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RESEARCH ARTICLE

The relative efficacy of different strain combinations of lactic acid bacteria in the reduction of populations of *Salmonella enterica* Typhimurium in the livers and spleens of mice

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lactic acid bacteria; multistrain; immune activity; *Salmonella* invasion.

Abstract

Multispecies probiotics have been reported to be more effective than monostrain probiotics in health promoting for the host. In this study, 12 lactic acid bacteria (LAB) strains were selected based on the level of induction of tumor necrosis factor (TNF)-α in RAW 264.7 macrophage cells. Their adherence to Caco-2 cells and inhibitory effects on *Salmonella* invasion of Caco-2 cells were compared. Strains with different probiotic properties were then combined and BALB/c mice were fed with LAB strains for 63 days; then the mice were challenged with *Salmonella* on day 64. For *Salmonella*-unchallenged mice that received a multistrain combination of LAB strains that have greater TNF-α production in macrophages, greater adherence and inhibit *Salmonella* invasion of Caco-2 cells to a greater extent, their peritoneal macrophages had greater phagocytic activity. For *Salmonella*-challenged mice, a significant reduction of *Salmonella* cells in the livers and spleens of the mice was observed 8 days post challenge. The addition of 12% skim milk powder together with LAB strain combinations significantly enhanced the reduction of *Salmonella* cells in the mice livers and spleens. In conclusion, we have shown that LAB strain combinations with particular probiotic properties when fed to mice can inhibit *Salmonella* invasion of the liver and spleen.

Introduction

Lactic acid bacteria (LAB) strains may have beneficial effects on human and animal health (Reid *et al.*, 2006) and thus have been widely used in yogurt processing, probiotic products and feed supplements (Sakamoto *et al.*, 2001; Koebnick *et al.*, 2003; Tsai *et al.*, 2005). Recently, the normal intestinal microbial community has been investigated as a potential target for the management of immune disorders. Various researchers have reported that LAB strains, such as *Lactobacillus* spp., *Bifidobacteria* spp., *Enterococcus* spp., *Lactococcus* spp., *Streptococcus* spp., etc. may possess immune-enhancing activities or are able to produce bacteriocins to protect the host from infection by pathogens (Gill *et al.*, 2001; Herich & Levkut, 2002; Cheikhyoussef *et al.*, 2008; Simova *et al.*, 2009). The possible mechanisms by which probiotics may offer protection against infection by gastrointestinal pathogens have been addressed in diverse patent applications and include the following: modification of the intestinal environmental; competition with pathogens for nutrients and colonization of adhesion sites in the intestinal environment; competition with pathogens for nutrients and sites on intestinal epithelium; production of antimicrobial metabolites; and modulation of immune and nonimmune defense mechanisms of the host (Timmerman *et al.*, 2004).

While the health effects of probiotics are genus, species and strain specific, research suggests that multistrain and/or multispecies probiotics are more effective than monostrain probiotics (Zoppi *et al.*, 2001; Shibolet *et al.*, 2002; Timmerman *et al.*, 2004). Multistrain or multispecies probiotics contain different strains with characteristics, and therefore, may be able to create an anaerobic probiotic niche that enhances the colonization and survival of probiotic strains at the expense of pathogens (Timmerman *et al.*, 2004). In addition, the probiotic properties of each strain, such as

colonization, biological activity and health-promoting properties, may be enhanced by the additive and synergistic effects that occur when these strains are used in combination (Ouwehand *et al*., 2000; Shihata & Shah, 2000). On the other hand, monostrain probiotics' effects are limited to strain-specific properties, and survival depends on the properties of each specific strain. With the market potential growing, increasingly more probiotic strains have been identified. For LAB strains used in multistrain formulations, however, the probiotic properties considered are, in general, merely their antimicrobial activity, bile tolerance, growth in milk and survival in the gastrointestinal tract (Casey *et al*., 2004). For example, a five-strain combination formula has been developed to reduce pathogen infections in weaned pigs (Casey *et al*., 2007). Another product, such as the commercially available product VSL#3, an eight-strain LAB formulation, was created by the addition of different strains to a formulation in the hope that it will benefit the host (Bibiloni *et al*., 2005). There are very few reports regarding multistrain probiotic products composed of LAB strains with selected functions, such as immunomodulation activity, adherence to host intestinal epithelium and inhibition of host invasion by pathogenic bacteria.

Previously, we have shown that the combination of different heat-killed strains of the same LAB species, i.e. *Lactobacillus acidophilus*, was able to retard the invasion of *Salmonella* Typhimurium in mice. This antagonistic effect may have been due to the immune-enhancing effects of the heat-killed LAB strains (Lin *et al*., 2007). In this study, we attempted to develop a LAB combination consisting of different species and to evaluate their effectiveness in reducing infections by *Salmonella* in mice. Such a preparation may be useful as a human probiotic or as an animal feed supplement. For this purpose, 146 different LAB strains, including *Lactobacillus* spp., *Enterococcus* spp., *Bifidobacterium* spp. and *Pediococcus* spp., were evaluated for their immunomodulating activity. Strains that increased tumor necrosis factor (TNF)-α production by macrophages the most were then evaluated for their probiotic properties including resistance to gastrointestinal conditions, adherence to Caco-2 cells, their antimicrobial activities against the growth of common pathogenic bacteria and their inhibitory effects on *Salmonella* invasion of Caco-2 cells. Strains were then selected and combined into several multispecies formulations and these formulations were compared for their effectiveness in protecting mice from *Salmonella* infection.

