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A Study on the Prevention of Salmonella Infection by Using the Aggregation Characteristics of Lactic Acid Bacteria

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Salmonella is one of the major pathogenic bacteria that cause food poisoning. This study investigated whether heat-killed as well as live *Lactobacillus* protects host animal against *Salmonella* infection. Live and heat-killed *Lactobacillusacidophilus* was administered orally to Sprague-Dawley rats for 2 weeks before the rats were inoculated with *Salmonella*. Rise in body temperature was moderate in the group that was treated with heat-killed bacteria as compared to the *Salmonella* control group. The mean amount of feed intake and water consumption of each rat in the heat-killed bacteria group were nearly normal. The number of fecal *Salmonellae* was comparable between the live and the heat-killed *L. acidophilus* groups. This finding shows that *L. acidophilus* facilitates the excretion of *Salmonella*. Moreover, the levels of pro inflammatory cytokines, including tumor necrosis factor (TNF)-alpha and interleukin (IL)-1 beta, in the heat-killed *L. acidophilus* group were significantly lower when compared to the levels in the *Salmonella* control group. These results indicate that nonviable lactic acid bacteria also could play an important role in preventing infections by enteric pathogens such as *Salmonella*.

***Key words*:** Salmonella, *Lactobacillus acidophilus*, Food poisoning, Heat-killed bacteria, Probiotics

INTRODUCTION

Salmonella is an enteric bacterial pathogen and a major pathogenic bacterium that causes food poisoning. Its routes of infection include contaminated foods and water. Salmonella is a gram-negative bacillus, causes paratyphoid fever, hematosepsis and gastroenteritis as food poisoning pathogens (1,2) and these pathogens often resist antibiotics such as tetracycline, trimethoprim-sulfamethoxazole and streptomycin (3,4). Salmonella has been known to have about 2,500 serotypes, including the most frequently found *Typhi* and *Typhimurium*. Typhi is a Salmonella serotype that causes Salmonellosis in humans. *Salmonella typhimurium*, which was used in this study, causes Salmonellosis in mice, so it is a useful strain that is frequently found in bacterial infections in animals (5).

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In the immune system, the macrophage is in charge of immune responses, including innate and adaptive immune responses against infections in all host defense systems. When the pathogen approaches the epithelial barrier, the macrophage produces cytokines to induce phagocytosis such as tumor necrosis factor (TNF)-alpha (6). In particular, TNF-alpha plays an important role in host immune responses and gram-negative bacterial infections (7). It is also used as an important parameter in animal models of Salmonella infection. The TNF-alpha level in infants infected with Salmonella is known to be high (8).

Salmonella inhibition studies using lactic acid bacteria (LAB) have often focused on the following subjects: treatments to food poisoning using bacteriocin that inhibits pathogens (9), the immunity acquired through the immunologic communications between LAB and intestinal epithelial cells (10), and the treatment or prevention of food poisoning by inhibiting the colon residence of the pathogens through the coaggregation, autoaggregation, intestinal cell adhesion and bacterial adhesion to hydrocarbons tests (11).

In this study, coaggregation of live and heat-killed (hk) LAB was performed using hydrophobicity, and the LABs with the best coaggregation ability were selected to investigate the prevention of Salmonella infection in animal model.

MATERIALS AND METHODS

LAB and S. typhimurium preparation. For the LAB, *Lactobacillus acidophilus* 11869BP that was used and orally administered once in a two day in this study was cultured from the CELLBIOTECH Co. Ltd. (Gimpo, Korea). The LAB was inoculated into an MRS broth (Difco, Detroit, MI, USA) and cultured at 37°C for 18~24 hr, and then washed twice with sterile saline to remove any metabolic substances associated with it. It was then killed by autoclaving at 121°C for 15 min. the hk LAB solution was freeze-dried and dissolved in saline before use (12). *Salmonella typhimurium* is NCCP 10725 was used throughout this study. This strainwasshaking-cultured (at 200 rpm) in brain heart infusion broth (Difco, Detroit, MI, USA) for 18-24 hr at 37°C under aerobic conditions, harvested by centrifugation (3200 × *g*, 4°C, 20 min), after which it was subshaking-cultured once in the same-type fresh media.

