

Enhancement of the immune response against *Salmonella* infection of mice by heat-killed multispecies combinations of lactic acid bacteria

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Heat-killed lactic acid bacteria (LAB) has advantages over live LAB in that it has a long shelf-life and is therefore easy to store and transport. From four LAB strains selected by immunomodulatory activity and adherent properties, we prepared the heat-killed multispecies combination of LAB (MLAB) and the cell walls from MLAB under two conditions (100 °C for 30 min and 121 °C for 15 min). Different effects on the adherent properties of these four LAB strains were observed, depending on the heating conditions. With mouse macrophage cells, the two heat-killed MLABs (HMLABs) showed significantly higher induction activities on the production of interleukin 12 (IL-12) than their individual strains did. Heat-killed MLABs and cell-wall preparations were able to reduce the *Salmonella* invasion of Caco-2 and mouse macrophage cells. Feeding mice with HMLAB could inhibit the *Salmonella* invasion of mice significantly. For these mice, the expression level of pro-inflammatory cytokines, such as TNF- α and IL-6, in mouse serum was reduced while that of the anti-inflammatory cytokine, i.e. IL-10, was enhanced. The HMLABs developed in this study showed higher protective effect against *Salmonella* invasion either of Caco-2 cells or of mice, relative to the heat-killed lactobacilli, which consisted of *Lactobacillus acidophilus* strains selected at random. In conclusion, the HMLABs were potentially useful for the protection of mice against *Salmonella* infection and the induced inflammation.

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INTRODUCTION

Strains of the favourable lactic acid bacteria (LAB), such as *Lactobacillus* spp., *Bifidobacterium* spp., *Enterococcus* spp. and *Lactococcus* spp. etc., have been used for probiotic preparation. The health effects of probiotics on host may include: modulating the immune system; increasing the protective effect against infection by pathogens; antiallergic effects; and preventing the cancer recurrence (Nonaka *et al.*, 2008; Yasuda *et al.*, 2008; Kobayashi *et al.*, 2011). To protect the host animal against the infection of pathogenic bacteria, competitive exclusion of pathogens by the adherence of LAB on host intestinal epithelium, and enhancement of the immunomodulatory activity of host by LAB, are two of the major factors (Hua *et al.*, 2010).

Upon infection by pathogenic bacteria, activated macrophages may phagocytize micro-organisms, secrete pro-inflammatory cytokines and nitric oxide, and present

Abbreviations: HMLAB, heat-killed multispecies combination of lactic acid bacteria; IL, interleukin; IFN, interferon; LAB, lactic acid bacteria; LPS, lipopolysaccharide; MLAB, multispecies combination of lactic acid bacteria.

antigens to helper T cells (Schreiber *et al.*, 1985; Celada & Nathan, 1994). Probiotics may induce Th1 response by increasing IL-10 and IL-12 production in dendritic cells and IFN- γ production in T cells (Chon *et al.*, 2009; Hua *et al.*, 2010). For IL-12, the ability of LAB to induce its production is a key point to examine LAB's strong immunoenhancing activity (Hirose *et al.*, 2010). For IL-10, it is a cytokine with potent immunoregulatory and anti-inflammatory properties and is produced by activated macrophages and T cells (Hammer *et al.*, 2005). To study the effect of LAB strains on the inflammation of *Salmonella* infected mice, the expression level of pro-inflammatory Th1 cytokine IL-6 and TNF- α and the anti-inflammatory Th2 cytokine IL-10 in mice could be used as an index (Cammarota *et al.*, 2009; Tsai *et al.*, 2011).

In recent years, reports regarding the probiotic functions of heat-killed LAB strains have been increasing. For example, heat-killed LAB strains may skew the immune system toward T helper 1 polarization in mouse splenocytes and dendritic cell-treated T cells (Chuang *et al.*, 2007), and intake of a heat-killed *Lactobacillus plantarum* strain, i.e. L-137, may augment acquired immunity in humans (Hirose

et al., 2006). Previously, we have developed a multispecies combination of LAB (MLAB) consisting of four LAB strains, i.e. *Lactobacillus acidophilus* (LASW); *Lactobacillus plantarum* (LPL05); *Lactobacillus fermentum* (LF33) and *Enterococcus faecium* (TM39), selected by immunomodulatory activities and adherent properties (Tsai *et al.*, 2011). Oral feeding of mice and chicks with this MLAB could enhance the protective effect in mice and broiler chicks against *Salmonella* infection and the induced inflammation (Tsai *et al.*, 2011; Chen *et al.*, 2012). In this study, we evaluated the adherent properties, immunomodulatory activities and the effect on *Salmonella* invasion of Caco-2 and mouse macrophage cells for the viable and heat-killed LAB strains, their viable and heat-killed strain combinations and cell walls. We then used the heat-killed multi-species combinations of LAB (HMLAB) to feed mice and evaluated their functions to protect the mice against *Salmonella* infection and the induced inflammation.

