Oral Administration of Heat-Killed *Lactobacillus plantarum* Strain b240 Protected Mice against *Salmonella enterica* Serovar Typhimurium

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The purpose of this study was to investigate the effects of heat-killed *Lactobacillus plantarum* strain b240 (b240) on systemic infection by *Salmonella enterica* serovar Typhimurium (S. Typhimurium) and to determine the mechanism by which b240 protects against infection. Mice were administered either b240 or saline orally for 3 weeks, and then inoculated with S. Typhimurium. The mice treated with b240 were significantly protected against S. Typhimurium as compared to those fed saline. Moreover, translocation of S. Typhimurium into each organ tested in the mice that received b240 tended to be less than in the control mice. An important mechanism of protection against infection was demonstrated by the ability of b240 to inhibit both binding by and invasion of S. Typhimurium into cells. These results indicate that nonviable lactic acid bacteria also play important roles in preventing infection by enteric pathogens.

**Key words:** probiotics; heat-killed *Lactobacillus*; *Salmonella* infection; inhibition of bacterial translocation

Probiotics are defined as “a live microbial feed supplement or components of bacteria which beneficially affect the host animal by improving its intestinal microbial balance.”1,2) A majority of the favorable bacteria used as probiotics in humans and animals belong to *Lactobacillus* spp. and *Bifidobacterium* spp., which produce large amounts of lactic acid and are normally non-pathogenic and non-toxic in the host gastrointestinal tract. One of the beneficial effects of probiotic bacteria is protection of the host against infection by various enteric pathogens, including *Salmonella enterica* serovar Typhimurium, Shiga toxin-producing *Escherichia coli*, and *Helicobacter pylori*.3–7) The mechanisms by which probiotics defend against enteric pathogen infection include not only the direct killing of pathogens by lactic acid and/or bacteriocin, but also antagonistic interference with adhesion and the invasion of pathogens into intestinal cells.8) In addition, several studies have shown recently that probiotic bacteria induce up-regulation of secretory IgA production on mucosal surfaces to eliminate pathogens.9)

While most studies use viable probiotic bacteria, heat-killed *Lactobacillus plantarum* strain b240 was examined in the present study. We have found that Peyer’s patch (PP) cells stimulated with heat-killed b240 produce higher amounts of IgA than those stimulated with 150 other heat-killed lactic acid bacteria, and that IgA production in such PP cells is comparable to that in cells stimulated with viable b240.10) In addition, heat-killed lactic acid bacteria have an extended shelf life, are easier to store and transport, and exhibit less interaction with other components of food products.

S. Typhimurium is a facultative intracellular pathogen that causes systemic infection in susceptible mice, associated with multiple organ failure and death. S. Typhimurium invades the host intestinal epithelium via the type III secretion system encoded on *Salmonella* pathogenicity island 1 (SPI-1).11,12) The SPI-1 type III secretion system forms a needle-like complex that enables direct injection of bacterial effector proteins into the host cell cytosol.13) Effector proteins induce rearrangement of the cytoskeleton in the host cell, which leads to bacterial engulfment, thereby making it possible for bacteria to enter the host through gut epithelial cells.14)

The results of the present study clearly indicate that nonviable heat-killed lactic acid bacteria are useful in generating efficient protection against enteric pathogen infection.

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* Abbreviations: Ig, immunoglobulin; PP, Peyer’s patch; SPI-1, *Salmonella* pathogenicity island 1; MLN, mesenteric lymph nodes; BSA, bovine serum albumin; PBS, phosphate buffered saline
Materials and Methods

Bacteria and heat-killed b240 preparation. Salmonella enterica serovar Typhimurium strain y3306 (S. Typhimurium), which possesses a virulent plasmid and resists nalidixic acid, was kindly provided by Dr. Hidenori Matsui of the Kitasato Institute. Lactobacillus plantarum strain b240 (ONRIC b0240; b240), kindly provided by Dr. Sanae Okada of Tokyo University of Agriculture, was isolated from fermented tea leaves. It was cultured in MRS broth (Beckton Dickinson, Sparks, MD) under aerobic conditions for 24 h at 33 °C, 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 heat-killed b240 solution was freeze-dried and dissolved in saline before use.