Cell cultures. The human colon adenocarcinoma cell line HT-29 (KCLB 30038; Seoul, Korea) cell lines were cultured in RPMI1640, which were supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA) and penicillin/streptomycin 1% (Invitrogen, Grand Island, NY, USA). Cells were cultured at 37°C in an atmosphere of 5% CO_2 and 95% air.

In vitro test of the coaggregation ability. The coaggregation analysis was performed according to Handley *et al*. (13). To measure the coaggregation abilities of the LAB live and hk bacteria and Salmonella, each OD of the LAB bacteria and Salmonella was prepared at 0.5. And the mixtures of the *Salmonella typhimurium* and the live or hk LAB bacteria were cultured for two hours to measure their OD levels (14). Then the coaggregation abilities were measured using the following equation.

$$\text{Coaggregation (\%)} = [\{(\text{Asal} + \text{Alac})/2 - (\text{Amix})/2\}/(\text{Asal} + \text{Alac})/2] \times 100$$

A: absorbance at 600 nm, sal: *Salmonella typhimurium*, lac: *lactobacillus acidophilus*

Adhesion assay. Adhesion assay was carried out as described from Jacobsen *et al*. (15). Each well of a 12-well tissue culture plate was seeded with HT-29 cells. 500 μl of DMEM without serum and antibiotics was added to each well and incubated at 37°C, CO_2 5% for 1 hr. Probiotics and *S. typhimurium* were grown overnight cultures of bacteria were appropriately diluted (10×) with DMEM to give a bacterial concentration of approximately 10^8 cells/ml. Simultaneously, Salmonella and live or hk LAB added for 1 hr incubation**.** After incubation for 1 hr, all of the dishes were washed three times with phosphate-buffered saline to release unbound bacteria. And then the well is stained with gram-staining kit (BD Biosciences, San Jose, CA, USA) and observed with a microscope (×1000).

Experimental animals. Eight-week-old white male Sprague-Dawley rats were purchased from Orient Bio (Seongnam, Korea). They were put in cages in groups of five. During the one-week adaptation period, the rats were induced to freely take pellet-type feeds and water under the conditions of a 24 ± 2°C temperature, a 40 ± 20% relative humidity and a 12 hr lighting cycle. While their health status was monitored, their feces were cultured in a Salmonella-shigella plate (Difco, Detroit, MI, USA) with *S. typhimurium* selective media before the uninfected rats were selected through a screening process.

Oral administration of LAB. To investigate the inhibition effects of the LAB oral administration on the pathogenic bacterial proliferation, six experimental groups were prepared, as shown in Table 1, and nine rats were assigned to each group. All group was treated to disrupt the original intestinal flora with the antibiotic process (ampicillin : 4 g/L) for three days. And then *L. acidophilus* live and hk bacteria were prepared. Starting two weeks before the administration of the pathogenic bacteria, 1×10^9 and 1×10^{10} CFU of 1 ml LAB were orally administered to the rats every day for a week.

Body weight and body temperature. After the one-week adaptation period, LAB was administered to the rats and their body weights were measured weekly. Before (0 hr) and after (24 hr) the Salmonella-induced diarrhea, the rats' body weights were measured to confirm their changes. The rats' body temperatures were measured before (0 hr) and after (1, 3, 6, 9, 12 and 24 hr) the Salmonella-induced

Table 1. Design of the experiment groups based on the administration of the probiotics

Groups	Condition of administration	Probiotics treatment
NC	Non-administration	-
SA	Non-administration, after inoculation of the pathogen	-
L.1.0E+9	Pre-administration, daily 2 wks before inoculation of the pathogen	live 10^9 CFU
L.1.0E+10	Pre-administration, daily 2 wks before inoculation of the pathogen	live 10^{10} CFU
hk.1.0E+9	Pre-administration, daily 2 wks before inoculation of the pathogen	heat-killed 10^9 CFU
hk.1.0E+10	Pre-administration, daily 2 wks before inoculation of the pathogen	heat-killed 10^{10} CFU

diarrhea using an animal rectal thermometer to confirm the changes.