Materials and methods

Bacteria strains and their growth conditions

A total of 146 LAB strains including *Lactobacillus* spp., *Enterococcus* spp., *Bifidobacterium* spp. and *Pediococcus* spp. were evaluated for their immunomodulating activity. The 12 strains that stimulated the highest TNF-α production in macrophages are shown in Table 1 and Fig. 1. The stock cultures of the 146 strains were maintained at −70 °C in 20% glycerol. Before experimental use, bacteria were propagated twice in Lactobacilli deMan Rogosa Sharpe (MRS) broth (Difco, Maryland) containing 0.05% L-cysteine for 24 h at 37 °C.

Other bacterial strains used in this study included *Staphylococcus aureus*, enterotoxigenic *Escherichia coli*, enterohaemorrhagic *E. coli*, *Salmonella enterica* serovar Enteritidis and serovar Typhimurium and are listed in Table 1. For the cultivation of these bacteria strains, one loopful of each strain was inoculated into 5 mL a tryptic soy broth and incubated at 37 °C for 12 h. Then, a portion of the culture was diluted and placed on a tryptic soy agar for future studies.

Culture of cell lines

Human epithelial-like cell line Caco-2 and mouse BALB/c macrophage RAW 264.7 cells were obtained from the Bioresource Collection and Research Center (BCRC), Hsin-Chu, Taiwan. Caco-2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% (v/v) fetal bovine serum and 1% 100X Penicillin-Streptomycin (HyClone, Logan, UT). RAW 264.7 cells were cultured in a monolayer with RPMI-1640 medium (HyClone) supplemented with 10% (v/v) fetal bovine serum, 1% 100× Penicillin–Streptomycin (HyClone) and 1% 100× MEM-nonessential amino acid solution (HyClone). For cell culture assay, cells were cultured in 75-cm^2 plastic tissue culture flasks (Nunc, Roskilde, Denmark). RAW 264.7 cells were used at the late confluence stage after 5 days of incubation at 37 °C and Caco-2 cells were used at the differentiated stage after 21 days of incubation at 37 °C. For both incubations, a 5% CO_2/95% air atmosphere was used.

Table 1. Bacterial strains used in this study

Strains and strain number
Lactobacillus spp.: *Lactobacillus acidophilus* LAP5; *L. brevis* LBR01 (BCRC12945); *L. fermentum* LF01; *L. fermentum* LF33; *L. fermentum* LF50; *L. plantarum* LPL05 (BCRC10069); *L. rhamnosus* LRH02; *L. reuteri* LRE01 (BCRC 14625)
Pediococcus spp.: *Pediococcus acidilactici* PA05; *P. inopinatus* PI08
Enterococcus spp.: *Enterococcus faecium* TM39; *E. casseliflavus* ECA01 (BCRC 14926)
Pathogenic bacteria: *Staphylococcal aureus* enterotoxin A (SEA, BCRC 13824); *S. aureus* enterotoxin B (SEB, BCRC 13825); *S. aureus* enterotoxin C (SEC, BCRC 13828); *S. aureus* enterotoxin D (SED, BCRC 13829); Enterotoxigenic *Escherichia coli* (LTIp, ATCC 37218); Enterohaemorrhagic *E. coli* O157:H7 (STI+SLTII, ATCC 43894); *Salmonella* Enteritidis ATCC 13076; *S.* Typhimurium ATCC 14082

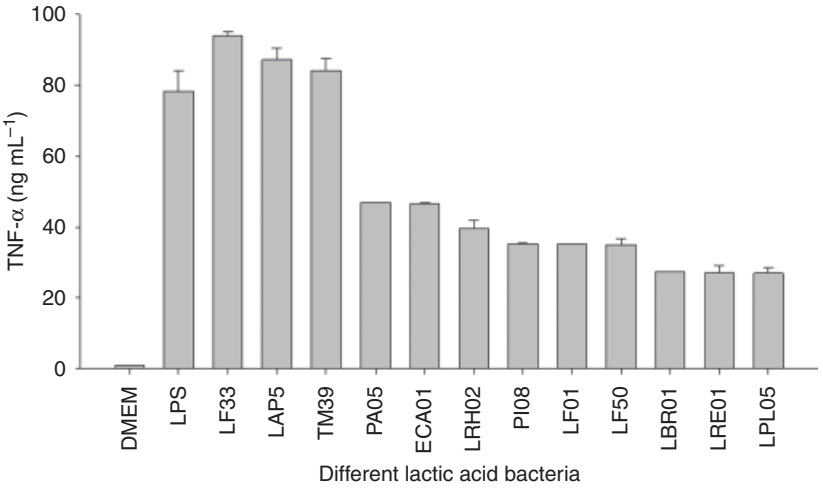

Fig. 1. TNF-α production by RAW264.7 macrophage cells after incubation with different LAB strains.