Mice. C57BL/6 female mice (5–6 weeks of age) were purchased from Oriental Yeast (Tokyo) and housed under specific pathogen-free conditions following the animal protocol guidelines of the Committee on Animal Care of Tokyo Medical University (protocol no. S-14).

Survival rate assay. Mice were administered 0.1, 1, or 10 mg of b240 or saline orally every day for 3 weeks before bacterial infection. They were inoculated with 1 × 10^4 cfu of S. Typhimurium orally on day 0, and their survival was monitored until 20 d after inoculation. Day 0 was defined as the day of S. Typhimurium inoculation throughout the experiments in this study.

Number of S. Typhimurium exhibiting translocation and secretory IgA production in mice. Mice were orally administered 10 mg of b240 or saline daily for 3 weeks before bacterial inoculation, and were inoculated with 1 × 10^4 cfu of S. Typhimurium orally on day 0, and their survival was monitored until 20 d after inoculation. Day 0 was defined as the day of S. Typhimurium inoculation throughout the experiments in this study.

Inhibition of adhesion and invasion of S. Typhimurium into HeLa cells by b240. HeLa cells were cultured at 5 × 10^5 cells/well in 12-well plates in serum-free and antibiotic-free DMEM medium for 18 h at 37 °C. Serial dilutions of heat-killed b240 were then added to wells in triplicate, and the wells were incubated for 15 min at 37 °C. After incubation, HeLa cells were cultured with 1 × 10^5 cfu of S. Typhimurium for 3 h at 37 °C, and then the cells in each well were washed with PBS 5 times. To measure the adherent S. Typhimurium, the cells were lysed with 0.1% Triton X-100 in PBS, and the lysates were cultured on nutrient agar plates (Eiken Chemical). At the same time, the small intestine was excised from the host mice to measure secretory IgA production. Total secretory IgA and S. Typhimurium-specific secretory IgA were evaluated as previously reported.

Inhibition of adherence or invasion was calculated as follows: inhibition rate (%) = [number of adherent or invasive bacteria (average number of b240-nontreated group – b240-treated group)/average number of b240-nontreated group] × 100.

Statistical analysis. The statistical significance of the findings was calculated using the Mantel-Cox log-rank test (adjustment for multiple comparisons was not performed) in the survival-rate experiments. An unpaired t-test was used for the in vitro assays. Dose-dependent responses in the in vitro assays were evaluated by regression analysis and were judged according to the p values for both linearity and lack of fit (LOF). Since statistically significant dose-dependent responses were observed in this analysis (linearity p < 0.01), the lower-tailed Williams test was performed for in vitro assays. p values of <0.05 were considered to indicate a significant difference. All values are mean ± standard deviation. Statistical analysis was performed using SAS software R 9.1 (SAS Institute, Tokyo) and Stat View Ver. 5 (SAS Institute, Cary, NC).

Results

Oral administration of heat-killed b240 protected mice against infection with S. Typhimurium

We first examined to determine whether oral administration of heat-killed b240 would affect the survival rate following S. Typhimurium infection. To find the effective dose roughly, we administered 0.1, 1, or 10 mg of b240 or saline to the mice. As shown in Fig. 1, the mice given saline and infected with a dosage of 1 × 10^3 cfu of S. Typhimurium began to die at about day 7, and their survival rate was less than 10% by the end of the observation period. Mice fed 1 or 10 mg of b240 exhibited significantly elevated survival rates, of 58.3% and 50.0% respectively, by day 20. However, when the mice were infected with a high dosage (1 × 10^5 cfu) of S. Typhimurium, b240 provided no protection from death caused by S. Typhimurium (data not shown).