Measurement of the intake of feed and consumption of water. Before the Salmonella oral administration, the amount of each rat's feed and water intake was confirmed. To compare the amount of the feed intake before and after the Salmonella-infected diarrhea, the feed amount was restricted to 200 g a day. Also the water amount was restricted to 500 ml a day, too.

Live Salmonella bacteria counts. To confirm the proliferation of the pathogenic bacteria, the feces of the experimental animals were aseptically collected at metabolic cage for 24 hr. One gram of the feces of each group of rats was homogenized in 9 ml of a saline solution and serial-diluted 10 times with PBS. After homogenization, fecal matter was serially diluted and plated on MacConkey agar (BD Biosciences, San Jose, CA, USA). Agar plates were incubated at 37°C for 24 hr and bacteria were counted as CFU/g of fecal matter. Morphology of Salmonella colony in pure culture and infected feces were similar (16).

Cytokine assay. Three hours after the oral administration of the pathogenic bacterium *S. typhimurium,* blood samples were collected from the orbits of the rats. The collected blood samples were left at room temperature for two hours, and then centrifuged (4°C, 1,500 × g, 15 min) to separate the serum. The samples were kept at −80°C until the cytokine analysis was conducted. The serum was thawed for the serum cytokine analysis, and the pro-inflammatory cytokine TNF-alpha and IL-1beta (R&D Systems, Minneapolis, MN, USA) were confirmed through ELISA. It was measured using an i-Mark instrument (Bio-Rad Laboratories, CA, USA).

Microscopic observation. For the preparation of the intestine samples for pathological examination, the intestine tissue was cut off, fixed in 10% formalin for 24 hr, and washed with water. The tissue was dehydrated in alcohol (for 1 hr each in 70, 80, 90, and 100%) and xylene (3 steps, 1 hr for each step), and was embedded into paraffin. The paraffin block was sliced at 7 μm thickness, stained with hematoxylin-eosin (H&E) (Sigma-Aldrich, St. Louis, MO, USA) and then stained again with gram-staining kit (BD Biosciences, San Jose, CA, USA) and observed with a microscope (×1000).

Statistical analysis. The data were processed with Graphpad Prism™ 5.0, and the statistical parameters, mean value, and standard deviation in a group were calculated and compared with those from other groups. The significance was determined via ANOVA ($p < 0.05$).

RESULTS

Comparison of the coaggregation and adhesion abilities of the probiotics. To confirm the coaggregation abilities of the Salmonella and LAB, the differences in the natural sedimentation of the LAB live bacteria, hk bacteria and Salmonella were investigated. The LAB live bacteria showed 43.5% ability, and the LAB hk bacteria showed 55% ability. As such, hk better than live bacteria have higher coaggregation with *Salmonella typhimurium* (Fig. 1).

For the adhesion test to confirm the residence ability, HT-29 cells with 40~50% confluence were prepared. After treat the *S. typhimurium*, simultaneously the live and hk LAB

Fig. 1. Effect of coaggregation with live and hk bacteria on *Salmonella typhimurium*. Coaggregation abilities of *Lactobacillus acidophilus* live or heat-killed strains with *Salmonella typhimurium* after 4 hr incubation at 37°C expressed as percentages. Values are the average ± SD from at least 3 experiments. $^{***}p < 0.001$.