METHODS

Bacterial strains and conditions of heat treatment on LAB strains. Four LAB strains including LASW, LF33, LPL05 and TM39 selected by immunomodulatory activity and adherent properties (Tsai *et al.*, 2011) were used in this study. The stock culture of each of these strains was maintained at -80°C in 20% glycerol. Before use, bacterial cells were propagated twice in Lactobacilli MRS broth (Difco) containing 0.05% L-cysteine, each time for 24 h at 37°C . Whole cells of each strain were obtained by centrifugation at 7000 g for 10 min and washed twice with sterilized PBS (pH 7.2). The LAB cells (10^9 c.f.u. ml^{-1} PBS) of each strain were heat-killed at 100°C for 30 min or at 121°C for 15 min. The surviving cells were then checked by plate counting. For HMLAB preparation, each of the LAB strains (10^9 cells ml^{-1}) was mixed in equal portion and heat-killed. Aliquots of 15 ml HMLAB preparation were pipetted into a sterile centrifuge tube and stored at 4°C . Normally, these samples were used immediately after preparation.

Salmonella typhimurium I50, a strain isolated from a patient with foodborne diarrhoea, was obtained from the National Center for Disease Control, Taipei, Taiwan. For the cultivation of this strain, one loopful was inoculated into 5 ml tryptic soy broth (Difco) and incubated at 37°C for 12 h. Then, a portion of the culture was diluted and placed on tryptic soy agar (Difco) for future study.

Cell culture. 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, Hsin-Chu, Taiwan. Culture conditions for these cell lines have been described previously (Tsai *et al.*, 2011).

Preparation of cell walls from MLABs. Cells in MLAB (10^9 c.f.u. ml^{-1}) were used for cell-wall preparation following rupture by a cell Ultrasonic (HOYU Technology) at 23 kHz for 6 h. The lysate was heated at 75°C for 30 min in order to inactivate the autolytic enzymes, and the unbroken cells and cell debris were harvested by centrifugation at 12000 g for 30 min at 4°C . At this stage, the cell walls of MLAB were present in the supernatant, which was then pipetted to a new centrifugation tube. The cell walls were then recovered from the supernatant by centrifugation at 20000 g for 30 min at 4°C , and the precipitate was suspended again in sterilized phosphate buffer (Chon *et al.*, 2009). The cell-wall preparations from

MLABs, either treated or not treated under two conditions, 100°C for 30 min or 121°C for 15 min, were used for future study.

Adhesion of heat-killed LAB to Caco-2 cells. Conditions for the culture and pre-treatment of Caco-2 cells, and methods for the evaluation of the adherent properties of live as well as heat-killed LAB cells, were as described previously (Tsai *et al.*, 2011). Briefly, Caco-2 cells were cultured in 75 cm^2 plastic tissue culture flasks. Prior to the adhesion test, each live or heat-killed LAB strain at 10^9 cell ml^{-1} was washed twice with PBS and centrifuged for 5 min at 5000 g. Bacterial cells of each strain were suspended in 1 ml Dulbecco's Modified Eagle Medium (DMEM, 1×10^8 cells ml^{-1}), and 100 μl of each suspension was transferred to the 24-well multidish containing Caco-2 cells (1×10^5 cells/per well) and then incubated for 2 h at 37°C in 5% CO_2 . 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 numbers of adhered LAB cells were counted according to the method of Gopal *et al.* (2001).

Effect of viable or heat-killed MLABs or cell walls on *Salmonella* invasion of Caco-2 and mouse macrophage cells.

The antagonistic effect of LAB strains against *Salmonella* invasion was investigated according to the method of Tsai *et al.* (2011) and Buckner *et al.* (2011). Prior to the *Salmonella* invasion, the MLAB or HMLABs, respectively, were washed twice with PBS and centrifuged for 5 min at 5000 g. The precipitate, which consisted of a total of 10^8 LAB cells ml^{-1} for MLAB or 10^8 heat-killed cells ml^{-1} for HMLAB, was then suspended in 1 ml DMEM. With regard to the heated or unheated cell walls, the earlier-described cell-wall suspensions, which were prepared from 10^8 cells ml^{-1} of MLAB, were centrifuged at 20000 g for 30 min to collect the cell walls. These cell walls were then suspended in 1 ml DMEM. One hundred microlitres of each of the above-described MLAB, HMLABs and cell-wall suspensions in DMEM were transferred to each well of the 24-well multidish containing cell cultures and then incubated for 2 h at 37°C in 5% CO_2 (for Caco-2 cells) or incubated for 20 h at 37°C in 5% CO_2 (for macrophage cells). *S. typhimurium* I50 cells (10^7 c.f.u. ml^{-1}) were suspended in 1 ml DMEM, and 100 μl of the suspension was pipetted into each well of the cell culture plate. The mixture in each well was incubated for 1 h (for Caco-2 cells) or 3 h (for macrophage cells) at 37°C in 5% $\text{CO}_2/95\%$ air atmosphere and then washed three times again with PBS. Gentamicin ($100\ \mu\text{g}\ \text{ml}^{-1}$) in fresh culture medium was added and incubated for 1 h in order to kill the extracellular *Salmonella*. Caco-2 or macrophage cells in culture plates were washed twice with sterile PBS and then lysed with 0.1% Triton X-100. Appropriate dilutions were pipetted to the plate count agar (Difco) to determine the number of invasive salmonellae. The *Salmonella* invasion assay was performed in triplicate.