Adhesion of LAB to the Caco-2 cells

The conditions described by Tsai *et al.* (2004b) for the adhesion study of LAB cells were followed. Caco-2 cells were transferred (5×10^4 cells mL^{-1}) to 24-well multidishes containing a fresh tissue culture medium without Penicillin–Streptomycin. The mixture was kept at 37 °C in a 5% CO_2/95% air atmosphere until cell lines grew to a monolayer in each well. Bacterial cells of each strain, after washing with phosphate-buffered saline (PBS), were resuspended in 1 mL DMEM, and 100 μL of each suspension was transferred to the 24-well multidish (1×10^8 CFU mL^{-1}) containing the Caco-2 cells and then incubated for 2 h. After incubation, cells were washed twice with PBS, fixed with 10% formalin for 30 min, washed four times with PBS and then stained with crystal violet for 5 min. The number of adherent LAB cells was counted according to the method of Gopal *et al.* (2001). Ten of the Caco-2 cells were used to calculate the average number of the adherent LAB cells per Caco-2 cell.

Assays for TNF-α production by macrophage RAW264.7 after stimulation with LAB strains

RAW 264.7 cells were cultured in triplicate, at a density of 1×10^6 cells mL^{-1} of RPMI-1640 medium without Penicillin–Streptomycin, in 24-well tissue culture plates. LAB cells were centrifuged at 7000 ***g*** for 10 min and the pellet was resuspended in RPMI-1640 medium containing RAW 264.7 cells to a final concentration from 10^8 to 10^9 CFU mL^{-1}. Lipopolysaccharide from *E. coli* O26:B6 (Sigma) was used as a positive control. After 24 h, 48 h and 72 h, the cytokines produced in these culture supernatants were analyzed.

Cytokines were measured using an ELISA kit purchased from Bender MedSystems Inc. (San Diego, CA) for mouse TNF-α. Ninety-six-well Immuno-Maxisorp plates (Nunc) were coated with monoclonal antibodies for TNF-α, and then diluted and placed in coating buffer (1 × PBS) overnight at 4 °C. Plates were blocked and washed. Culture medium was added to the plates and they were incubated for 2 h at room temperature. Plates were then washed again, and biotinylated anti-mouse TNF-α and horseradish peroxidase-conjugated streptavidin were added for the detection of TNF-α, followed by incubation for 1 h at room temperature. The chromogenic reactions were developed with the 3,3′,5,5′-tetramethylbenzidine substrate for 30 min at room temperature and were terminated with 2 N H_2SO_4 and the $A_{450\,nm}$ was measured. Equivalent levels of TNF-α were calculated by comparison with a reference curve generated using TNF-α standards. The results were expressed as the concentration of the cytokine in serum (pg mL^{-1}).

Acid and bile resistance of the LAB

To evaluated acid and bile resistance among the LAB strains, the methods described by Tsai *et al.* (2005) were used. In general, 100 μL of each culture containing about 10^8–10^9 CFU mL^{-1} of LAB was suspended in PBS. The pH was adjusted to 2.0, 2.5 and 3.2 using 0.1 N HCl and the mixture was incubated at 37 °C for 3 h. Control incubations of each LAB strain were performed at pH 7.2 under the same conditions. After incubation, viable bacterial counts were determined by plating serial dilutions of the culture in PBS (pH 7.2) on MRS agar (Merck, Darmstadt, Germany). LAB that survived the acid tolerance study were centrifuged (5000 ***g***, 5 min), washed with PBS (pH 7.2) and then grown in 9 mL MRS broth with and without 0.3% (w/v) oxgall bile (Sigma). Viable bacterial counts were determined by plating serial dilutions on MRS agar.

Antimicrobial activity assay

The antibacterial activity of LAB strains was studied using the agar diffusion test. Strains of LAB were grown overnight (20 h) in an MRS broth at 37 °C. The cultures were centrifuged and the supernatants were recovered. Pathogenic

bacterial strains were grown on Luria–Bertani agar (LA). Then, wells were hollowed out of the LA plates and 100 μL of the spent culture supernatant (LAB-SCS) was dropped into each well and the culture was incubated at 37 °C for 14 h. The antimicrobial activity was determined by measuring the clear zone around the wells. LAB strains with inhibition zones < 11, 11–16, 17–22 and > 23 mm were classified as strains of no $-$; mild $+$; strong $++$; and very strong $+++$ inhibition, respectively (Rammelsberg & Radler, 1990).

Inhibition of *Salmonella* invasion of Caco-2 cells by LAB strains

The Caco-2 cells were transferred (1×10^5 cells mL^{-1}) with 0.05% trypsin to 96-well tissue culture plates containing a fresh tissue culture medium and cultured for 20–22 h. The wells were then washed three times with PBS and about N ($N = 1$–9) $\times 10^7$ CFU per well of each LAB strain was added and incubated for 1 h at 37 °C in a 5% CO_2 incubator. About 5×10^7 CFU per well of *Salmonella* cells were added to wells containing the Caco-2 cell culture monolayers and the cells were incubated for 2 h at 37 °C in a 5% CO_2 atmosphere. The wells were then washed three times with PBS and incubated for another 1 h in a fresh DMEM medium containing 100 μg gentamicin mL^{-1}. After incubation, each well was again washed with PBS three times and then Caco-2 cells were lysed with 100 μL of 1% Triton X-100. Appropriate dilutions were spread onto a plate count agar and the plates were incubated at 37 °C overnight. The bacterial counts were calculated to measure *Salmonella* invasion.