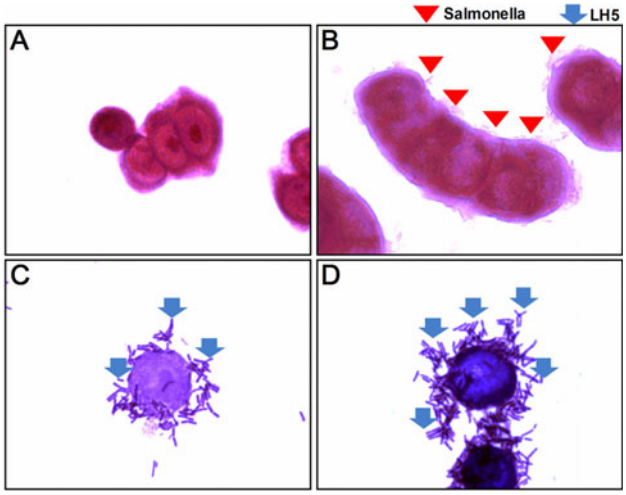

Fig. 2. Effect of adhesion assay with *Salmonella typhimurium*, live and hk bacteria on HT-29 cell lines. The cells were treated in the absence (A) or presence of *S. typhimurium* alone (B), live LAB (C), hk LAB (D) cultured for 1 hr.

were treated for 1 hr, the LAB that was residing in the intestinal epithelial cells was investigated. In much of the microscopic field, the hk probiotics showed better adhesion ability than the live probiotics (Fig. 2).

Comparison of the body temperature and the body weight. After the Salmonella infection, the changes in the body temperature were monitored for 0, 1, 3, 6, 9, 12 and 24 hr through a rectal investigation. In the negative control (NC) group to which Salmonella was not administered, the body temperature was consistently maintained, whereas in the *S. typhimurim* administration (SA) group to which Salmonella was administered, the mean body temperature of six rats decreased by −1.7°C after six hours. Nine hours later, the body temperature abruptly increased by 2.7°C due to an immune response. In comparison, the LAB group showed a decrease in body temperature from 0 hr to 6 hr, and the decrease was significantly less than that in the SA body temperature. Particularly, the 10^{10} of the hk bacteria treatment group showed the least difference in the body temperature decrease. Since then, no abrupt increase in the body temperature caused by immune responses was observed (Fig. 3A). In terms of the change in the body weight after the Salmonella infection, the NC group that did not receive Salmonella treatment showed an increased body weight, whereas the other groups that received Salmonella treatments showed a decreased body weight. Particularly, the SA group to which Salmonella was administered showed a 16.6 g reduced weight loss of 5.4% decrease in body weight, whereas the hk 10^9 group showed a 2.5 g reduced the least weight loss 1.9% decrease (Fig. 3B).

Measurement of the feed intake and the water consumption. In terms of the daily amount of the feed intake, the NC group showed 34.4 g of 24 hr intake, whereas the SA group showed a significantly different 10.3 g. In comparison, the live or hk LAB group reported a 24 hr intake of 24.8~27.9 g, which is closer to that of the NC group than of the SA group (Fig. 4A). In terms of the amount of purified water intake, the NC group reported 29.3 ml, whereas the