Effect of viable or heat-killed LAB strains or MLABs or the cell-wall preparations from MLAB, respectively, on the expression of TNF- α and IL-12 in mouse macrophage cells.

Mouse macrophage RAW 264.7 cells were cultured in triplicate, at a density of 1×10^6 cells ml^{-1} of DMEM without penicillin-streptomycin, in each well of the 24-well tissue culture plates. Aliquots of 100 μl of each of these viable or heat-killed LAB strains or MLABs, each consisting of 10^6 cells ml^{-1} or cell walls prepared from 10^8 cells ml^{-1} of MLAB, were pipetted to stimulate the macrophages. Lipopolysaccharide ($20\ \mu\text{g}\ \text{ml}^{-1}$) (LPS, from *Escherichia coli* O26:B6; Sigma) was used as a positive control. The macrophage cells were co-incubated with each of the above-described LAB preparations or LPS, at 37°C for 20 h (Liu *et al.*, 2011). The levels of cytokines produced in these culture supernatants were analysed. Cytokines of TNF- α and IL-12 were measured using an ELISA kit (BD OptEIA™ Set; BD Biosciences), according to the manufacturer's suggestions. The 96-well Immuno-Maxisorp plates (Nunc) were filled with 100 μl coating

buffer (1 × PBS) containing monoclonal antibodies for TNF- α or IL-12, then diluted and placed overnight at 4 °C. Afterwards, plates were blocked and washed. One hundred microlitres of the culture medium was then pipetted to the well in the plate and incubated for 2 h at room temperature (25 °C). Plates were then washed again and biotinylated anti-mouse TNF- α or IL-12, and horseradish peroxidase-conjugated streptavidin were added for the detection of TNF- α or IL-12, followed by incubation for 1 h at room temperature. The chromogenic reactions were developed with 3',3',5,5'-tetramethylbenzidine substrate for 30 min at room temperature and were terminated with 1 M H₂SO₄ and absorbance measured at 450 nm. Equivalent levels of TNF- α or IL-12 were calculated by comparison with a reference curve generated using TNF- α or IL-12 standards. The results were expressed as the concentration of cytokine in the cell culture medium (pg ml⁻¹).

Oral feeding of mice with HMLAB and *Salmonella* challenge.

Specific mice free from pathogens were used in this study. They were male inbred strain BALB/c mice weighing 24–28 g and aged 8 weeks, purchased from the National Laboratory for Animal Breeding and Research Center, Taipei, Taiwan. These mice were raised at 25 °C, 60 % of RH%, and 12 h light cycle, with feed stuff (1314/1324 total pathogen-free feeding stuff) manufactured and gamma irradiation sterilized by Altromin Spezialfutter. Prior to the experiment, mice were fed *ad libitum* for 1 week. On treatment day 0, mice were randomly allocated into four groups of five mice each. For HMLAB feeding and *Salmonella* challenge, conditions described by Tsai *et al.* (2005) were used. HMLAB was prepared as described earlier and adjusted to a cell density of 10⁹ cells ml⁻¹ for each LAB strain. Each mouse was given a single 200 μ l dose of HMLAB preparation daily by oral administration for 7 consecutive days. Each mouse in the control group was given a 200 μ l dose of sterile normal saline. On day 8, each mouse was challenged with *S. typhimurium* I50 by oral administration of a single 200 μ l dose of *S. typhimurium* I50 with a cell count of 3.0 × 10⁷ c.f.u. ml⁻¹.

Assessment of TNF- α , IL-6 and IL-10 in mouse serum and enumeration of the *Salmonella* cells invaded in mouse liver and spleen.

Blood samples were collected from the orbital vascular plexus of each mouse 3 and 6 days post *Salmonella* challenge. Blood serum was obtained after incubation for 1 h at room temperature followed by centrifugation (2000 g, 10 min). Sera were stored at -80 °C until assay. The cytokines IL-6, TNF- α and IL-10 were measured with commercial ELISA kits according to the instructions (BioLegend). Three and 6 days post *Salmonella* challenge, *Salmonella* cells that had invaded the spleens and livers of mice were enumerated. Cell suspensions were diluted with PBS and the numbers of invaded *Salmonella* cells were counted with selected Brilliant Green agar (Difco) after 48 h incubation at 37 °C.

