Prevention of Pathogenic *Escherichia coli* Infection in Mice and Stimulation of Macrophage Activation in Rats by an Oral Administration of Probiotic *Lactobacillus casei* I-5

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Lactic acid bacteria (LAB) belong to a group of bacteria which have a variety of health benefits. The administration of LAB contained in fermented foods, especially dairy products, has been found to exhibit a range of physiological and therapeutic effects on the consumer, including enhancement of non-specific and specific immune responses, suppression of intestinal infection, alleviation of food allergies, and anticarcinogenic activity.1–3 These are called probiotics4 and are generally regarded as safe (GRAS-status) since they hardly cause any infection in the host.5 Therefore, probiotic LAB have been commercially developed as ingredients of functional foods.

Probiotic LAB have recently been expected to become a useful tool that could be used as a preventive substance instead of antibiotics. Infections by enteropathogenic bacteria frequently cause serious gastrointestinal diseases. The current means to control such infections rely heavily on the use of antibiotics. However, heavy use of antibiotics has become a major problem, since it results in drug-resistant bacteria.6 Thus, alternative and non-pharmaceutical strategies for controlling enteropathogenic bacterial infections have been sought. Some probiotic LAB have been known to show protective effects against pathogens. Infection by oral challenge with such enteropathogens as *Salmonella typhimurium*, *Escherichia coli*, *Shigella sonnei*, and *Listeria monocytogenes* has been efficiently suppressed by probiotic LAB feeding to rodents.7–11 Rotavirus-vaccinated mouse dams fed with probiotic LAB could increase virus-specific IgA levels, and this conferred an increased degree of passive protection to nursing pups against rotavirus diarrhea.12 Feed containing probiotic LAB could suppress the introduction of *Salmonella* in broilers due to their resistance to infection.13 Pre-feeding piglets with probiotic LAB, prior to weaning under conditions of being exposed to environmental pathogens (mainly non-specific rotavirus and *E. coli*) could effectively reduce the cumulative morbidity index of diarrhea, and the piglets consequently exhibited a higher feed conversion efficiency than non-probiotic-fed controls.14 It was thought that probiotic LAB would become a potent alternative to antibiotics.

However, the protective and immune-enhancing effects of probiotic LAB are known not as genus- or species-specific, but as strain-specific, the effects depending on the LAB strains.1,3 Accordingly, probiotic LAB strains have become very important in the fields of

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Key words: alcohol fermentation by-product; *Lactobacillus casei*; pathogenic *Escherichia coli*; macrophages; NF-κB

*Lactobacillus casei* I-5 isolated from an alcohol fermentation broth enhanced immunity and prevented pathogenic infection as a probiotic. Mice fed with I-5 cells for 11 days prior to an intraperitoneal challenge with pathogenic *Escherichia coli* Juhl exhibited a high survival rate compared with the control group. Rats fed with I-5 cells for 10 days significantly increased the phagocytosis of peritoneal macrophages. In a cell culture system employing peritoneal macrophages from rats, the I-5 administration activated NF-κB stimulated by LPS. It also enhanced LPS-stimulated IL-12 and TNF-α production, but not IL-6 production. These results show that *L. casei* I-5 effectively prevented infection by pathogenic *E. coli* possibly through the activation of peritoneal macrophages. The strain would be useful to prevent pathogenic microbial infections in humans and farm animals.
nutrition, health, and food for research and commercial development. Probiotic LAB have mostly been found from animal sources, dairy products or human and animal intestines. While plant materials, fruit juices and vegetables are fermented with LAB, probiotic LAB found from plant sources have yet to be studied.

Contamination of LAB has also occurred in the fermentation process for producing rectified alcohol for industrial use from cane molasses. However, some studies have been limited to preventing decreased alcohol productivity by using microbiological control.\(^{15-17}\) In order to obtain probiotic LAB from occasional contaminants in our alcohol fermentation process, we have isolated bacteria from the fermentation broth and characterized them as Lactobacilli (Ishida-Fujii et al., unpublished results). One of the LAB strains, L. casei I-5, exhibited a protective effect against a pathogenic E. coli strain in mice.