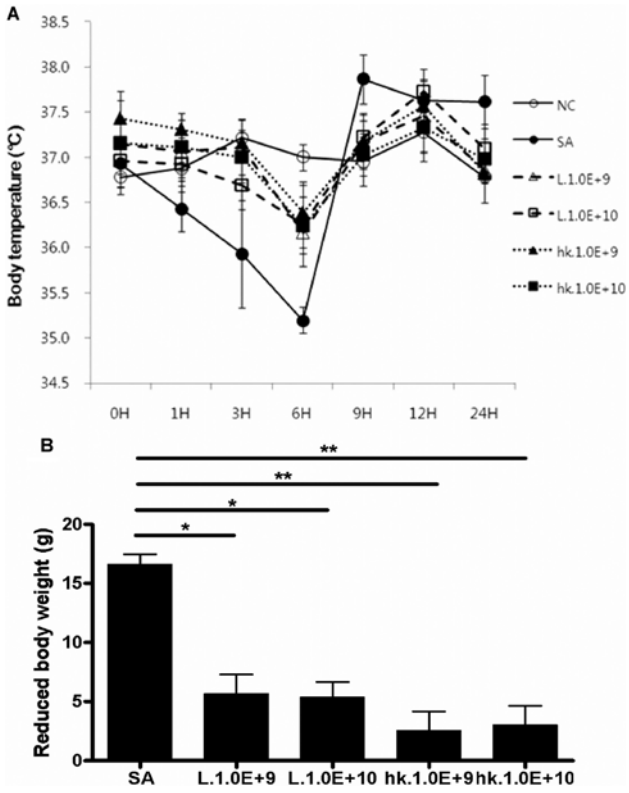

Fig. 3. Physiologic changes after *Salmonella typhimurium* infection. (A) Effect of infection with Salmonella on rectal temperature with live or hk Lactobacillus. (B) Changes Oral administration of live or hk probiotics measure a reduced body weight. The results are expressed as means ± SD (n = 9). $^{*}p < 0.05$, $^{**}p < 0.01$ vs. SA. SA, *Salmonella typhimurium* administration; L, live; hk, heat-killed.

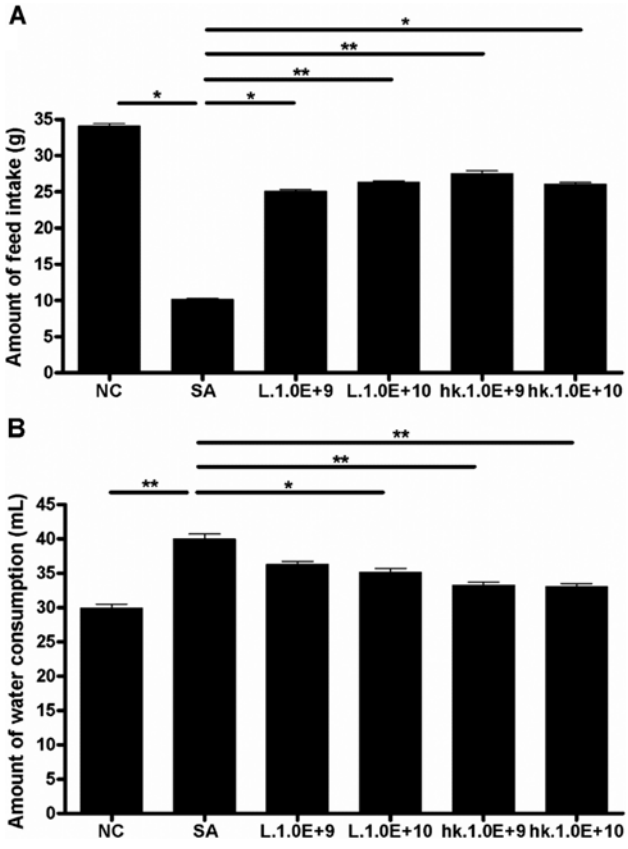

Fig. 4. Clincal sign of *Salmonella typhimurium* with live or hk probiotics. (A) After *S. typhimurium* infection, live or hk probiotics effects on feeding differences. (B) After *S. typhimurium* infection, live or hk probiotics effect on dringking differences. The results are expressed as means ± SD (n = 9). $^{*}p < 0.05$, $^{**}p < 0.01$ vs. SA. NC, negative control; SA, *Salmonella typhimurium* administration; L, live; hk, heat-killed.

SA group reported 39.3 ml, which means that the amount of water intake increased due to the Salmonella infection. The hk bacteria and live bacteria groups showed a 32.6~36.7 ml purified water intake, which was less than the SA group (Fig. 4B).