Statistical analysis. All data were subjected to one-way analysis of variance using SPSS 17.0 for Windows (SPSS). Mean values of treatment groups were compared using Duncan's multiple range test and differences were considered statistically significant at $P < 0.01$ and $P < 0.05$.

RESULTS

Heat treatment conditions and induction of TNF- α and IL-12 production in mouse macrophage 264.7 cells

Under the heat treatment conditions (100 °C for 30 min or 121 °C for 15 min), the four LAB strains used for HMLAB

preparations were all inactivated. No viable cells were observed.

The immunomodulatory activities of the viable and heat-killed LAB strains, the MLAB and HMLAB, each at cell concentration of 10⁶ cells ml⁻¹ or the cell walls prepared from 10⁸ cells ml⁻¹ of MLAB, either heated or unheated, were evaluated. Results showed that all these viable or heat-killed cells, or the cell wall preparations, were able to induce the release of TNF- α from mouse macrophage cells. The levels of TNF- α expression induced with viable or heat-killed MLAB were higher than those of each strain and the cell-wall preparations (Fig. 1a). In addition, MLAB, especially HMLAB, induced higher IL-12 production than those induced by each strain or the cell-wall preparations. Compared with live MLAB, HMLAB induced a higher expression level of IL-12. Thus, the immune effect of MLAB, based on the production of IL-12, could be enhanced by heat treatment.

Adherent properties of heat-killed LAB cells

The adherent abilities of live and heat-killed LAB strains were evaluated with Caco-2 cells. The heat-killed LAB strains, treated at either 100 °C for 30 min or 121 °C for 15 min, showed adherent capability. For strains treated at 100 °C for 30 min, their adherent levels were similar or near to those of their live strains (Table 1). However, for strains treated at 121 °C for 15 min, more than 50 % of their adherent capability was lost, except for strain *Lactobacillus fermentum* LF33 (Table 1).

Inhibition of *Salmonella* invasion of cultured Caco-2 and mouse macrophage cells by live or heat-killed LAB cells or their cell walls

The protective effect of viable and heat-killed MLABs and their cell walls against *Salmonella* invasion of Caco-2 and macrophage cells was evaluated. Significant protective effects against *Salmonella* invasion of Caco-2 and macrophage cells were observed (Fig. 2). The two HMLABs, either prepared at 100 °C for 30 min or 121 °C for 15 min, were able to reduce the numbers of invading *Salmonella* cells. The *Salmonella* counts invading were reduced from 4.05 to 2.6–3.2 log c.f.u. well⁻¹ in Caco-2 cells and from 4.8 to 3.2–3.3 log c.f.u. well⁻¹ in macrophage cells. Cell walls, either the heat-treated or untreated preparations, showed a significant protective effect against *Salmonella* invasion of Caco-2 and macrophage cells. Also, heat treatment of the cell-wall preparation further enhanced its protective effect (Fig. 2).

Salmonella invasion of mice fed with HMLABs

Since the results in Figs 1 and 2 indicate that heat-killed MLABs are able to induce the highest expression of IL-12 and reduce the *Salmonella* invasion of Caco-2 and macrophage cells, we attempted to evaluate the efficacy