The aim of this study was to examine the protective effect of administering L. casei I-5 cells to mice that had been challenged by pathogenic E. coli Juhl. The effects of LAB on the cellular and humoral immune responses in mice and rats were also investigated.

**Materials and Methods**

*Microorganisms.* L. casei I-5 was isolated from an alcohol fermentation broth which had been generated from sugarcane and orange molasses. The strain had been stored at the laboratory of R & D Center of Japan Alcohol Corporation (J.alco, Chiba, Japan). Pathogenic E. coli Juhl used in the mice experiments had been stored at Mercian Bioresource Laboratories (Shizuoka, Japan). I-5 was aerobically incubated in a 2-fold-diluted concentrated broth (pH 6) at 37°C for 3 days. The concentrated broth was a distillation residue concentrated by about 2.5 fold from the alcohol production process at the J.alco plant in Izumi (Kagoshima, Japan) that uses sugarcane and orange molasses. The I-5 cells were centrifuged at 6,760 × g for 10 min at 4°C, and suspended with the supernatant in a 20-fold cell concentration, the cells then being administered to the experimental animals. E. coli Juhl was incubated on an SCD agar medium (Nissui Pharmaceutical, Tokyo, Japan) at 31°C for 20 h.

*Animal experiments.* All procedures using animals were in accordance with the “Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Science,” and were approved by the Animal Use and Care Committee at Mercian Cleantec Corporation. Male ICR mice (SPF, 3.5 weeks of age) were obtained from Nihon SLC (Shizuoka, Japan) and housed for 4 days. The mice were kept under SPF conditions (room temperature, 24 ± 3°C; relative humidity, 55 ± 15%; 12 h light/12 h dark illumination cycle) and fed on a standard germ-free feed (CMF, Oriental Yeast, Tokyo, Japan) with free access to water at all times. They were randomly allocated to four groups (n = 10), two groups being orally administered with I-5 (7–9 × 10^4 cells/d) for 11 days. All the control groups in these experiments were administered with the same volume of distilled water. All mice (5.5 weeks of age) were challenged intraperitoneally with pathogenic E. coli Juhl (3 × 10^7 or 1 × 10^6 cells/mouse). The challenged mice were kept for 7 days on the CMF diet, and their health was monitored twice a day throughout the experimental period. Male SD rats (SPF, 6 weeks of age) were obtained from Nihon SLC and housed for 4 days. The rats were kept under SPF conditions (room temperature, 24 ± 3°C; relative humidity, 55 ± 15%; 12 h light/12 h dark illumination cycle), and were fed with a standard feed (MF, Oriental Yeast) with free access to water at all times. The rats were randomly allocated to two groups (n = 8), one group being orally administered with I-5 (5 × 10^8–1 × 10^11 cells/rat) and the other being the control group.

*Measurements of cytokines and immunoglobulins in mouse blood plasma.* Another group of mice (n = 10), from which blood plasma was taken, was fed with I-5 (6 × 10^5 cells/mouse) or distilled water for 7 days prior to the E. coli challenge (3 × 10^7 cells/mouse). The experimental conditions for these mice were the same as those already described. At the end of the experiment (1 week post-challenge), the mice were subjected to ether anesthesia. Blood samples (1 ml) were taken through cardiac puncture with sodium heparin. The blood was centrifuged at 4,000 rpm for 10 min at 4°C, and then the supernatant (blood plasma) was stored at −80°C for measurements of antibodies and cytokines. The total immunoglobulin A (IgA) antibody was measured by using a mouse ELISA kit (Bethyl Labo., TX, USA), and gamma-interferon (IFN-γ) and interleukin-6 (IL-6) were also measured by using mouse ELISA kits (Endogen, IL, USA).

