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Safety Assessment of *Lactobacillus fermentum* PL9005, a Potential Probiotic Lactic Acid Bacterium, in Mice

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Abstract We recently isolated a novel probiotic strain, *Lactobacillus fermentum* PL9005 (KCCM-10250), from infant feces and showed that it had a potential immunoenhancing effect. In the present study, a safety assessment of the bacteria was performed using a BALB/c mouse model. Mice were administered with *L. fermentum* PL9005 daily for 28 days. There were no detectable changes in body weight, feed intake, or clinical signs, and no significant difference in hematological parameters or blood biochemistry between the *L. fermentum* PL9005-fed and control groups. Bacterial translocation was detected in the mesenteric lymph nodes, liver, and spleen of some mice with and without *L. fermentum* PL9005 feeding, however, the organisms were not related to ingestion of *L. fermentum* PL9005; this was confirmed by PCR using a species-specific primer. No gross lesions were detected in the liver, spleen, or intestine of *L. fermentum* PL9005-fed or control mice. Mucosal thickness in the ileum, cecum, and colon of *L. fermentum* PL9005-fed mice was not significantly different from that of corresponding organs in control mice. No inflammation or epithelial cell degeneration in the intestines was observed in any mice. These results indicate that ingestion of *L. fermentum* PL9005 is safe in mice and can be applied in the functional food market.

Key words: *Lactobacillus fermentum* PL9005, immunoenhancing effect, safety assessment, mice

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. It is well-known that some LABs have health-promoting attributes, including antimicrobial properties [13, 19, 20, 21, 29, 30], immunomodulation [12, 25, 26], lactose intolerance alleviation [9], antitumor properties [18, 26], and hypocholesterolemic effects [5, 8]. These findings have caught the attention of nutrition, health, and food scientists. Consequently, new LABs with these health-promoting properties have been identified and successfully introduced into the food and pharmaceutical markets [22, 27].

Several studies have revealed that LABs are associated with clinically pathological conditions such as bacteremia [2], and they are occasionally associated with endocarditis and abscess as well [1, 11]. However, it is unlikely that LAB was the causative agent in these cases [11, 16]. Taking this into account, safety assessment is regarded as the first and most important step before these strains are incorporated into food products [4, 17].

We recently isolated a novel probiotic strain, *Lactobacillus fermentum* PL9005 (KCCM-10250), from infant feces and showed that its dietary intake suppresses type 2 helper T cell production in antigen-primed mice splenocyte [23] and had a potential immunoenhancing effect [28]. In the present study, the safety on long-term ingestion of *L. fermentum* PL9005 was assessed in a BALB/c mouse model with some modifications of methods used previously [37].

Materials and Methods

Bacterial Preparation

L. fermentum PL9005 (KCCM-10250), previously isolated from infant feces, was used in this study. The bacteria were inoculated on MRS agar (Difco, Sparks, MD, U.S.A.) and incubated at 37°C for 36–48 h. A single colony was

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inoculated in 10 ml of MRS broth (Difco) and incubated overnight at 37°C. Bacterial cells were collected by centrifugation at 2,700 ×*g* for 10 min and washed twice with sterile phosphate-buffered saline (PBS, pH 7.2). The cells were resuspended in PBS at three different concentrations (2×10^7, 2×10^9, and 2×10^{10} CFU/ml), based on the OD value (at 600 nm) evaluated preliminarily.

Animals

Five-week-old specific pathogen-free (SPF) BALB/c female mice (Biogenomics, Seoul, Korea) were used after one week of acclimation. Mice were housed in individual cages at 22±2°C under a 14/10 h light/dark cycle. Food and water were provided *ad libitum* and removed from the cages five hours prior to inoculation of *L. fermentum* PL9005. All animal experiments were performed in accordance with laboratory animal guidelines of Seoul National University.

Experimental Design

This study consisted of four groups containing nine mice each; three groups were allocated as experimental groups while one group served as control. The mice in the experimental groups were administered intragastrically with 0.5 ml of different concentrations (10^7, 10^7, and 10^{10} CFU/0.5 ml) of *L. fermentum* PL9005, once a day for 28 days, and control mice were treated similarly with PBS. Body weights were measured weekly, and feed intake was measured daily. Clinical signs, such as diarrhea, ruffled fur, and lethargy, were checked twice a day.