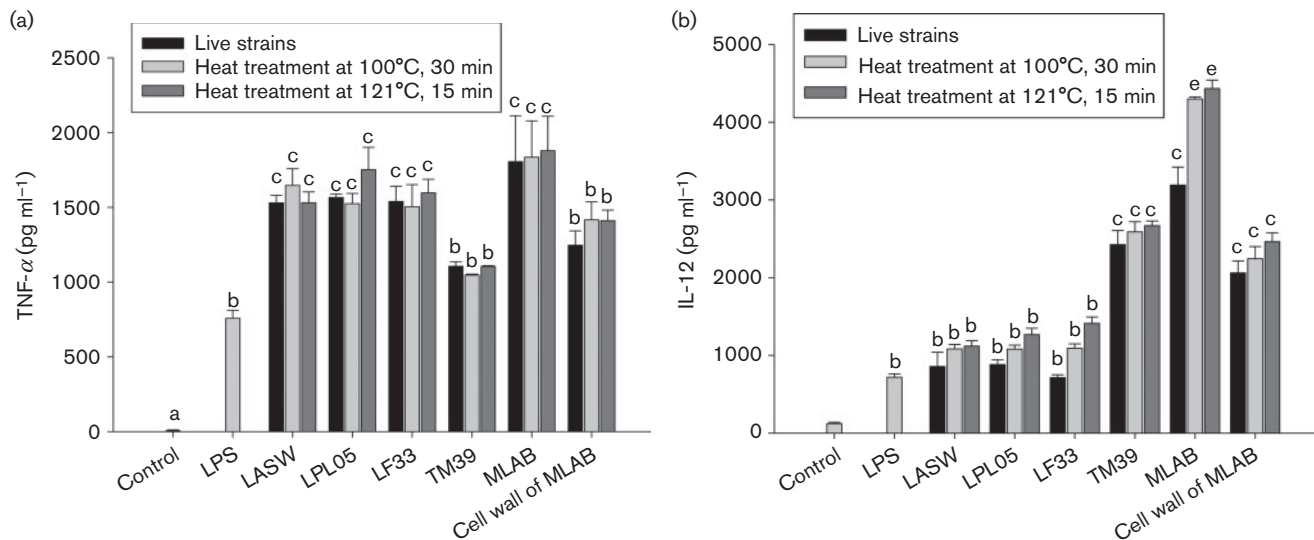


Fig. 1. (a) TNF- α , (b) IL-12 production from murine macrophage Raw 264.7 cells after stimulation for 20 h with viable and heat-killed LAB strains, MLAB and HMLABs, each at 10^6 cells ml⁻¹, or cell walls prepared from 10^8 cells ml⁻¹ of MLAB, either heated or unheated. Negative control (PBS) and positive control with LPS ($20 \mu\text{g ml}^{-1}$) were also performed. Each value represents the mean value \pm SD from three trials. Different letters above bars indicate significant differences between treatments ($P < 0.05$).

of feeding HMLABs on the reduction of *Salmonella* invasion of mice. Under the conditions described, results showed that *Salmonella* counts in the livers and spleens of the mice fed with either of the two HMLABs were significantly lower than those of the mice without HMLAB feeding (Fig. 3). Significant reduction of *Salmonella* counts (from 3.0 to 1.3~1.1 log c.f.u. per liver and from 4.0 to 1.8 log c.f.u. per spleen) were observed 3 days post *Salmonella* challenge. Although multiplication of the invaded *Salmonella* cells in mouse liver and spleen was observed, significant reduction of the *Salmonella* counts in these organs was also observed 6 days post *Salmonella* challenge (Fig. 3).

Assay of the cytokines IL-6, TNF- α and IL-10 in mouse serum

Blood samples of mice fed with sterilized water or HMLABs for 7 days followed by *Salmonella* challenge were assayed at 3 and 6 days post-challenge for their serum levels of cytokines IL-6, TNF- α and IL-10. Mice fed with both HMLABs showed lower levels of TNF- α and IL-6 in their livers and spleens as compared with those of the challenged mice without HMLAB feeding (Fig. 4a, b). For cytokines TNF- α and IL-6, high expression levels were observed in mice 6 days post *Salmonella* challenge (Fig. 4a, b). For example, from 3 to 6 days post-challenge, the TNF- α and IL-6 levels, respectively, boosted from 86

Table 1. Adhesion of lactic acid bacteria (LAB) to Caco-2 cells

Strain	LAB cells adhered per Caco-2 cell*		
	Viable†	Heat-killed‡	Heat-killed§
<i>Lactobacillus acidophilus</i> LASW	132 \pm 1.94	104 \pm 2.82	58 \pm 3.42
<i>Enterococcus faecium</i> TM39	125 \pm 2.21	131 \pm 2.11	49 \pm 6.94
<i>Lactobacillus fermentum</i> LF33	103 \pm 2.26	87 \pm 1.89	75 \pm 9.41
<i>Lactobacillus plantarum</i> LPL05	133 \pm 1.89	125 \pm 2.71	38 \pm 3.32

*Ten of the Caco-2 cells were used to calculate the mean number of the adhering LAB cells per epithelial cell.

†Viable strains were treated under the same condition as heat-killed LAB preparation except for heat treatment.

‡Heat-killed LAB cells were prepared according to the conditions (100 °C, 30 min) described in Methods.

§Heat-killed LAB cells were prepared according to the conditions (121 °C, 15 min) described in Methods.