*Natural killer cell cytotoxicity assay.* At the end of the I-5 administration, blood samples (6 ml) were taken from the rats (n = 8) through cardiac puncture with sodium heparin. Peripheral blood mononuclear cells were separated from the heparinized blood using Ficoll-Conray (Isolymph, Gallard-Schlesinger Industries, NY, USA). The isolated cells were washed twice with phosphate-buffered saline (PBS), counted and adjusted to 5 × 10^6 cells/ml. The non-adherent mononuclear cells were used as effector cells (E) for an NK cell cytotoxicity assay. The cytotoxicity of peripheral blood NK cells was quantified by using a 51Cr-release assay according to the procedure described previously.\(^{18}\) A K562 tissue culture cell line (human chronic myelocytic leukemia, Dainippon Sumitomo Pharma, Osaka, Japan) was used to provide the target cells (T) which were labeled with 100 µCi of 51Cr at 37°C for 1 h. The labeled target cells were washed three times with PBS and adjusted to 1 × 10^5 cells/ml, after which they were
cultured with the effector cells (E/T = 50:1) at 37 °C for 4 h. A 100-μl amount of the supernatant was used for isotope counting. The maximum 51Cr release was determined by using 1 mol/l of HCl instead of the effector cells. The cytotoxicity percentage was calculated as follows:

$$\text{[(experimental } 51\text{Cr release} - \text{spontaneous } 51\text{Cr release})/(\text{maximum } 51\text{Cr release} - \text{spontaneous } 51\text{Cr release})] \times 100.$$  

Measurements of the 51Cr release were carried out in triplicate.

**Macrophage phagocytosis assay.** Peritoneal cells were collected from rats (n = 8) after removing blood samples. Ice-cold PBS (20 ml) was intraperitoneally injected into the rats, and the liquid containing the peritoneal cells was taken out through the abdominal cavity, centrifuged (120 × g, at 4 °C for 10 min) and suspended in an ice-cold RPMI 1640 medium (Dainippon Sumitomo Pharma) containing 10% fetal calf serum (FCS, Invitrogen, CA, USA). The cells were adjusted to 1 × 10⁶ cells/ml, 200 μl of the cell suspension was plated on a Lab-Tek chamber slide (8-well, Nunc, Roskilde, Denmark). After 1 h of incubation at 37 °C in a CO₂ incubator, the non-adherent cells were removed by rinsing them with the pre-warmed RPMI 1640 medium. The adhered cells in the medium were used as macrophages. The RPMI 1640 medium containing 0.02% latex beads (1.1 μm, Sigma-Aldrich, St. Louis, MO, USA) was added to each chamber, and the slides were incubated at 37 °C for 30 min in the CO₂ incubator. The cells adhering to the slides were washed twice with PBS and fixed in methanol. After being stained with Giemsa, 100 cells of macrophages per chamber were observed under a microscope. Three chambers were analyzed per rat. The phagocytic index (%) was calculated as the average percentage of the number of macrophages containing one or more latex beads.

**Measurements of the NF-xB activity and cytokines by a cell culture system using rat peritoneal macrophages.** Peritoneal cells were collected from the rats (n = 8) as already described. The cells were adjusted to 2 × 10⁶ cell/ml with an ice-cold RPMI 1640 medium containing 10% FCS, and 1000 μl of the cell suspension was plated in 24-well plates (Sumitomo Bakelite, Tokyo, Japan). The plates were incubated in a CO₂ incubator at 37 °C for 1 h, and the adhering cells were identified as macrophages. One hundred μl of lipopolysaccharide (LPS, 10 μg/ml, E. coli 055:B5, Sigma-Aldrich) or PBS was mixed with 900 μl of the RPMI 1640 medium containing 10% FCS, and the mixture added to each well. The plates were cultured in the CO₂ incubator for 19 h, and then centrifuged (600 × g, 10 min). The macrophage cells were used to prepare nuclear proteins, and the supernatant was used to determine the level of cytokines. Tumor necrosis factor-alpha (TNF-α), IL-6, and interleukin-12 (IL-12) in the supernatant were measured with rat ELISA kits (Endogen), and nuclear proteins were extracted with a nuclear extraction kit (Chemicon International, Temecula, CA, USA), before being stored at −80 °C. The amount of nuclear-localized nuclear factor-kappa B (NF-xB) was determined with a NF-xB p50/p65 transcription factor assay kit (Chemicon International), employing an electromophoretic mobility shift assay (EMSA) coupled with ELISA. The amounts of NF-xB p50/p65 were calculated as relative indices (%) of the absorbance of a sample compared with that of the control group.