Salmonella cell count in the feces. To investigate the Salmonella changes in the feces of the experimental animals, the total number of fecal bacteria was confirmed using a plate. The total numbers of bacteria in 1 g of feces were: NC, 1.5×10^9; SA, 5.68×10^9; live (10^9), 4.95×10^9; live (10^{10}), 5.55×10^9; hk (10^9), 4.69×10^9; and hk (10^{10}), 4.13×10^9. These results confirmed that the Salmonella infection group showed a statistically significant increase in the total number of bacteria compared with the non-infection group. The measurement of the Salmonella live bacteria showed the following results in 1 gram of feces in each group: NC, 1×10^4; SA, 3.85×10^8; live (10^9), 9.25×10^7; live (10^{10}), 5.1×10^7; hk (10^9), 2.35×10^8; and hk (10^{10}), 2.73×10^7. When 5×10^{10} of *S. typhimurium* per rat were orally administered, the two live groups showed 24% and 13.2% discharge rates of Salmonella, respectively. In contrast, Salmonella were discharged by 61% and 70.8% in two hk LAB groups, respectively. These results suggest that the live probiotics act to inhibit the growth of *S. typhimurium* in the intestines and the coaggregation properties of hk probiotics serve to discharge the intestinal harmful bacteria, such as *S. typhimurium* (Fig. 5).

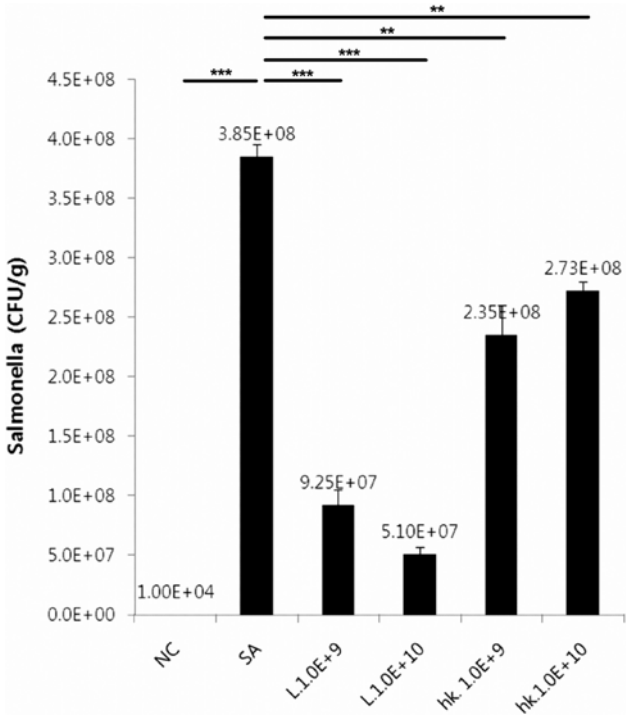

Fig. 5. Effect of live and hk probiotics on Salmonella infection fecal shedding of mice. Fecal shedding was examined in *S. typhimurium* infected animals. Data are shown as mean ± SD of mean log10 CFU per gram of feces (n = 9 mice/diet group), and data are representative of three independently conducted experiments. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. SA. NC, negative control; SA, *Salmonella typhimurium* administration; L, live; hk, heat-killed.

Pro-inflammatory cytokine in serum. In the serum analysis to confirm the effects of intestinal protection and diarrhea prevention after the infection, the levels of the pro-inflammatory cytokine TNF-alpha and IL-1 beta in all the LAB groups were lower than in the SA group. Considering that the expression level was lower in the live bacteria than in the hk bacteria, the LAB administration was confirmed to have reduced the development of intestinal mucosal inflammation caused by Salmonella. *S. typhimurium* by the coaggregation ability the removal of the as a pro-inflammatory cytokines that lowers the results showed (Fig. 6).

Bacterial detection in the colon. To confirm the presence of LAB and Salmonella in the intestinal tissues of the rats that were sacrificed after the experiments, micro-sectioning and gram staining were conducted. The presence of Salmonella was confirmed only in the SA group. In live probiotics group, the adhesion to the intestine was confirmed. Whereas, in hk Probiotics groups, the adhesion to the intestine was not confirmed as expected (Fig. 7).