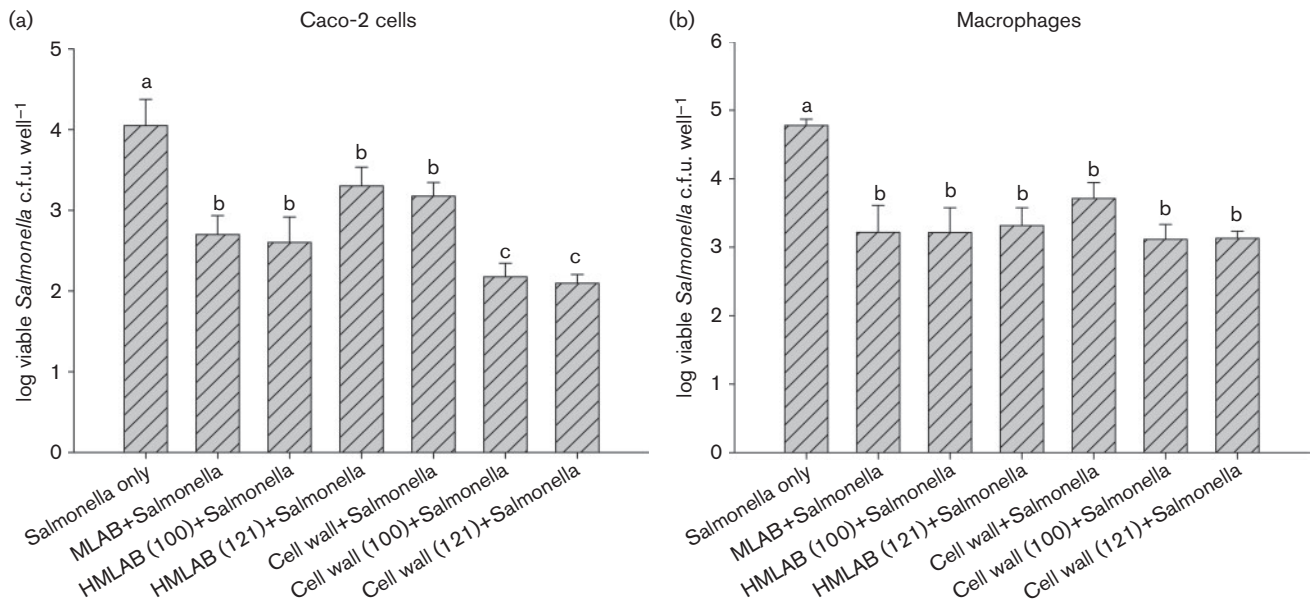


Fig. 2. Effect of MLAB, HMLABs and cell-wall preparations on *Salmonella* invasion of Caco-2 cells (a) and macrophage RAW 264.7 cells (b). The MLAB, HMLABs and cell walls from MLAB were prepared under the experimental conditions described in Methods. Each vertical bar represents the mean \pm SD from three trials; different letters above bars indicate significant differences between treatments ($P < 0.05$).

to 392 pg ml⁻¹ and from 48 to 504 pg ml⁻¹ in mouse serum, respectively. Under such conditions, feeding HMLABs could reduce the expression level of both cytokines significantly (Fig. 4). Mice receiving HMLAB

prior to *Salmonella* challenge showed higher levels of serum IL-10 as compared with those of the challenged mice without HMLAB feeding (Fig. 4c). These results indicate that feeding mice with HMLAB was able to

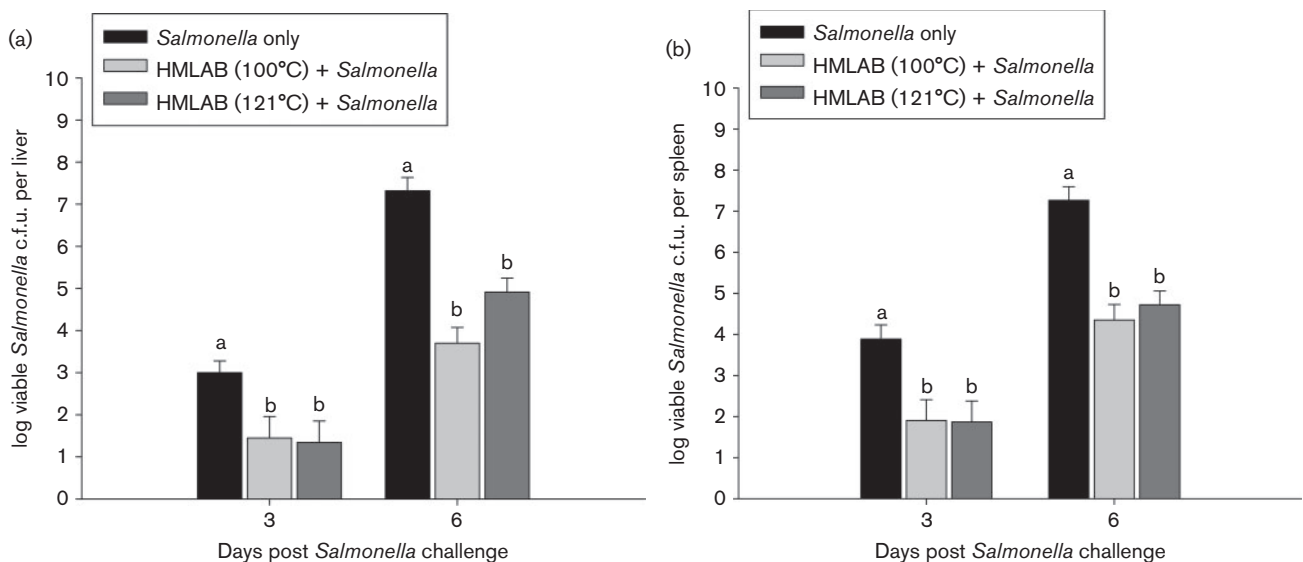


Fig. 3. Effect of two HMLABs prepared under two conditions on the *Salmonella* cells invading the (a) livers and (b) spleens of the mice which had been orally administered sterile deionized water daily or HMLABs for 7 days prior to the oral administration of *Salmonella* cells (3.0×10^7 c.f.u. ml⁻¹). Each vertical bar represents the mean \pm SD ($n=5$); different letters above bars indicate significant differences between treatments ($P < 0.05$).