Hematology

Blood samples were obtained by cardiac puncture and collected in heparin-treated tubes. White blood cells (WBC), red blood cells (RBC), hemoglobin concentration (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were determined.

Blood Biochemistry

Blood samples (400 μl) were separately collected in new 1.5-ml microtubes and centrifuged at 3,000 rpm for 20 min in a microcentrifuge. The plasma layer was collected, and amounts of total protein, albumin, total cholesterol, and glucose were analyzed using a serum chemistry analyzer (Hitachi Ltd., Tokyo, Japan).

Bacterial Translocation

The spleen, liver, and mesenteric lymph nodes were aseptically removed, weighed, and electrically homogenized (Ultraturrax T8, IKA, Heidelberg, Germany) in PBS (1 ml/0.01 g tissue). The homogenates were serially diluted ten-fold with PBS and inoculated onto MRS agar. The plates were then incubated anaerobically at 37°C for 48 h. To perform the PCR assay to identify organisms detected in tissue samples, each colony was separately inoculated and cultured in MRS broth. Total genomic DNA of each culture was prepared by a method described previously [34]. The primer set used (F; 5'-GCCGCCTAAGGTGGGACAGAT-3', R; 5'-CTGATCGTAGATCAGTCAAG-3') was specific to *L. fermentum*, as it was designed from the 16S-23S ribosomal RNA intergenic spacer region of *L. fermentum* [34]. PCR conditions were as follows: 3 min of denaturation at 95°C followed by 35 cycles consisting of 30 sec of denaturation at 95°C, 30 sec of annealing at 53°C, and 1 min of extension at 72°C before a final 7 min of extension at 72°C. The PCR product was detected by the electrophoresis of 9 μl of reaction solution in 1.5% agarose gel at 100 V in 1× Tris-Acetate-EDTA buffer for 30 min, followed by staining with ethidium bromide. The PCR product bands were visualized on a UV transilluminator.

Positive bands were confirmed by sequencing. A pGEM®-T Easy Vector System (Promega, Madison, WI, U.S.A.) was used to clone the PCR products. The PCR products were purified using a QIAquick™ Gel Extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Purified PCR product was ligated with 50 ng of pGEM®-T Easy vector overnight at 4°C and transferred into JM109-competent *Escherichia coli* cells (Promega) by heat shock at 42°C for 30 sec. Luria-Bertani agar plates containing ampicillin (100 μg/ml) were coated with 0.5 mM IPTG (isopropyl-BD-thiogalactopyranoside) and 80 μg/ml X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside), dried at 37°C for 30 min, and used to select clones. Plasmid DNA was isolated from *E. coli* using a QIAprep Spin Miniprep Kit (Qiagen). Nucleotide sequencing of the PCR product was performed by a DNA sequencing service center (Geno Tech Corp., Taejon, Korea) with a DNA sequencer (model; Applied Biosystems 3730xl DNA Analyzer, Applied BioSystems, Inc., Foster City, CA, U.S.A.). The sequence analysis was resolved with ABI BigDye terminator (Perkin-Elmer, Foster City, CA, U.S.A.).

Histology

The gross lesions of visceral organs in each mouse were checked and recorded. Spleen weight index was expressed as the actual spleen weight (mg) divided by body weight (g). Intestine samples were fixed in 10% buffered formalin for over 24 h. The ileum, cecum, and colon were trimmed, dehydrated in alcohol-xylene series, and embedded in paraffin wax. From each paraffin block, 2 μm thickness sections were prepared and stained with hematoxylin and eosin. Histological observations, including mucosal thickness, inflammation, and epithelial degeneration or necrosis, were carried out.

Statistical Analysis

Significant differences between the experimental and control groups were determined using Duncan's Multiple Range

Test (SAS ver. 8.1, SAS Institute Inc., Cary, NC, U.S.A.). Values of $p<0.05$ were considered significant.