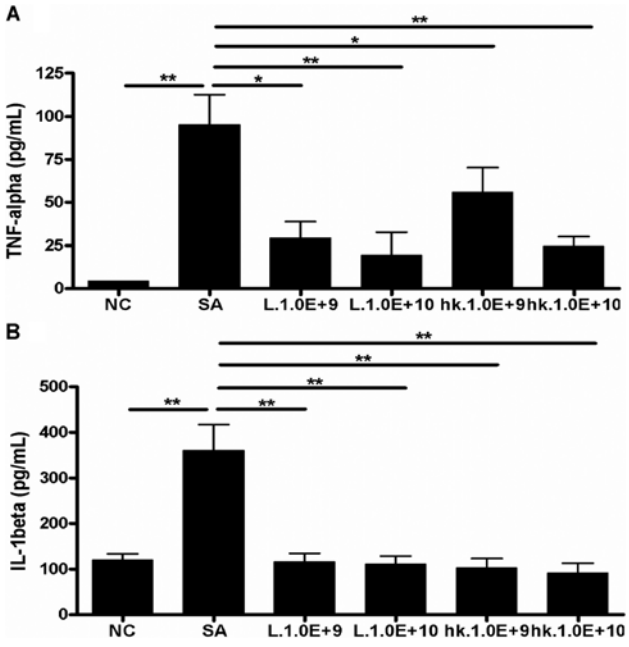

Fig. 6. Effects of live and hk LAB administrations on serum levels of Salmonella infection mice. (A) TNF-alpha (B) IL-1beta were measured by ELISA. The results are expressed as means ± SD (n = 9). $^{*}p < 0.05$, $^{**}p < 0.01$ vs. SA. NC, negative control; SA, *Salmonella typhimurium* administration; L, live; hk, heat-killed.

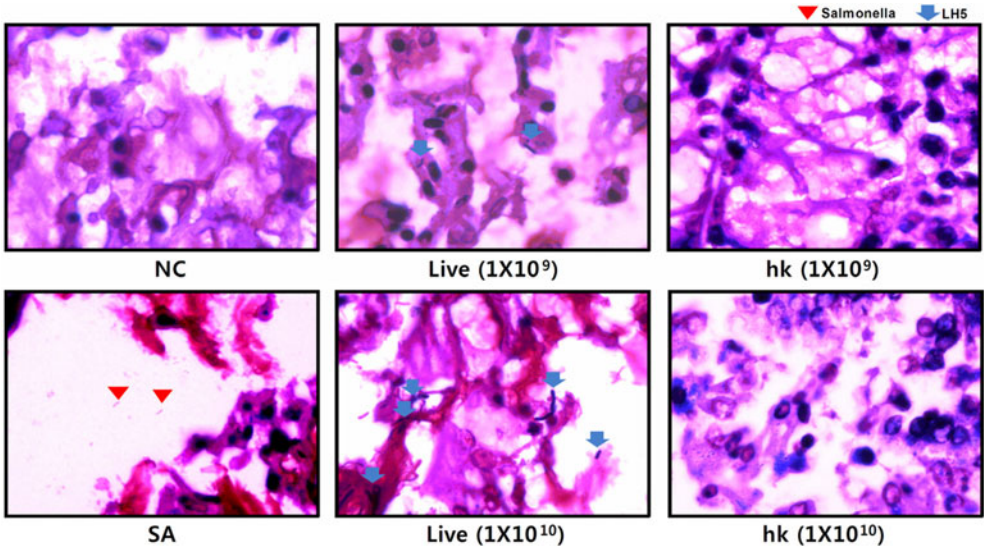

Fig. 7. Effects of repeated administration of live and hk LAB on intestinal tissues in mice. Histopathologic features of colon tissues by *S. typhimurium* infection mice. Magnification of hematoxilin & eosin (H&E) staining were ×1000. And then gram staining conducted. NC, negative control; SA, *Salmonella typhimurium* administration; L, live; hk, heat-killed.