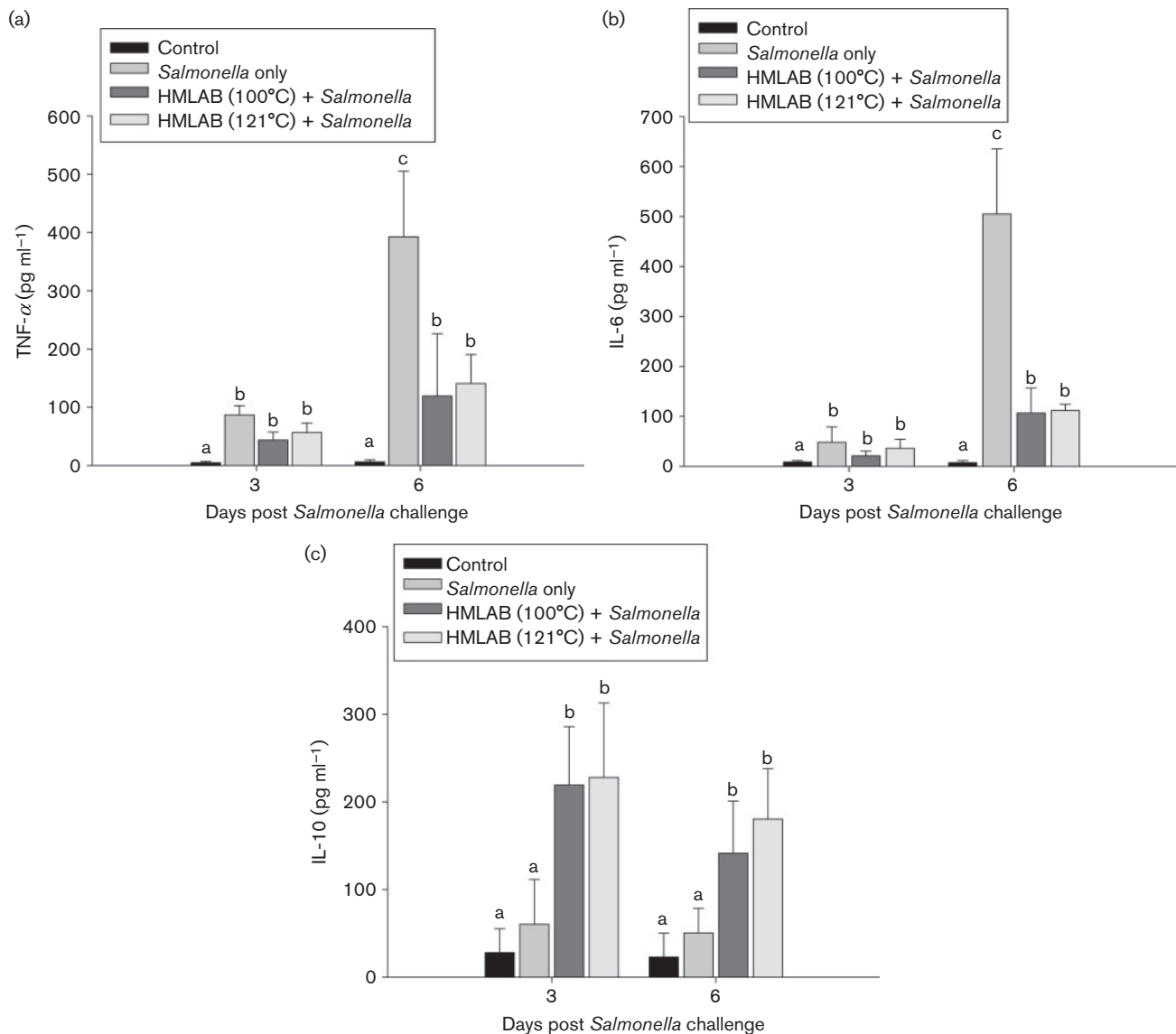


Fig. 4. (a) TNF- α , (b) IL-6 and (c) IL-10 level in sera of mice fed with sterile deionized water or HMLABs for 7 days followed by *Salmonella* challenge. In the control group, mice were not challenged with *Salmonella*. Each vertical bar represents the mean \pm SD ($n=5$); different letters above bars indicate significant differences between treatments ($P < 0.05$).

enhance the anti-inflammatory cytokines and reduce the inflammatory response of mice.