Systemic infection with S. Typhimurium was inhibited by oral administration of b240

Mice infected with S. Typhimurium died as a result of systemic bacterial infection and subsequent failure of multiple organs. The ability of b240 to inhibit bacterial translocation into the organs was therefore examined. The numbers of bacteria that had translocated into each organ tested were almost beneath the limit of detection on days 1 and 3 (data not shown). In addition, the number of bacteria in the feces was comparable to that in the b240 and saline groups on days 1 and 3 (log_{10} cfu/g: saline vs. b240: 2.5 ± 0.9 vs. 2.4 ± 0.9 on day 1, 2.6 ± 0.8 vs. 2.3 ± 0.6 on day 3). However, on day 6, the number of bacteria in each tested organ of the mice fed b240 was approximately 10- to 100-fold less than that for the saline group, although there were no statistically
significantly different, except in the number of bacteria in the feces in experiment 1 (Fig. 2). Similar results were obtained in two independent experiments.

Effects of oral administration of b240 on secretory IgA production

Since secretory IgA plays a role in protection against pathogenic infection on mucosal surfaces, the effects of oral administration of 10 mg of heat-killed b240 on secretory IgA production were investigated. Unexpectedly, the amounts of both total secretory IgA and S. Typhimurium-specific IgA on days 1, 3, and 6 after S. Typhimurium infection in the mice fed b240 were comparable to those in those fed saline, although the secretory IgA levels in both groups tended to increase after S. Typhimurium infection (data not shown).

Inhibition of adhesion and invasion of S. Typhimurium by b240

To explore the mechanisms of the observed inhibition of bacterial translocation by oral b240, we examined the effects of b240 on adhesion and invasion of S. Typhimurium into HeLa cells by in vitro assay. A good correlation between in vitro HeLa cells and the in vivo model of rabbit ileal loops is argued in an S. Typhimurium invasion. The number of adherent S. Typhimurium was significantly dose-dependently decreased and significantly decreased in the presence of 1 and of 10 mg/ml of b240 as compared with the absence of b240, respectively ($p < 0.01$, Fig. 3A). Correspondingly, as shown in Fig. 3B, the number of S. Typhimurium invading cells treated with b240 was also significantly decreased in the presence of 10 mg/ml of b240 as compared with the absence of b240 ($p < 0.01$), and this decrease was significantly dose-dependent. In the case of the cells treated with 10 mg/ml of b240, the inhibition rates of adhesion (Fig. 3C) and invasion (Fig. 3D) were 94.8 ± 3.6% and 90.2 ± 5.3% respectively. In addition, the antibacterial effect of b240 was estimated in vitro. When $1 \times 10^7$ cfu/ml of S. Typhimurium was incubated with 10 mg/ml of b240 or BSA for 3 h, b240 had no antibacterial effect against S. Typhimurium ($\log_{10}$ cfu/ml: b240 5.4 ± 0.1 vs. BSA 5.1 ± 0.2 after incubation for 3 h).

Discussion

Administration of probiotics is a beneficial means of maintaining and improving host health. Viable lactobacilli are used in most probiotic studies, while few studies using heat-killed probiotic bacteria have been reported. Our previous study demonstrated that heat-killed b240 stimulation of PP cells derived from BALB/c mice increased IgA production to a level comparable to that observed under viable b240 stimulation. Hence, we evaluated the role of heat-killed L. plantarum strain b240 in mice infected with S. Typhimurium.

Various mechanisms of inhibition by viable lactic acid bacteria of adhesion and invasion of pathogens have been demonstrated in various studies. First, lactic acid and bacteriocin-like compounds produced by probiotic bacteria kill enteric pathogens directly in the gastrointestinal tract. Secondly, probiotic bacteria enhance host intestinal immunity by increasing secretory IgA production to eliminate enteric pathogens. Thirdly, probiotic bacteria competitively inhibit binding to receptors used by pathogens on epithelial cells such as mannose and glycoproteins. The b240 we used was thoroughly washed with sterile saline to remove lactic acid and metabolites secreted by b240 before autoclaving. The heat-killed b240 itself had no direct lethal effect on S. Typhimurium in vitro. Secretory IgA is abundantly produced on mucosal surfaces, and contributes to host defense against both pathogenic adhesion and invasion.