Mice

The animal experiments were approved by the Institutional Animal Care and Use Committee of Hung Kuang University, Taichung County, Taiwan (approval No. 96022). Mice free from specific pathogens were used in this study. All were male inbred strains of BALB/c mice weighing 20–22 g that were 8 weeks old when purchased from the National Laboratory for Animal Breeding and Research Center, Taipei, Taiwan. These mice were raised at 20 ± 2 °C, 55 ± 5% of RH%, under a 12-h light cycle. They were fed laboratory rodent diet 5001 (manufactured by PMI Nutrition International Inc. Richmond, IN) sterilized by γ irradiation. Mice were fed *ad libitum* for 1 week before being used in the study. On treatment day 0, the mice were randomly allocated into six groups of 10 mice. The feed intake and body weight of each mouse was measured weekly.

Multistrain combinations of LAB strains

LAB strains in the multistrain groups were individually grown and then mixed. All probiotic preparations were cultured to check their viability and for enumeration.

Table 2. Effect of LAB culture on the invasion of Caco-2 cells by *Salmonella* Typhimurium

Lactic acid bacteria strains	*Salmonella* invasion (log CFU mL^{-1})‡
Control DMEM medium	4.50 ± 0.15
L. fermentum LF01	2.48 ± 0.00*
E. casseliflavus ECA01	2.30 ± 0.00*
L. fermentum LF33	2.18 ± 0.15*
E. faecium TM39	1.65 ± 0.15*
L. brevis LBR01	1.00 ± 0.00*
L. fermentum LF50	2.00 ± 0.00*
P. acidilactici PA05	4.57 ± 0.55
L. plantarum LPL05	< 1*
L. acidophilus LAP5	< 1*
P. inopinatus PI08	< 1*
Formula I (*Lactobacillus acidophilus* LAP5+*Enterococcus faecium* TM39+*L. fermentum* LF33+*L. plantarum* LPL05)	1.18 ± 0.15*
Formula II (*E. casseliflavus* ECA01+*P. inopinatus* PI08+*L. fermentum* LF01+*P. acidilactici* PA05)	2.30 ± 0.15*
Formula III (*L. fermentum* LF50+*L. brevis* LBR01+*P. acidilactici* PA05+*L. fermentum* LF01)	3.91 ± 0.33

*Value indicates a significant difference ($P < 0.05$) from the PBS-negative control.

†Experimental conditions are described in Materials and methods. The inoculum level of *Salmonella* Typhimurium ISM50 was 7.6 ± 0.2 log CFU mL^{-1}.

‡Intracellular *Salmonella* numbers in the suspension of the lysed cells (see Materials and methods).

Strains used in multistrain probiotic preparations were either randomly selected or were selected based on their probiotic properties, including enhancing TNF-α production in macrophages, inhibition of the growth of pathogenic bacteria and adherence to Caco-2 cell lines. Strains in multistrain Formula I (F I) were LAP5, TM39, LF33 and LPL05. Strains in F II were ECA01, PI08, LF01 and PA05 and strains in F III were LF50, LBR01, PA05 and LF01 (Table 2). For F I and II, in addition to the LAB cells, the effects of 12% skim milk powder (SMP) were also investigated.

LAB feeding, *Salmonella* challenges and assays of the phagocytic activity of peritoneal macrophages from mice

The methods of LAB feeding and pathogen challenge described by Hudault *et al.* (1997) were used. LAB strains were grown in an MRS broth (Difco) at 37 °C for 20 h. After centrifugation (5000 ***g***) at 4 °C, bacterial cells were resuspended in ddH_2O and adjusted to a cell density of 1×10^{10} CFU mL^{-1}. Each mouse was fed a single 0.2-mL dose of a known concentration of LAB strains (1×10^{10} CFU mL^{-1}) daily for 28 consecutive days and a 0.1-mL dose of LAB strains (1×10^{10} CFU mL^{-1}) daily from the 29th to the 63rd day. Mice were offered a basal diet (MF-18, Oriental Yeast Co. Ltd) and water *ad libitum*. The

six groups of mice (10 mice each group) were fed with F I, F II, F III and ddH_2O, respectively. For F I- and F II-fed mice, the effects of 12% SMP in the multistrain preparation were also investigated. During the 63-day feeding period, the growth rate of each mouse was measured weekly. After 63 days, five mice in each group were challenged with *S.* Typhimurium ISM 50 by an oral administration of a single 0.2-mL dose of *Salmonella* cells (5×10^7 CFU mL^{-1}). Strain ISM50 was isolated from a patient with salmonellosis by the National Institute of Preventive Medicine, Department of Health, Taipei, Taiwan. Before *Salmonella* challenge, another five mice in each group were killed and the phagocytic activity of peritoneal macrophage cells of the BALB/c mice was assayed. For the *Salmonella*-challenged mice, on day 8 after challenge, the numbers of *Salmonella* in the livers and spleens of the mice were determined using the method of Tsai *et al.* (2005). Five mice of each of the challenge groups were killed by cervical dislocation. The spleens and livers were removed aseptically. These organs were mixed in sterile water to a final volume of 5 mL and were homogenized. Cell suspensions were serially diluted with PBS and 1 mL of the dilution was plated on brilliant green agar. After 48 h of incubation at 37 °C, *Salmonella* colonies (CFU) were enumerated.