RESULTS AND DISCUSSION

We previously reported that *L. fermentum* PL9005, isolated from infant feces, had a potential immunoenhancing effect. Feeding with *L. fermentum* PL9005 led to increases of IgA^+ B cells in the small intestine, CD4+ T lymphocytes in peripheral blood, and lymphocyte proliferation response to stimulation of mitogens (concanavalin A and lipopolysaccharide). Before a novel probiotic strain is introduced into the market, however, the clarification of its safety is needed. Several standards on safety assessment of probiotic strains have recently been recommended [6, 33, 35, 37, 38]. In the present study, based on the protocol of Zhou *et al.* study [37], the safety of *L. fermentum* PL9005 was assessed using a mouse model.

Appetite, activity, and weight gain are the most general indicators of health status for animals. In the present study, no significant difference was detected in body weight ($p<0.05$, Fig. 1) or feed intake between *L. fermentum* PL9005-fed groups and the control group. Throughout the experimental periods, there was no noticeable change in clinical signs, such as diarrhea, ruffled fur, and lethargy, in any of the groups of mice.

Biochemical assays can be used for detecting a deficiency of nutrients or an imbalance in nutrient metabolism [31]. To detect adverse effects of *L. fermentum* PL9005 feeding, a range of hematological and blood biochemical parameters was monitored (Table 1). All hematological parameters, including WBC, RBC, HGB, HCT, MCV, MCH, and MCHC, in mice of *L. fermentum* PL9005-fed groups did not significantly differ from those in mice of the control group ($p<0.05$). The levels of total protein, albumin, glucose,

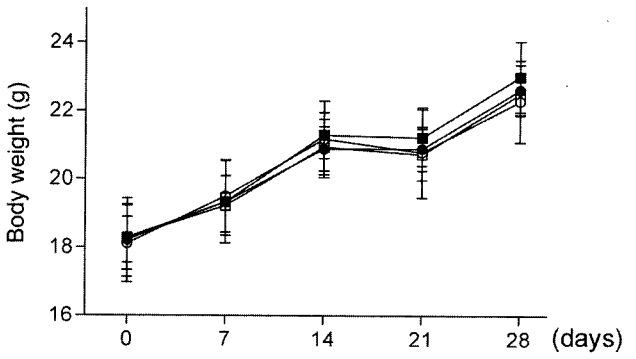

Fig. 1. Body weight of mice intragastrically administered with *L. fermentum* PL9005 at three doses (10^7 [-●-], 10^9 [-○-], 10^{10} [-□-] CFU0.5 ml) or PBS (control [-■-]) for 28 days.
There was no significant difference in body weight between the groups ($p<0.05$).

and total cholesterol in the plasma samples of the mice were also measured, however, there was no significant difference in the blood biochemistry profiles between the *L. fermentum* PL9005-fed and control mice ($p<0.05$). This suggests that administration with *L. fermentum* PL9005 for a 4-week period has no adverse effect on hematology and blood biochemistry in mice. Similar to these results, several studies also showed that administration of LAB had no effect on plasma glucose, total protein, and albumin [32, 38].

For probiotic safety assessment, it is strongly recommended that bacterial translocation is examined because it is a prerequisite in the pathogenicity of most opportunistic gut pathogens [7]. In the present study, positive translocation tissue was defined by the presence of any microorganism morphologically similar to *L. fermentum* PL9005 on MRS plate. The incidence of translocation of bacteria from each visceral organ is shown in Table 2. To differentiate suspected organisms from *L. fermentum* PL9005, a PCR

Table 1. Hematology and blood biochemistry measurements† of mice*.

	Control	*L. fermentum*-fed groups (CFU)		
		10^7	10^9	10^{10}
WBC (10^3/μl)	6.78±2.68	5.62±2.19	7.64±2.98	6.54±2.21
RBC (10^6/μl)	7.57±0.29	7.05±0.40	7.49±0.30	7.66±0.64
HGB (g/dl)	12.8±0.51	12.58±0.53	12.76±0.48	12.84±0.90
HCT (%)	37.56±2.04	37.14±0.86	37.14±1.71	36.98±2.05
MCV (fl)	47.78±1.59	47.22±0.52	47.74±1.25	46.58±1.13
MCH (pg)	16.92±0.46	16.80±0.39	17.04±0.29	16.78±0.22
MCHC (g/dl)	34.10±1.39	34.22±1.18	34.38±0.86	34.48±0.44
Total plasma protein (g/dl)	3.52±0.19	3.62±0.22	3.43±0.36	3.42±0.16
Albumin (g/dl)	1.28±0.07	1.31±0.08	1.24±0.10	1.24±0.07
Glucose (mg/dl)	68.87±8.90	70.93±8.02	69.40±10.88	71.37±5.21
Total plasma cholesterol (mg/dl)	508.00±96.73	523.56±71.04	595.78±90.25	560.22±86.08

†Mean±standard deviation.
*The mice were administered with PBS (control) or suspensions of *L. fermentum* PL9005 intragastrically for 28 days.

