. aureus. With the API 50CH kit, the bacterium in the overnight culture was identified as $L$. fermentum with 93.2% similarity and was designated $L$. fermentum PL9005. Identification was confirmed with full DNA sequencing of the 16S rRNA gene, which showed 99.19% similarity to $L$. fermentum.

Body weight gain, food intake, and clinical signs in mice. There was no significant difference in body weight gain and food intake between the mice fed $L$. fermentum PL9005 and the control mice (data not shown). No clinical signs such as diarrhea, ruffled fur, and lethargy were observed in either treated or control mice.

Determination of IgA-positive cells in the mucosa of mouse small intestine. IgA-positive cells were assayed in the histological samples of the small intestines of mice by direct immunohistochemistry with an anti-mouse IgA antibody. Intragastric administration of $L$. fermentum led to a significant increase of IgA-positive cells in the lamina propria of the small intestine ($P < 0.05$, Fig. 1).

Lymphocyte subsets. Table 1 shows the percentages of lymphocyte subsets in the peripheral blood of the mice

<table>
<thead>
<tr>
<th>Group $a$</th>
<th>CD4$^+$</th>
<th>CD8$^+$</th>
<th>ConA</th>
<th>LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>46.95 ± 3.64</td>
<td>8.52 ± 1.11</td>
<td>5.44 ± 1.85</td>
<td>2.55 ± 1.46</td>
</tr>
<tr>
<td>$L$. fermentum PL9005</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$10^7$ CFU</td>
<td>57.37 ± 6.51$^c$</td>
<td>11.02 ± 2.99</td>
<td>5.62 ± 1.93</td>
<td>4.83 ± 1.71$^c$</td>
</tr>
<tr>
<td>$10^9$ CFU</td>
<td>49.48 ± 8.39</td>
<td>11.09 ± 5.04</td>
<td>6.18 ± 2.06</td>
<td>5.27 ± 1.44$^c$</td>
</tr>
</tbody>
</table>

$^a$ Each group consisted of five mice.

$^b$ Results are expressed as absorbance of mitogen-treated wells divided by the absorbance of control wells at 450 nm in an ELISA.

$^c$ Significantly different from control mice ($P < 0.05$).
fed *L. fermentum* PL9005 and the control mice. The percentages of CD4⁰ T lymphocytes were significantly higher in blood samples of mice fed 10⁷ CFU *L. fermentum* compared with control mice (*P < 0.05*). Mean percentage of CD4⁰ T lymphocytes in mice fed 10⁹ CFU *L. fermentum* was also slightly higher than that in the control group, although the means were not significantly different. The percentage of CD8⁰ T lymphocytes was not significantly different between treated and control mice.

**Proliferation assay.** The proliferative responses of spleen cells to stimulation with ConA in mice fed *L. fermentum* PL9005 were slightly but not significantly higher than those in control mice (Table 1). However, the response to stimulation with LPS was significantly higher in mice fed *L. fermentum* PL9005 than in control mice (Table 1).

**Assay of lactic acid concentration in mouse small intestine.** Lactic acid concentrations increased dose dependently in the small intestines of the mice fed *L. fermentum* PL9005 and were significantly higher in the mice receiving 10⁹ CFU than in control mice (*P < 0.05*, Fig. 2).

**DISCUSSION**

LAB can exhibit antagonistic activity toward various human intestinal pathogens. Certain LAB synthesize antimicrobial compounds that are related to the bacteriocin family (16). Others compounds are well-known metabolic end products of lactic acid fermentation, such as lactic and acetic acids and hydrogen peroxide (28, 31) or are unidentified (5). However, the spectrum of antibacterial activity is very specific to each strain. For example, *L. fermentum* ATCC 14931 did not show any inhibitory activity against *E. coli* (3) or *Salmonella Enteritidis, Salmonella Typhimurium,* and *S. aureus* (20). Compared with these findings, *L. fermentum* PL9005 in the present study showed a broad spectrum of antibacterial activity.

In the present study, several immune indices such as number of IgA-positive cells in the small intestines, the CD4⁺ T lymphocyte ratio in blood, and the lymphocyte proliferation response to LPS stimulation were enhanced in mice fed *L. fermentum* PL9005.