To assay the phagocytic activity of peritoneal macrophage cells in BALB/c mice fed with different multistrain formulas, the methods described and modified by Chow *et al.* (2010) were used. Macrophage cells (1×10^6) from the mice were seeded in a 96-well plate and incubated overnight at 37 °C. The cells were washed three times with PBS and then exposed to BioParticals® FITC-conjugated *E. coli* at a multiplicity of infection of 10 : 1. The plates were centrifuged at 4 °C, 250 ***g***, for 5 min to facilitate contact between bacteria and peritoneal macrophage cells before incubating at 37 °C. After 2 h of incubation, cells were washed three times with PBS and then mixed with 50 μL 2 × trypan blue solution in 50 μL analysis buffer (10% fetal bovine serum and 1% 100 × MEM-non-essential amino acid solution in RPMI-1640 medium) before being analyzed by flow cytometry using a FACStarPLUS.

Statistical analysis

Data were expressed as mean ± SD and the statistical significance was determined using a one-way ANOVA, followed by Duncan's new multiple range test using the Statistical Analysis System SAS Enterprise Guide 2.1.40. Statistical significance was considered as $P < 0.05$.

Results

Selection of LAB strains by assay with TNF-α

In order to screen the LAB strains with immunomodulating activity, the enhancement of TNF-α production by RAW 264.7 macrophage cells following coincubated with each LAB strain was measured. A total of 146 LAB strains were screened, and the TNF-α production levels for the top 12 LAB strains were all higher than 30 ng mL^{-1} (Fig. 1). The sources of these 12 LAB strains are given in Table 1. The TNF-α level of 80 ng mL^{-1} for RAW 264.7 cells was obtained with lipopolysaccharide (10 μg mL^{-1}) (Sigma), which served as the positive control while the undetectable level of TNF-α for macrophage cells in DMEM medium only was used as the negative control (Fig. 1). LAB strains LF33, LAP5 and TM39 enhanced TNF-α production more than lipopolysaccharide did in the macrophage cells. The TNF-α levels for the other 9 LAB strains (strain PA05 to LPL05) were between 30 and 50 ng mL^{-1} (Fig. 1). The TNF-α levels for these 12 LAB strains are shown in Fig. 1. All of the remaining 134 LAB strains enhanced TNF-α production to levels < 20 ng mL^{-1} (data not shown). These strains were not studied further.

Adherence and acid and bile tolerance among LAB strains

The 12 LAB strains that enhanced TNF-α production the most were examined for their ability to adhere to the human colon cell line Caco-2 (Table 3). Five strains (PI08, LAP5, TM39, LF33 and LPL05) showed strong adherence to Caco-2 cells (> 200 CFU LAB per Caco-2 cell). Strain PA05 showed moderate adherence (about 110 CFU LAB per Caco-2 cell). The rest of the strains showed lower adherence (from 18 to 78 CFU per Caco-2 cell). Based on the methods described for the assay of the acid and bile resistance of the LAB, all of the 12 selected LAB strains were acid tolerant to pH ≥ 2.5 and less tolerant at pH 2.0. For bile tolerance, strains of animal origins, such as LAP5, TM39 and LF33, were tolerant to bile while strains of vegetable origin were generally less tolerant (Tanaka *et al.*, 1999; Tsai *et al.*, 2004b).

Table 3. Adhesion of LAB to Caco-2 cells

Strain	Average number of adherent bacteria per Caco-2 cell*
Pediococcus inopinatus PI08	271 ± 2.22
Lactobacillus acidophilus LAP5	263 ± 1.43
Enterococcus faecium TM39	247 ± 1.63
L. fermentum LF33	207 ± 2.63
L. plantarum LPL05	206 ± 2.57
P. acidilactici PA05	110 ± 2.36
L. brevis LBR01	78 ± 2.82
L. reuteri LRE01	59 ± 1.60
L. rhamnosus LRH02	45 ± 1.84
L. fermentum LF50	36 ± 1.43
L. fermentum LF01	19 ± 0.88
E. casseliflavus ECA01	18 ± 0.92

*Method for the calculation of the average number of the adherent LAB cells per Caco-2 cell is shown in Materials and methods.