Table 2. Incidence of bacterial translocation in different tissue sites of mice administered with PBS (control) or *L. fermentum* PL9005 for 28 days.

	Control	*L. fermentum*-fed groups (CFU)		
		10^7	10^9	10^{10}
Spleen	0/9	1/9	0/9	0/9
Liver	0/9	1/9	0/9	0/9
Mesenteric lymph nodes	3/9	3/9	0/9	1/9

assay was performed using a species-specific primer set. Two major PCR products were produced in the DNA sample of *L. fermentum* PL9005 (Fig. 2). Each fragment was cloned and sequenced. One amplified gene (total 471 bp) corresponded partially to the 16S, 23S rRNA gene and 16S-23S rRNA intergenic spacer region of *L. fermentum* (GenBank database accession no. AF080099) with 98.9% homology (271/274; subjected sequence nos. 119–267 and 270–394). The other gene of 277 bp, which was expected to be amplified by PCR using the species-specific primer in this study, also corresponded with 99.3% homology (275/277). However, no detectable fragment was produced in DNA samples of suspected organisms. This means that the suspected organisms were not *L. fermentum* PL9005, and that feeding of *L. fermentum* PL9005 (10^7, 10^9, 10^{10} CUF/day) for 28 days did not result in bacterial translocation from the gut to other visceral organs in the mice. The translocation of normal indigenous intestinal bacteria in healthy mice has been reported by several groups [3, 15, 24]. In addition, Gordon *et al.* [14] reported that 50% of normal mice contained bacteria or even occasionally bacteremia, in their mesenteric lymph nodes, liver, or spleen.

Macroscopic examinations revealed enlargement, inflammation, and necrosis of the liver, spleen, and intestine. Specifically, splenomegaly and hepatomegaly are indirect indicators of infection. However, in the present study, no gross lesions were observed in the organs of *L.*

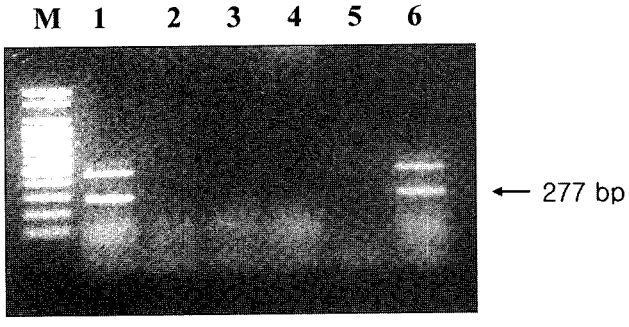

Fig. 2. Agarose gel electrophoresis of PCR products amplified with a species-specific primer [34].
M, 100 bp DNA ladder; lanes 1 and 6, DNA of *L. fermentum* PL9005; lanes 2–5; DNA of suspected organisms.

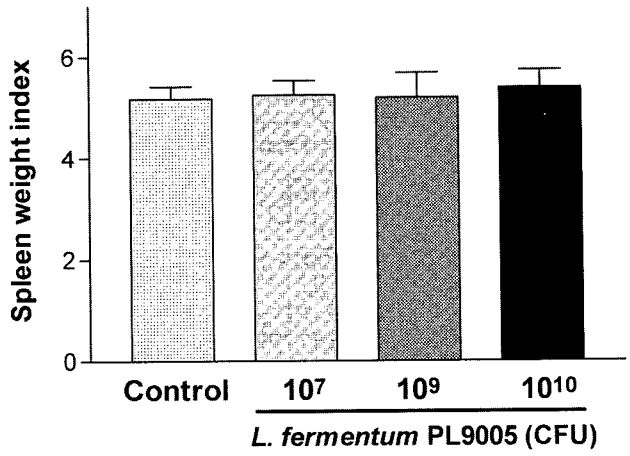

Fig. 3. Spleen weight index (SWI) of mice intragastrically administered with *L. fermentum* PL9005 at three doses (10^7, 10^9, 10^{10} CFU/0.5 ml) or PBS (control) for 28 days.
SWI was expressed as the actual spleen weight (mg) divided by body weight (g). There was no significant difference in SWI between the groups ($p<0.05$).