Increased numbers of IgA+ cells in the lamina propria in BALB/c mice after oral administration of various lactobacilli, including L. plantarum have been reported. However, no enhancement of total or S. Typhimurium-specific secretory IgA production was found to be associated with oral administration of b240 in C57BL/6 mice for 3 weeks before bacterial inoculation. This finding can be ascribed to the different mouse strains used. When PP cells obtained from various strains of mice were stimulated with b240 in vitro, the cells derived from C57BL/6 mice were more resistant to the induction of secretory IgA than those derived from other mouse strains, such as BALB/c mice, CBA/J mice, and dDY mice, although the reasons for this remain unclear (unpublished results). This might account for the

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Fig. 2. Translocation of S. Typhimurium Was Inhibited by Oral Administration of b240.
Mice were treated orally with 10 mg of b240 or saline daily for 3 weeks and then inoculated with $1 \times 10^4$ cfu of S. Typhimurium. Organs were removed 6 d after bacterial inoculation. [ ] saline ($n = 10$); [ ] b240 ($n = 10$) in experiment 1 and [ ] saline ($n = 9$); [ ] b240 ($n = 9$) in experiment 2. Similar results were obtained in two independent experiments. * $p < 0.05$ compared with the group fed saline, by unpaired t-test.
discrepancy in findings between the present and previous studies. Several studies have reported that probiotic bacteria can adhere to intestinal epithelial cells and can competitively inhibit adhesion and invasion by enteric pathogens. Moreover, heat-killed probiotic bacteria retain the ability to bind to cultured Int-407 and Caco-2 cells as well as viable probiotic bacteria. The capacity of \textit{L. plantarum} to adhere to mouse intestinal epithelial cells is strongest among lactic acid bacteria isolated from fermented vegetables, and \textit{L. plantarum} shows a more potent antagonistic effect against invasion by \textit{S. Typhimurium} than other lactic acid bacteria. However, it remains unclear whether the potent antagonistic effect of \textit{L. plantarum} strain b240 observed in the present study is superior to that of other strains of \textit{L. plantarum} in vivo or in vitro, and whether b240 has a larger number of antagonistic components against adhesion, because b240 was selected as the strain with the highest IgA induction ability among 150 lactic acid bacteria studied in an in vitro screening test. To elucidate the precise mechanism underlying the protective effect of b240, it is important to compare the antagonistic effect of b240 with that of other lactic acid bacteria, including \textit{L. plantarum}.

Effector protein translocation from bacteria into cells via the type III secretion system have been found to be responsible for the process of \textit{S. Typhimurium} translocation by inducing physiological changes in host cells. Recently, Wu et al. found that probiotic bacteria decreased the expression of EspB protein in \textit{Citrobacter rodentium}, one of the component proteins of the type III secretion system, resulting in a reduction in Tir protein secretion and translocation, but it is not known whether heat-killed b240 affects the expression of the effector and/or component proteins of the \textit{S. Typhimurium} type III secretion system. To extend the finding that heat-killed b240 reduced the translocation of \textit{S. Typhimurium} into the organs, it is task to investigate the effects on the barrier function of mucosal tissues. Pathological bacterial translocation as a marker of impaired barrier function can also be effectively reduced by probiotic therapy.

In conclusion, oral administration of heat-killed b240 was found to reduce \textit{S. Typhimurium} infection effectively through inhibition of \textit{S. Typhimurium} adhesion and invasion of intestinal epithelial cells, which normally leads to systemic infection associated with multiple organ failure. Heat-killed b240 thus appears to play a valuable role in preventing the lethality of \textit{S. Typhimurium} infection in mice. Investigation of interaction with the \textit{S. Typhimurium} type III secretion system and purification of active components of b240 are interesting subjects for future research.

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