Table 4. Antimicrobial activity of SCS of LAB against pathogenic bacteria

LAB strains	Staphylococcal enterotoxin (SE) *S. aureus* SEA BCRC 13824	SEB BCRC 13825	SEC BCRC 13828	SED BCRC 13829	Enterotoxigenic *E. coli* (ETEC) ATCC 35401	Enterohaemorrhagic *E. coli* (EHEC) ATCC 43894	*S.* Enteritidis ATCC 13076	*S.* Typhimurium ATCC 14028
L. fermentum LF50	−*	−	−	+	+	+	−	−
P. acidilactici PA05	+	+	−	++	+	+	+	+
L. plantarum LPL05	+	+	−	++	++	−	+	+
P. inopinatus PI08	+	+	+	++	++	+	−	+
L. acidophilus LAP5	+	+	+	+	++	+	+	+
E. faecium TM39	−	−	−	−	++	+	+	−
L. brevis LBR01	−	−	−	+	+	−	−	−
L. fermentum LF01	−	−	−	+	+	+	+	−
L. fermentum LF33	++	+	++	+++	+++	++	++	++
L. rhamnosus LRH02	−	+	+	++	++	+	+	++
L. reuteri LRE01	−	+	+	+	+	+	−	+
E. casseliflavus ECA01	−	−	−	−	+	−	−	−

*LAB strains with inhibition zones < 11 mm, 11–16 mm, 17–22 mm and > 23 mm were classified as strains of no −; mild +; strong ++; and very strong +++ inhibition, respectively.

Effects of SCS of LAB strains on the growth of pathogenic bacteria

When *E. coli*, *Salmonella* Typhimurium, enterotoxigenic *S. aureus* and *Bacillus cereus* (Table 1) were used as indicator bacteria, most of the above-mentioned 12 LAB strains were able to inhibit the growth of these pathogenic bacteria although some, such as LF50, TM39, LBR01, LF01 and ECA01, showed low or no antimicrobial activity against certain bacteria strains, such as enterotoxigenic *S. aureus*. The levels of antimicrobial activity for these 12 LAB strains are shown in Table 4.

Inhibitory effects of LAB strains and multistrain formulations on the *Salmonella* invasion of Caco-2 cells

LAB cultures were used to study the inhibitory effects of LAB on the *Salmonella* invasion of Caco-2 cells. Table 2 shows that the invasion rate of Caco-2 cells by *S.* Typhimurium decreased significantly when salmonellae were coincubated (2 h) with the cultures of certain LAB strains. Strains LPL05, LBR01, LAP5 and PI08, LBR01 and TM39 significantly inhibited the *Salmonella* invasion of Caco-2 cells as compared with the control without LAB.

Based on the above characteristics for individual LAB strains, multistrain formula I (F I) was prepared using strains with strong adherence (> 200 CFU per Caco-2 cell), high TNF-α-enhancing activity (Fig. 1), high antimicrobial activity and the most inhibitory effects on *Salmonella* invasion. Meanwhile, two other multistrain formulas, each containing strains with different levels of probiotic properties, were used for comparison. Formula II (F II) consists of strains with medium TNF-α-enhancing activity and strains with high or low adherence. Formula III (F III) consists of strains with low or medium adherence as well as TNF-α-enhancing activity. As stated above, F I also inhibited *S.* Typhimurium invasion of Caco-2 cells to the greatest extent (Table 2).

Uptake of labeled *E. coli* by BALB/c mice macrophage cells

The effects of LAB on the phagocytosis of macrophage cells from BALB/c mice were determined. Each day, the mice were orally administered with water (control), or F I, II (with or without 12% SMP) or III [N ($N = 1$–9) $\times 10^9$ CFU each LAB per mouse] for a period of 64 consecutive days. Statistical analysis indicated a significant increase ($P < 0.05$) in the phagocytotic activity (%) of macrophage cells from mice fed with F I, followed by F II when flow cytometry with FACStarPLUS was used for the measurement of phagocytosis (Fig. 2). In order to evaluate the effect of 12% SMP on the phagocytosis, the effect on the phagocytosis with F II plus SMP was assayed. Phagocytosis was not affected by the addition of SMP (data not shown).

Inhibitory effects of multistrain formulas with or without SMP on the invasion of *Salmonella* to mouse liver and spleen

After feeding with multistrain formulas for 63 days, mice were orally challenged with *S.* Typhimurium (ISM 50, 1×10^7 CFU per mouse) and after another 8 days, they were killed and analyzed for the counts of viable *Salmonella* cells in their spleens and livers. Viable *Salmonella* counts in the spleens and livers of the mice fed with F I significantly decreased ($P < 0.05$) compared with the counts obtained from the

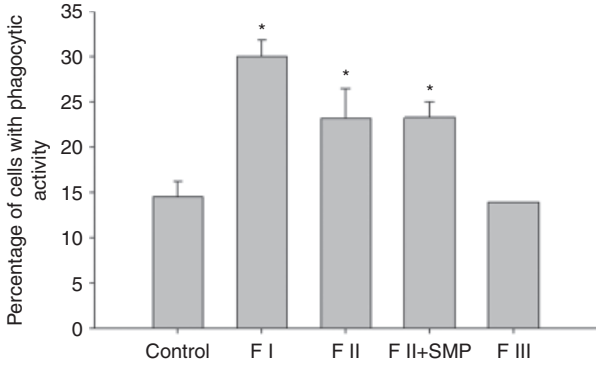

Fig. 2. Effect of different LAB multistrain formulas on the phagocytic activity of peritoneal macrophage cells from BALB/c mice. Multistrain formulas used for F I, F II and F III, respectively; F I, LAB strains LAP5, TM39, LF33 and LPL05; F II, LAB strains ECA01, PI08, LF01 and PA05; and F III, LAB strains LF50, LBR01, PA05 and LF01. F II+SMP means that in F II, 12% SMP was added. *Significant difference.