fermentum PL9005-fed and control mice. There was no significant difference in spleen weight index between *L. fermentum* PL9005-fed and control mice ($p<0.05$, Fig 3). Moreover, microscopic examination revealed no inflammation or epithelial cell degeneration in any mice (data not shown), thus indicating that administration with *L. fermentum* PL9005 for 4 weeks does not lead to infections in any organs of mice.

Integrity of the gut mucosa is important in host defense [10]. The ability to disturb this mechanical barrier is an indicator of potential pathogenicity for most facultative pathogens. Ma *et al.* [24] demonstrated the association in mucosal structure and high translocation rate of *L. murinus* in mice. However, in the present study, the mucosal thickness of the ileum, cecum, and colon of *L. fermentum* PL9005-fed mice was not significantly different from that of corresponding organs of control mice (Table 3), suggesting that *L. fermentum* PL9005 has no harmful effect on the gut mucosa of mice.

In conclusion, twenty-eight days of feeding with three different doses (10^7, 10^9, and 10^{10} CFU/animal) of *L. fermentum* PL9005 did not influence body weight, feed intake, clinical signs, or gross and histopathological findings in mice. Hematological parameters (WBC, RBC, HGB, HCT, MCV, MCH, and MCHC) and blood biochemistry (total plasma protein, albumin, glucose, and cholesterol) were also not significantly different between *L. fermentum* PL9005-fed and control mice. Based on these results, long-term ingestion of *L. fermentum* PL9005 in mice appears to be safe, and can be applied in the functional food market. Furthermore, the methods described in this study can be used as a guideline for safety assessment on newly introduced probiotic strains.

Table 3. Mucosal thickness* of the ileum, cecum, and colon of mice administered with PBS (Control) or *L. fermentum* PL9005 for 28 days.

	Control	*L. fermentum*-fed groups (CFU)		
		10^7	10^9	10^{10}
Ileum	260.30±12.71	266.58±19.43	264.42±11.79	267.63±13.70
Cecum	99.15±7.24	103.25±10.26	101.58±9.08	102.00±8.37
Colon	193.92±10.70	197.08±12.13	197.50±13.34	197.50±12.03

*The thickness (mm) was measured from ten randomly-selected mucosal layers of each sample and expressed as mean±standard deviation.