**Measurement of total IgA in rat feces.** At the end of the I-5 administration, three-hour fecal samples were taken from the rats (n = 8). Fecal extracts were prepared as previously described. In brief, 0.1 g of the fecal sample was suspended in 0.9 ml of PBS containing 0.1 mg/ml of a trypsin inhibitor, 0.05 mol/l of EDTA, and 0.001 mol/l of phenylmethylsulfonyl fluoride, before being crushed after keeping it at 4 °C for 1 h. The suspension was vigorously mixed, and the supernatant was collected as the fecal extract after centrifugation (600 × g, 10 min, 4 °C), before being stored at −80 °C. Total IgA was measured by using a rat ELISA kit (Bethyl Labo.).

**Statistical analysis.** The results of the experiments were each expressed as the mean ± SE. The statistical significance of the difference was determined by Student’s t-test (Stat View, Abacus, USA). P values lower than 0.05 were considered to be statistically significant.

**Results**

**Effect of I-5 administration on pathogenic E. coli Juhl in mice**

The protective effect of LAB strain I-5 against pathogenic E. coli infection in mice was examined. ICR mice were fed with a standard germ-free diet (CMF), and strain I-5 (7–9 × 10⁹ cells/mouse) was orally administered for 11 successive days prior to a challenge with pathogenic E. coli Juhl (3 × 10⁹ or 1 × 10⁹ cells/mouse). E. coli Juhl was intraperitoneally injected into mice that had been administered with I-5 and their survival was monitored. The intraperitoneal infection by E. coli Juhl was acute, and 92% of the mice died within 2 days of the challenge. At an infection level of 1 × 10⁹ cells/mouse, the survival rate of the mice administered with I-5 was markedly higher than that of the control group 7 days after the challenge (Fig. 1). I-5 was very effective for preventing the E. coli Juhl infection in mice.

**Effect of I-5 administration on the cytokines and immunoglobulins in mouse blood**

To examine the effect of I-5 on pathogenic E. coli infection which could have been due to immune
modulation, we determined the levels of cytokines and antibodies in the plasma of the mice that had been challenged with pathogenic *E. coli* Juhl. ICR mice were fed with or without I-5, and then challenged by a low dose (3 × 10⁷ cells/mouse) of pathogenic *E. coli* Juhl. After 7 days, the levels of the key immune molecules, total IgA, IFN-γ and IL-6, were determined in the peripheral blood plasma of the mice. The mice (n = 6) fed with I-5 seemed to show a slightly higher IFN-γ production (762.8 ± 374.7 pg/ml) than the control group (434.3 ± 136.5 pg/ml), and a slightly lower IL-6 production (15.5 ± 5.7 pg/ml) than the control group (23.6 ± 11.3 pg/ml). In contrast, there was no difference in the total IgA production between the I-5-fed group (1320.1 ± 27.9 ng/ml, n = 6) and the control group (1304.8 ± 25.2 ng/ml, n = 6). These results suggest that the administration of I-5 did not significantly enhance the humoral immunity.

**Effect of I-5 administration on the phagocytosis and NK cell cytotoxicity in rats**

The effect of I-5 on the activation of macrophages and NK cells was examined, since these cells play critical roles in the natural immune system. Rats were used since they contain enough of these cells to use in examining the immune function, although the roles of these cells in the immune systems of the mouse and rat are basically the same. The phagocytic activity of peritoneal macrophages was determined by measuring the phagocytosis of latex beads in SD rats fed with or without I-5. The mean (n = 8) phagocytic index of the I-5-administered group was significantly (P < 0.001) higher than that of the control group (Fig. 2). There was no significant difference in the NK cell cytotoxicity of peripheral blood mononuclear cells between the I-5-fed group (3.2 ± 0.5%, n = 8) and the control group (3.3 ± 0.8%, n = 8). These results indicate that the administration of I-5 enhanced the activity of the peritoneal macrophages in rats, but not the NK cell cytotoxicity.