Mucosal immunity is essential for protection against pathogens in the gastrointestinal tract, and secretory IgA constitutes almost 80% of all antibodies produced in mucosa-associated tissue (24, 25). Matar et al. (22) found that ingestion for 3 days of milk fermented by *L. helveticus* increased the number of IgA-positive cells in intestinal and bronchial tissues. These authors suggested that the peptides released from the milk proteins might be responsible for the increase in number of IgA-positive cells. However, in the present study *L. fermentum* PL9005 was administered as a PBS suspension, not in milk, which suggests that the increase of IgA-positive cells in the small intestine may be caused by intact *L. fermentum* PL9005 itself or partially degraded *L. fermentum* PL9005 antigens rather than fermented derivatives.

To have good stimulation of intestinal mucosa, the number of IgA-positive lymphocytes must be only slightly higher than that found in normal control mucosa (7). High numbers of IgA-positive cells could have harmful effects on the host and favor an increase in intestinal permeability, which occurs with celiac disease (29). In the present study, the number of IgA-positive cells was significantly higher (approximately three times) in mice fed *L. fermentum* PL9005 than in the control mice. Because no clinical signs, including diarrhea, were observed in mice fed *L. fermentum* PL9005, there may be few adverse effects of the increase in IgA-positive cells caused by ingestion of *L. fermentum* PL9005 in normal mice. Nevertheless, studies are needed on the appropriate dose or inoculation period sufficient to increase the number of IgA-positive cells without inducing adverse effects.

T cells are the main effectors and regulators of cell-mediated immunity. In the present study, ingestion of *L. fermentum* PL9005 resulted in an increase in CD4⁺ T cells in blood that was not dependent on the inoculation dose. Thus, *L. fermentum* PL9005 might be able to induce systemic upregulation of CD4⁺ cells. Gill et al. (12) found a slight decrease in the percentage of CD4⁺ cells in mice fed LAB for 28 days. However, these authors did not refer to the precise mechanism causing such an event. CD4⁺ T cells probably are altered differently depending on the LAB strains administered. In the present study, the populations of natural killer cells or B lymphocytes in blood were not examined.

Lymphocyte proliferation responses to mitogens are widely used to assess T- and B-cell function. In the present study, ingestion of *L. fermentum* PL9005 led to a significant increase in B-cell mitogens, which is the lymphocyte proliferation response to LPS stimulation. Although not significantly different, T cell mitogens, the response to the stimulation with ConA, were also slightly higher in mice fed *L. fermentum* PL9005 than in control mice. Kirjavainen et al. (18) assessed the lymphocyte proliferation response in the presence of mitogens of three different concentrations. The tendency for a significant difference between groups differed slightly according to the mitogen concentrations used. In the present study, we used only one con-
centration of mitogen (ConA, 2.5 μg/ml; LPS, 5.0 μg/ml). The concentration of ConA used probably is inappropriate for finding a significant difference in lymphocyte proliferation response between mice fed L. fermentum PL9005 and control mice. Although additional tests are needed for confirmation, we expect that ingestion of L. fermentum PL9005 may lead to the enhancement of lymphocyte proliferation responses to stimulation by both ConA and LPS.

LAB exert antimicrobial activity via production of hydrogen peroxide, lactic acid, and antimicrobial chemicals such as bacteriocins (2). Assays of lactic acid concentrations in the small intestines of mice in the present study revealed that ingestion of L. fermentum PL9005 induced dose-dependent increases in lactic acid production in the small intestine. L. fermentum PL9005 probably produces this effect for protection against enteric pathogens in the small intestine. In the present study, the culture extract prepared to avoid the adverse effects of hydrogen peroxide and lactic acid had inhibitory activity with a broad spectrum. Because most bacteriocins produced by LAB inhibit gram-positive bacteria, this result was unexpected. We are now in the process of isolating and characterizing these inhibitory chemicals.

Several studies have revealed that LAB are associated with pathological changes such as bacteremia (4) and occasionally endocarditis and abscesses (1, 10), although it is unlikely that LAB were the causative agents in these cases (10, 14). Therefore, LAB safety assessments are regarded as the most important step prior to the incorporation of these organisms into food products (6, 15). In the present study, body weight measurements, food intake, and observations for clinical signs indicated that L. fermentum PL9005 is nonpathogenic in mice. However, other important criteria such as bacterial translocation to mesenteric lymph nodes, liver, and spleen and the results of blood chemistry and hematology analyses must be thoroughly assessed.

Nutrition and food scientists are largely concerned with finding new strains of LAB with potential probiotic activities. The results of this study suggest that L. fermentum PL9005 can be probiotic, as indicated by its immunoenhancing effects, although it is not clear that its probiotic ability is greater than those for other bacteria such as Lactobacillus rhamnosus (HN001) and Lactobacillus acidophilus (HN017).

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