DISCUSSION

Adherent property and immunomodulatory activity of the viable and heat-killed LAB strains may play major roles to protect the cell cultures, such as Caco-2 and macrophage cells, and mice against the *Salmonella* invasion (Lin *et al.*, 2007; Ishikawa *et al.*, 2010). Since the adherent properties of heat-killed LAB strains are strain dependent (Lin *et al.*, 2011), we thus attempted to evaluate the adherent properties of the heat-killed LAB strains. For viable LAB

strains used in this study, all strains have high adherent capabilities. Heating these LAB strains at 100 °C for 30 min seems not to affect their adherent properties significantly. However, after heating at 121 °C for 15 min, significant reduction of their adherent capabilities was observed (Table 1). Regarding the adhesion of heat-killed LAB cells to the intestinal mucus and the induced immunomodulatory activity of the host, the non-protein cell-wall components, such as lipoteichoic acid and exopolysaccharides, are both important (Perea Vélez *et al.*, 2007; Hirose *et al.*, 2010; Izumo *et al.*, 2011). Both factors have been reported to affect *Lactobacillus* adhesion to the gastrointestinal tract. Dramsi *et al.*, (2005) reported that bacterial surface proteins constitute a diverse group of molecules

with important functions, such as adherence, invasion and interaction with the host immune system or the environment.

The immunomodulatory effects of live and heat-killed *Lactobacillus* strains have been demonstrated by Izumo *et al.* (2011), who reported that a heat-killed strain of *Lactobacillus pentosus* SPT84 was more effective than its live strain in *in vitro* induction of IL-12 or IFN- γ production. Sashihara *et al.* (2007) reported that the IL-12-inducing activity of heat-killed *Lactobacillus gasseri* OLL2809 was higher than that of its live strain if cultured with immune cells under a neutral pH condition; a condition similar to what we used in this study. They concluded that such phenomena might be induced by the peptidoglycan conformation or degradation of the cell wall that is associated with autolysis of the cells. The amount of peptidoglycan in bacterial cells has been reported to be correlated with the production of IL-12 from immune cells (Izumo *et al.*, 2011).

It has been reported that various beneficial effects may be obtained by heat-killed LAB (Izumo *et al.*, 2011). In this study, enhanced levels of IL-12 were found with live or heat-killed MLAB (Fig. 1a). For live MLAB, additive, synergistic and symbiotic effects of different strains with specific properties have been reported (Timmerman *et al.*, 2004). In this regard, the HMLABs, either prepared at 100 °C or 121 °C, also showed significant enhanced levels of IL-12. Since additive and symbiotic effects of the multistrain combination of LAB are associated with the viability of LAB cells, for heat-killed MLABs, the synergistic effect might play a major role.

Under the experimental conditions, when live and heat-killed MLABs, with cell concentration of 10^8 cells ml⁻¹ and their cell walls prepared from such cell quantity, were evaluated for their protective activities against *Salmonella* invasion of Caco-2 and macrophage cells, significant reduction of invaded *Salmonella* cells was observed (Fig. 2). Under the same conditions with the same cell concentration (10^8 cells ml⁻¹), a heat-killed multistrain combination which consisted of three *Lactobacillus acidophilus* strains, selected at random, could not reduce the *Salmonella* invasion of Caco-2 cells (Lin *et al.*, 2007). In addition, for our *in vivo* study with mice, each mouse was given a daily single 200 μ l dose of 10^9 cells ml⁻¹ HMLAB, which was only one tenth of the daily feeding dose, (200 μ l of 10^{10} cells ml⁻¹) reported by Lin *et al.* (2007). Even under such conditions, significant reductions of *Salmonella* invasion of mouse liver and spleen were observed 3 days post *Salmonella* challenge (Fig. 3). In comparison of the results obtained 3 and 6 days post *Salmonella* challenge, it seems to be possible that our HMLABs are able to inhibit the intracellular growth of *Salmonella* cells invaded in the mouse organs (Fig. 3). Thus, to prepare the heat-killed LAB products, use of multispecies strains selected by immunomodulatory activity and adherent property is important.

Various natural *Lactobacillus* strains have been shown to have anti-inflammatory effects (Lebeer *et al.*, 2012), and

surface layer proteins of *Lactobacillus acidophilus* and bacterial component of *Lactobacillus* strains have been suggested to be potential mediators of the anti-inflammatory effect (Chon *et al.*, 2009; Lebeer *et al.*, 2012). The HMLABs, either prepared under 100 °C for 30 min or 121 °C for 15 min, were able to reduce the levels of pro-inflammatory cytokines, such as TNF- α and IL-6, and enhance the level of anti-inflammatory cytokine, such as IL-10, in *Salmonella*-challenged mice (Fig. 4).

Conclusions

Under two heat treatment conditions, we developed the HMLABs, which consisted of strains of four different LAB species. Enhanced immunomodulatory activity in mouse macrophages was observed, compared to the live MLAB. Oral feeding the mice with such HMLABs could reduce the *Salmonella* invasion of mice and the induced inflammation. These HMLABs may have potential for use as effective supplements to reduce the *Salmonella* infection and the induced inflammation of humans and animals.

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