**Effect of I-5 administration on the LPS-stimulated NF-κB activation in rat peritoneal macrophages**

The nuclear translocation of NF-κB in rat peritoneal macrophages was examined, because NF-κB is a key molecule that transduces signals from toll-like receptors (TLRs). The amount of nuclear translocation of NF-κB p50/p65 in the peritoneal macrophages of rats (n = 8) fed with I-5 and incubated with or without LPS was
measured. The relative amount (n = 8) of the LPS-stimulated nuclear proteins of NF-κB p50/p65 was significantly higher (P < 0.001) in the I-5-fed group than in the control group (Fig. 3). This result indicates the enhanced activity of NF-κB in the peritoneal macrophages of rats administered with I-5.

**Effect of I-5 administration on the LPS-stimulated cytokine production in rat peritoneal macrophages**

To determine the effect of I-5 administration on the immune modulation through the activation of macrophages, the cytokine production in rat peritoneal macrophages was measured. Macrophages collected from SD rats (n = 8) that had been fed with I-5 were incubated with or without LPS. After this incubation, cytokines were determined by using the cell supernatant of the macrophages. Without LPS-stimulation, IL-12 production was significantly higher (P < 0.05) in the I-5-fed group than that of the control group (Fig. 4). There was no difference in TNF-α and IL-6 production between the two groups. On the other hand, with LPS stimulation, IL-12 and TNF-α production in the I-5-fed group was significantly higher (P < 0.001 and 0.01 respectively) than in the control group, whereas the production of IL-6 remained similar (Fig. 4). These results clearly indicate that I-5 enhanced the production of Th1-type cytokines, but not of Th2-type cytokines.

**Effect of I-5 administration on total IgA in rat feces**

The total IgA production in feces collected from SD rats (n = 8) fed with I-5 was no different from that from the control (Fig. 5). This result suggests that I-5 had no effect on the total IgA production in rat feces.

**Discussion**

We found in the present study that the administration of *L. casei* I-5 isolated from an alcohol fermentation broth was very effective for preventing a pathogenic *E. coli* Juhl infection in mice. Since the effect of I-5 indicated a practical probiotic use, we further investigated the molecular mechanism for the enhanced immunity. We examined the key immune molecules in the process of the protective effect of I-5. I-5 feeding slightly increased the IFN-γ production and decreased IL-6 production in the plasma of mice infected by pathogenic *E. coli* Juhl, whereas it had no significant effect on the total IgA production. These results suggested that the administration of I-5 might not enhance humoral immunity.
The immune responses of rats were then examined to obtain further insight into the process of the protective effect of I-5. First, the involvement of macrophages and NK cells in this phenomenon was investigated, since these cells play critical roles in a primary natural immune system against microbial infection. The administration of I-5 significantly enhanced the phagocytic activity of peritoneal macrophages, but did not enhance the cytotoxicity of NK cells in rats. The activation of peritoneal macrophages seemed to contribute to the protective effect of I-5 administration against pathogenic E. coli.

We next investigated the activation of NF-κB and the production of cytokines in LPS-stimulated peritoneal macrophages from rats fed with I-5 to confirm whether the adaptive immunity was stimulated through the activation of peritoneal macrophages. The administration of I-5 with LPS significantly activated NF-κB p50/p65 in macrophages. NF-κB is a key transcriptional factor on the TLR signaling pathway, and triggers the gene expression of inflammatory cytokines which subsequently induce an adaptive immune response.20,21) Furthermore, I-5 feeding significantly increased the LPS-stimulated Th1-cytokine production (IL-12 and TNF-α), but did not affect the Th2-cytokine production (IL-6). I-5 also enhanced IL-12 production without LPS, indicating I-5 to be a strong probiotic in enhancing the production of Th1-cytokines. The I-5 administration with LPS increased the production of Th1-cytokines which would lead to Th1 cell differentiation. These results indicate that I-5 would stimulate adaptive immunity by activating NF-κB and Th1-cytokines with LPS.