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REFERENCES

1. Aguirre, M. and M. D. Collins. 1993. Lactic acid bacteria and human clinical infection. *J. Appl. Bacteriol.* **75:** 95–107.
2. Bayer, A. S., A. W. Chow, D. Betts, and L. B. Guze. 1978. Lactobacillemia - report of nine cases, and important clinical and therapeutic considerations. *Am. J. Med.* **64:** 808–813.
3. Berg, R. D. 1983. Translocation of indigenous bacteria from the intestinal tract, pp. 333–352. *In* R. J. Hentges (ed.). *Human Intestinal Microflora in Health and Disease.* Academic Press, London.
4. Boyd, H. B. 1993. Safety assessment of novel foods. *Intern. Food Ingred.* **5:** 9–11.
5. De Rodas, B. Z., S. E. Gilliland, and C. V. Maxwell. 1996. Hypocholesterolemic action of *Lactobacillus acidophilus* ATCC 43121 and calcium in swine with hypercholesterolemia induced by diet. *J. Dairy Sci.* **79:** 2121–2128.
6. Donohue, D. C., S. Salminen, and P. Marteau. 1998. Safety of probiotic bacteria, pp. 307. *In* S. Salminen and A. von Wright (eds.) *Lactic Acid Bacteria.* Marcel Dekker, New York, U.S.A.
7. Ford, H. R., A. Avanoglu, P. R. Boechat, R. Melgoza, R. S. LumCheong, P. Boyle, M. Garrett, and M. I. Rowe. 1996. The microenvironment influences the pattern of bacterial translocation in formula-fed neonates. *J. Pediat. Surg.* **31:** 486–489.
8. Fukushima, M. and M. Nakano. 1996. Effects of a mixture of organisms, *Lactobacillus acidophilus* or *Streptococcus faecalis* on cholesterol metabolism in rats fed on a fat- and cholesterol-enriched diet. *Brit. J. Nutr.* **76:** 857–867.
9. Fuller, R. 1991. Probiotics in man and animals. *J. Appl. Bacteriol.* **66:** 365–378.
10. Fuller, R. 1992. History and development of probiotics, pp. 1–8. *In* Fuller, R. (ed.), *Probiotics - The Scientific Basis.* Chapman and Hall, London.
11. Gasser, F. 1994. Safety of lactic acid bacteria and their occurrence in human clinical infections. *Bull. Pasteur Instit.* **92:** 45–67.
12. Gill, H. S., K. J. Rutherfurd, J. Prasad, and P. K. Gopal. 2000. Enhancement of natural and acquired immunity by *Lactobacillus rhamnosus* (HN001), *Lactobacillus acidophilus* (HN017) and *Bifidobacterium lactis* (HN019). *Brit. J. Nutr.* **83:** 167–176.
13. Gill, H. S., Q. Shu, H. Lin, K. J. Rutherfurd, and M. L. Cross. 2001. Protection against translocating *Salmonella typhimurium* infection in mice by feeding the immuno-enhancing probiotic *Lactobacillus rhamnosus* strain HN001. *Med. Microbiol. Immunol.* **190:** 97–104.
14. Gordon, L. E., D. Ruml, H. J. Hahne, and C. P. Miller. 1995. Studies on susceptibility to infection following radiation. IV: The pathogenesis of endogenous bacteremia of mice. *J. Exp. Med.* **102:** 413–424.
15. Hale, P. and A. Hill. 1973. The recovery of *Lactobacillus* sp. from the livers of healthy mice. *Lab. Anim.* **7:** 119–124.
16. Holzapfel, W. H., P. Haberer, J. Snel, U. Schillinger, and J. H. J. Huis in't Veld. 1998. Overview of gut flora and probiotics. *Int. J. Food Microbiol.* **41:** 85-101.
17. Hull, R. 1995. Probiotics and human health. *Proc. Nutr. Soc. Aust.* **19:** 88–93.
18. Kato, J., K. Endo, and S. Rybka. 1997. Effects of oral administration of *Lactobacillus casei* on antitumor responses induced by tumor resection in mice. *Int. J. Immunopharmacol.* **16:** 29–36.
19. Kim, H. J., J. H. Kim, J. H. Son, H. J. Seo, S. J. Park, N. S. Paek, and S. K. Kim. 2004. Characterization of bacteriocin produced by *Lactobacillus bulgaricus*. *J. Microbiol. Biotechnol.* **14:** 503–508.
20. Kim, S. H., S. J. Yang, H. C. Koo, W. K. Bae, J. Y. Kim, J. H. Park, Y. J. Baek, and Y. H. Park. 2001. Inhibitory activity of *Bifidobacteria longum* HY8001 against vero cytotoxin of *Escherichia coli* O157:H7. *J. Food Prot.* **64:** 1667–1673.
21. Kim, S. M., K. H. Lee, N. K. Lee, C. J. Kim, C. H. Kim, and H. D. Park. 2004. Antagonistic activity of polyfermenticin SCD against *Helicobacter pylori* KCTC 2948. *J. Microbiol. Biotechnol.* **14:** 148–152.
22. Klein, G., A. Pack, C. Bonaparte, and G. Reuter. 1998. Taxonomy and physiology of probiotic lactic acid bacteria. *Int. J. Food Microbiol.* **41:** 103–125.
23. Lee, H. Y., J. H. Park, S. H. Seok, S. A. Cho, M. W. Baek, D. J. Kim, Y. H. Lee, and J. H. Park. 2004. Dietary intake of