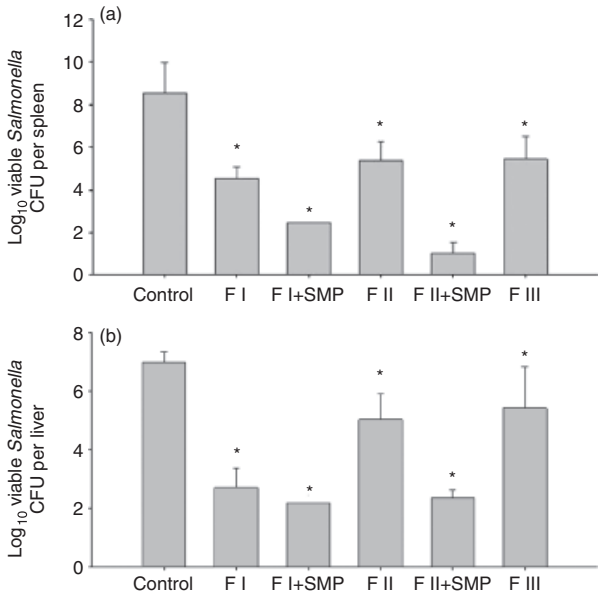

Fig. 3. *Salmonella* Typhimurium ISM 50 infection at day 8 after *Salmonella* challenge in the (a) spleens and (b) livers of the mice. These mice were fed daily with the multistrain combination or 1 × PBS (negative control) for 63 days before the oral administration of 0.2 mL of *Salmonella* cells (5×10^7 CFU mL^{-1}). Mice were fed with multistrain F I, II and III, with or without SMP, respectively, which consisted of LAB strains LAP5, TM39, LF33 and LPL05 (F I); ECA01, PI08, LF01 and PA05 (F II); and LF50, LBR01, PA05 and LF01 (F III). SMP means 12% SMP.

control, F II and F III mice (Fig. 3). For F I mice, *Salmonella* counts in the livers and spleens decreased 4–4.5-logs compared with those from the control group. F II and F III mice showed less inhibitory effects against *Salmonella* invasion.

The addition of 12% SMP to the multistrain mixture enhanced the inhibitory effects on *Salmonella* invasion significantly. A decrease in *Salmonella* cells in mouse liver and spleen of 5–6 logs was observed for F I and F II mice with SMP-fed mice compared with the control group (Fig. 3).

Discussion

There is evidence that LAB strains play a role in immunomodulation and protect the host through anti-infection, anti-inflammatory and antitumor effects (Jones & Versalovic, 2009). Studies have shown that some LAB strains, such as *Lactobacillus reuteri* (Jones & Versalovic, 2009), *Lactobacillus rhamnosus* GG (Schultz *et al.*, 2003), *Lactobacillus paracasei* (Tsai *et al.*, 2008), *L. acidophilus* (Bleau *et al.*, 2007), *Lactobacillus casei* (Ya *et al.*, 2008) and *Lactobacillus plantarum* (Pathmakanthan *et al.*, 2004), can enhance immunity and modulate the immune response in the host. LAB strains have been shown to stimulate immune cells to release proinflammatory cytokines such as TNF-α and interferon-γ (Schoenborn & Wilson, 2007). Macrophages initiate the innate immune response by recognizing pathogens, phagocytosing them and secreting inflammatory mediators. Recent reports have focused on the possibility that LAB may activate macrophages and enhance phagocytosis and may modulate the immune function of an immunocompromised host in a strain-dependent manner (Schiffrin *et al.*, 1997; Blum & Schiffrin, 2003; Lin *et al.*, 2007). In this study, we selected LAB strains with the ability to induce high levels of TNF-α in macrophage cells RAW264.7. For phagocytic assays, multistrain F I, which consisted of strains with high TNF-α-inducing activity in macrophages, showed the highest phagocytic activity among the three multistrain formulas (Fig. 2). Enhanced phagocytic activity of macrophage cells from animals given LAB has been demonstrated (Paubert-Braquet *et al.*, 1995; Gill *et al.*, 2001). The level of enhancement appears to depend on the strain, dose and viability of the LAB used (Gill, 1998). In this study, similar results were obtained. The phagocytic activity of macrophage cells from mice fed with multistrain F I was higher than that of macrophage cells from mice fed with other multistrain formulas. Because the LAB strains used in F I were able to induce high levels of TNF-α production the most in macrophage cells and enhance the phagocytic activity of macrophages, these results suggest that the increased TNF-α production and phagocytotic ability are two factors that may be involved in the ability of LAB to inhibit the *Salmonella* invasion of cultured Caco-2 cells as well as the survival of this pathogen in the livers and spleens of mice. Once across the intestinal epithelium in the host, *Salmonella* encounter the submucosal macrophages (Ohl & Miller, 2001). Therefore, the ability of LAB to enhance TNF-α production as well as the phagocytic activity of macrophages may play a critical role in the reduction of populations of *S.* Typhimurium in the livers and spleens of mice.