Adhesion to the intestinal mucosal cells is the first step in immune modulation by probiotic bacteria. In fact, Perdigon et al.22) have shown that the cells of L. casei adhered to intestinal mucosal cells and were transferred via M and follicle-associated epithelial cells (FAE) in Peyer’s patches of the small intestine, such that the internal LAB cells should contact the immune cells in Peyer’s patches. Perdigon et al.23) have also demonstrated that the ability for the adhesion of viable L. casei cells was stronger than that for dead ones because of countering the peristaltic action of the intestines and subsequent long-term survival in the intestinal tract. These results suggested that the administered I-5 cells might have existed live for a long time and been in contact with macrophages after internalization via M and FAE cells in the small intestine. These reports could explain the protective effect of viable I-5 cells being stronger than that of dead cells killed by heat as revealed in our experiments (data not shown). Furthermore, the immune stimulation of I-5 with LPS might have been via TLR of macrophages, because NF-κB-transducing signals from TLR were activated during I-5 administration.

I-5 feeding showed no effect on the total IgA production in rat feces. Furthermore, we found that the administration of I-5 had no effect on the total- and specific-IgM and IgG production in the serum of piglets immunized with 4-hydroxyl-3-nitrophenylacetyl-conjugated keyhole limpet hemocyanin (NP-KLH) instead of a vaccine (data not shown). These results suggested that I-5 had no effect on humoral immunity.

We thus propose a possible model for the protective effect of I-5 in Fig. 6. The administration of I-5 would exhibit a protective effect against pathogenic infection...
by enhancing both the natural and adaptive immunity. The natural immunity consisted of peritoneal macrophage phagocytosis, and the adaptive immunity involved the cellular immunity of Th1 cell differentiation via the activation of NF-κB and the production of Th1-cytokines in macrophages. The administration of both I-5 and LPS activated peritoneal macrophages and in turn activated NF-κB, TNF-α and IL-12, leading to Th1 cell differentiation. Finally, the Th1 cells attacked pathogenic E. coli, thus initiating the protective effect of I-5. The function of I-5 seems to have had no influence on the health of the hosts, because none was induced when administered alone. Therefore, I-5 is considered to be a reasonable probiotic.

It can be expected that I-5 will have the protective effect against microbial infection of both humans and farm animals, as well as laboratory animals. In fact, we tested the protective effect on chickens against oral infection with Salmonella enteritidis, and found a positive result (data not shown). As already mentioned, I-5 did not inhibit any humoral immune response, and thus did not decrease the effects of vaccines. This result suggests that the administration of I-5 may have highly protective effect against microbial infection and work as a complement to vaccines. Neonatal diarrhea caused by E. coli is a serious problem for infants in developing countries and for the breeding of farm animals, especially calves and piglets. I-5 may be practically useful to prevent diarrhea in humans and farm animals.

Probiotic LAB originating from a plant source of an alcohol-fermented broth of sugar cane and orange molasses has not previously been reported. Contaminants in alcohol-fermented broth are known to have alcohol resistance. Similarly, I-5 grew well in the medium containing a high concentration of ethanol or at a high temperature (Ishida-Fujii et al., unpublished results). In addition, L. casei exhibiting high stress resistance modified the structural properties of its cell wall. The cell-wall components of LAB have been reported to stimulate the immune system through interaction with the leucocyte pattern-recognition receptors, TLRs. These observations suggested that I-5 might have different immunogenicity caused by the cell-wall structure from that of LAB found from animal sources. The contaminants in an alcohol-fermented broth may be interesting as a source of probiotic LAB with various immune modulating functions.

The oral administration of probiotic L. casei I-5 prevented pathogenic E. coli infection in mice and stimulated macrophage activation in rats. We believe that this work will benefit humans, farmers, drug-producing companies, and subject-related researchers.

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