various lactic-acid bacteria suppresses type 2 helper T cell-production in antigen-primed mice splenocyte. *J. Microbiol. Biotechnol.* **14:** 167–170.
24. Ma, L., E. Deitch, R. Specian, E. Steffen, and R. Berg. 1990. Translocation of *Lactobacillus murinus* from the gastrointestinal tract. *Curr. Microbiol.* **20:** 177–184.
25. Marteau, P. and J. C. Rambaud. 1993. Potential of using lactic acid bacteria for therapy and immunomodulation in man. *FEMS. Microbiol. Rev.* **12:** 207–220.
26. Matar, C., J. C. Valdez, M. Medina, M. Rachid, and G. Perdigon. 2001. Immunomodulating effects of milks fermented by *Lactobacillus helveticus* and its non-proteolytic variant. *J. Dairy Res.* **68:** 601–609.
27. Matsuzaki, T. 1998. Immunomodulation by treatment with *Lactobacillus casei* strain Shirota. *Int. J. Food Microbiol.* **41:** 133–140.
28. Park, J. H., Y. Lee, E. Moon, S. H. Seok, S. A. Cho, M. W. Baek, H. Y. Lee, D. J. Kim, and J. H. Park. 2005. Immunoenhancing effects of a new probiotic strain, *Lactobaeillus fermentum* PL 9005. *J. Food. Prot.* **68:** 571–576.
29. Perdigon, G., M. E. N. de Marcias, S. Alvarez, G. Oliver, and A. P. de Ruiz Holgado. 1990. Prevention of gastrointestinal infection using immunobiological methods with milk fermented with *Lactobacillus casei* and *Lactobacillus acidophilus*. *J. Dairy Res.* **57:** 255–264.
30. Shu, Q., K. J. Rutherfurd, S. G. Fenwick, J. Prasad, P. K. Gopal, and H. S. Gill. 2000. Dietary *Bifidobacterium lactis* (HN019) enhances resistance to oral *Salmonella typhimurium* infection in mice. *Microbiol. Immunol.* **44:** 213–222.
31. Swendseid, M. E. 1987. Biochemical assessment of protein and amino acid status, pp. 205–211. *In* Livingston, G. E. (ed.), *Proceedings of a National Conference Sponsored by the Food and Nutrition Council of the American Health Foundation, Nutritional Status Assessment of the Individual.* Food and Nutrition Press, New York, U.S.A.
32. Tortuero, F., J. Rioperez, E. Fernandez, and M. L. Rodriguz. 1995. Response of piglets to oral administration of lactic acid bacteria. *J. Food Prot.* **58:** 1369–1374.
33. van der Kamp, J. W. 1996. Safety considerations regarding probiotic strains - conclusions of two specialist workshops. *Int. Dairy Feder. Newslett.* **5:** 27–28.
34. Walter, J., G. W. Tannock, A. Tilsala-Timisjarvi, S. Rodtong, D. M. Loach, K. Munro, and T. Alatossava. 2000. Detection and identification of gastrointestinal *Lactobacillus* species by using denaturing gradient gel electrophoresis and species-specific PCR primers. *Appl. Environ. Microbiol.* **66:** 297–303.
35. Ward, L. J. H., J. C. S. Brown, and G. P. Davey. 1995. Detection of dairy *Leuconostoc* strains using the polymerase chain reaction. *Lett. Appl. Microbiol.* **20:** 204–208.
36. Willem, M., R. Fonden, U. Svensson, M. Saxelin, L. Morelli, K. Collins, D. Brassard, E. Shiffrin, G. Mogensen, B. Grenov, S. Salminen, E. Isolauri, A. von Wright, and T. M. Sandholm. 1997. Recommendations for safety of probiotic cultures for traditional or novel foods. *Demonst. Nutr. Functional. Probio. Food Newslett.* **2:** 2.
37. Zhou, J. S., Q. Shu, K. J. Rutherfurd, J. Prasad, P. K. Gopal, and H. S. Gill. 2000. Acute oral toxicity and bacterial translocation studies on potentially probiotic strains of lactic acid bacteria. *Food Chem. Toxicol.* **38:** 153–161.
38. Zhou, J. S., Q. Shu, K. J. Rutherfurd, J. Prasad, M. J. Birtles, P. K. Gopal, and H. S. Gill. 2001. Safety assessment of potential probiotic lactic acid bacterial strains *Lactobacillus rhamnosus* HN001, *Lb. acidophilus* HN017, and *Bifidobacterium lactis* HN019 in BALB/c mice. *Int. J. Food Microbiol.* **56:** 87–96.