DISCUSSION

The most serious problem in antibiotics treatment to Salmonellosis is the secondary damage caused by the dead Salmonella (17). Antibiotics-resistant Salmonella poses another problem (18). To overcome these problems, antibiotics should be used discreetly, and the secondary damage from the dead Salmonella should be overcome, in addition to the removal of the Salmonella. In this study, the performance of the LAB that met the aforesaid conditions in the inhibition and removal of Salmonella was confirmed for the prevention and treatment of Salmonella using LAB.

In the investigation of the feed intake amount, the amount in the SA group abruptly decreased. This result coincided with that in the previous study (19). In the case of the LAB live and hk bacteria groups, the decrease in the feed intake amount was mild, having remained almost at the same level as that of the normal group. According to the study of Wang *et al*. in 1993 (20), the amount of water intake after Salmonella administration increased due to the effects of the Salmonella endotoxins (20). In this study, each SA group rat took in 39.3 ml of water, whereas each Salmonella and hk (10^{10}) LAB group rat took in 32.6 ml, which confirm that the water intake amount after Salmonella infection normalized. This result indicates that the LAB hk bacteria removed the Salmonella and almost of by-products of Salmonella due to the coaggregation properties, so the increase in the amount of water intake was compromised.

The most notable result of this study was the difference in the number of Salmonella live bacteria in the feces of the live and hk LAB groups. In the previous studies, the growth of Salmonella under the acid stress conditions of lactic acid was inhibited (21). In this study, the fecal Salmonella live bacteria in the LAB live bacteria treatment group were more significantly inhibited than in the SA group. This could be related to the *L. acidophilus* that was used in this study produced lactic acid to change the intestinal pH and to inhibit the Salmonella proliferation. As a result of the observation of the change in the number of fecal Salmonella live bacteriain the LAB hk bacteria treatment group, a large amount of Salmonella survived in the feces. The Salmonella that stayed in the intestine were considered to have been excreted due to the hk LAB aggregation. As mentioned, the antibiotics resistance in Salmonella treatments and the Salmonella endotoxins are considered to have been overcome.

To investigate the inflammation level in the rats after the Salmonella administration, their serum TNF-alpha levels were compared. The TNF-alpha level of the LAB live and hk bacteria group more significantly decreased than that in the SA group. Especially, In the 10^{10} live and hk LAB group showed a decrease inthe level than the 10^{9} LAB group. According to the previous study on the clinical trial using *Lactobacillus GG*, LAB administration helped enhance the expression of the receptors that were involved in immunological enhancement (22). The LABs that were administered to the animal models were thought to have contributed to the immunological enhancement of the intestine and to the inhibition of the increase in the inflammatory cytokine TNF-alpha level in the case of the Salmonella infection. Therefore, live bacteria were considered more closely associated with the intestinal immune system than hk bacteria.

In summary, when the LAB live and hk bacteria were compared in terms of their prevention of Salmonella infec-

tion, the live bacteria group of the LAB 10^9 group was confirmed to have excelled in controlling the expression level of the serum TNF-alpha and IL-1beta that are known as a representative inflammatory cytokine; and in the LAB 10^{10} group, the levels of the live and hk bacteria were similar. This was due to the temporary increase in the immunological enhancement after the LAB live bacteria administration; and when more LAB was administered, a similar immunological enhancement occurred. In the previous study, the Salmonella endotoxins were reported to have induced thirst in the rats (20). Therefore, the Salmonella group that was anticipated to have had the most Salmonella endotoxins reported the highest amount of water intake, whereas the Salmonella and LAB group showed a decreased amount. Considering the significant decrease in the water intake particularly in the hk LAB group, the decreased thirst caused by LPS was more effective in the LAB hk bacteria group.

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