LAB strains with adhesive abilities may prevent contact between intestinal epithelial cells and pathogenic bacteria by mechanisms such as nonspecific steric hindrance of the receptors for pathogens (Bernet *et al*., 1994; Coconnier *et al*., 2000) or by the competitive exclusion of adherent pathogenic bacteria (Velraeds *et al*., 1996). The adherence of LAB to host intestinal epithelial cells may play an important role in preventing *Salmonella* invasion. For example, multistrain F II, which contained a *Pediococcus* strain, i.e. *Pediococcus inopinatus* PI08, which is able to strongly adhere to Caco-2 cells, also showed a significant reduction in the level of *Salmonella* invasion of Caco-2 cells (Table 2). The importance of LAB adherence in inhibiting *Salmonella* invasion may also play a role in reducing the number of *Salmonella* cells found in mice that were fed with multistrain F I or II plus 12% SMP (Fig. 3). Although the addition of 12% SMP to the multistrain formula did not enhance phagocytosis of macrophages (Fig. 2), it significantly enhanced the inhibitory effects of LAB strains on *Salmonella* numbers in the livers and spleens of mice (Fig. 3). This may be due to an increase in the milk calcium ion concentration, which enhanced the adherence of LAB strains to intestinal epithelium (Venegas *et al*., 2006). In addition, LAB strains were shown to inhibit the growth of specific pathogenic bacteria (Table 4). This could be due to the production of lactic acid and/or bacteriocins (Makras *et al*., 2006; Simova *et al*., 2009). LAB strains used for multistrain preparations were selected based on their ability to inhibit the growth of pathogenic bacteria and to adhere to intestinal epithelium cell line Caco-2 in addition to their immunomodulatory activity.

According to Nauciel & Espinasse-Maes (1992), the effects of infection of mice with sublethal doses of *S*. Typhimurium can be divided into three main phases. In the first phase, *S*. Typhimurium grows exponentially and invades the spleen and liver. After 7 days, growth inhibited with this second phase is characterized by a plateau in *Salmonella* that lasts about a week. During the third phase, the number of bacteria progressively declines in both organs. In this study, the maximum counts of viable *Salmonella* in the livers and spleens of mice were determined in the second (plateau) phase, 8 days after the *Salmonella* challenge. It was found that multistrain F I showed the greatest inhibitory effect on the *Salmonella* counts in the mouse livers and spleens (Fig. 3).

For strains in multistrain F I, strains *L. acidophilus* LAP5, *Lactobacillus fermentum* LF33 and *Enterococcus faecium* TM39 have been shown to be acid and bile tolerant and able to adhere to the cultured human intestinal epithelial cell line Caco-2 (Table 3). These strains have also been shown able to adhere to the intestinal epithelial cells isolated from swine, poultry and mice (BALB/c) (Tsai *et al*., 2005). In addition, strains LAP5, LF33 and LPL05 in F I are generally regarded as safe strains (Reid *et al*., 2006). Toxicity evaluations for strains of LAP5 as well as TM39 *in vitro* and *in vivo* have also been performed and the results suggest that these strains are safe to use in humans and animals (Tsai *et al*., 2004a, c). Although single strains, such as LPL05, LAP5 and *P. inopinatus* PI08, respectively, also show inhibitory effects on *Salmonella* invasion of Caco-2 cells just as the F I strains did (Table 2), it should be kept in mind that in the gastrointestinal tract of the host, multistrain probiotics may serve additional functions, such as creating an anaerobic probiotic niche that enhances the colonization and adhesion of strains, and enhancing the additive and synergistic effects due to the combination of multistrain-specific properties (Ouwehand *et al*., 2000; Shihata & Shah, 2000; Timmerman *et al*., 2004). The probiotic properties of LAB strains, such as antimicrobial activity, bile tolerance, growth in milk and survival in host gastrointestinal tracts, demonstrate the beneficial effects of multistrain probiotics. The commercially available probiotic VSL#3 is an eight-strain formulation; although the mechanisms of action for the individual strains in VSL#3 are not known, positive clinical outcomes have been obtained for patients with inflammatory bowel disease (Bibiloni *et al*., 2005). Also, a five-strain probiotic combination including *Lactobacillus* and *Pediococcus* spp. has been shown to reduce pathogen shedding and to decrease signs of disease in pigs challenged with *S*. Typhimurium. In this case, the LAB strains used were selected for their general probiotic properties, such as antimicrobial activity, bile and acid tolerance, growth in milk, survival in host gastrointestinal tracts, etc. (Casey *et al*., 2007).

In this study, the strains used for the multistrain formulation were those with known specific functions, such as immunomodulating activity, adherence to intestine epithelium and inhibition of *Salmonella* invasion of Caco-2 cells. We showed that their ability to reduce the population of *S*. Typhimurium in the livers and spleens of mice was correlated with the results of *in vitro* assays. In conclusion, multistrain probiotics, such as F I, which contain strains with specific functions and mechanisms of action, together with additional advantages, such as the clonal and the syngestic effects derived from the combination of these individual strains, may protect the host; this includes human and domestic animals. These LAB strains may prove to be useful as animal feed supplements.

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Authors' contribution

C.-C.T. and H.-Y.T. contributed equally to this work.

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