

Probiotic *Lactobacillus casei* strain Shirota prevents indomethacin-induced small intestinal injury-Involvement of lactic acid

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Running head: Prevention of enteropathy by probiotic

Abbreviations used in this study: NSAID, non-steroidal anti-inflammatory drug; PPI, proton pump inhibitors; COX, cyclooxygenase; TLR, Toll-like receptor; LPS, lipopolysaccharide; NF- κ B, nuclear factor- κ B; LcS, *Lactobacillus casei* strain Shirota; RT-PCR, reverse transcription-polymerase chain reaction; iNOS, inducible nitric oxide synthase; I- κ B, inhibitory- κ B.

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ABSTRACT

Inflammatory responses triggered by activation of the lipopolysaccharide (LPS)/Toll-like receptor (TLR) 4 signaling pathway are a key mechanism in non-steroidal anti-inflammatory drug-induced enteropathy. The aim of this study was to investigate the probiotic effect of *Lactobacillus casei* strain Shirota (LcS) on indomethacin-induced small intestinal injury. Rats pretreated with viable LcS or heat-killed LcS once or once daily for a week were administered indomethacin by gavage to induce injury. Anti-inflammatory effects of L-lactic acid (1-15 mM) were evaluated *in vitro* using THP-1 cells. One-week treatment with viable LcS prevented indomethacin-induced intestinal injury with increase in the concentration of lactic acid in small intestinal content, and inhibited increases in myeloperoxidase activity and expression of mRNA for tumor necrosis factor- α (TNF- α) while affecting neither TLR4 expression nor the number of Gram-negative bacteria in intestinal content, while neither heat-killed LcS nor a single dose of viable LcS inhibited intestinal injury. Prevention of this injury was also observed in rats given L-lactic acid in drinking water. Both L-lactic acid and LcS culture supernatant containing 10 mM lactic acid inhibited NF- κ B activation and increases in TNF- α mRNA expression and TNF- α protein secretion in THP-1 cells treated with LPS. Western blot analyses showed that both L-lactic acid

and LcS culture supernatants suppressed phosphorylation and degradation of I- κ B- α induced by LPS without affecting expression of TLR4. These findings suggest that LcS exhibits a prophylactic effect on indomethacin-induced enteropathy by suppressing the LPS/TLR4 signaling pathway, and that this probiotic effect of LcS may be mediated by L-lactic acid.

Keywords: probiotics, enteropathy, lactic acid, Toll-like receptor, signal transduction.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are used worldwide by millions of individuals for the treatment of musculoskeletal pain. Since serious upper gastrointestinal events such as gastroduodenal ulceration, perforation, and bleeding often limit the use of these drugs, strategies for prevention of such complications have been developed. Co-administration of gastroprotective drugs such as proton pump inhibitors (PPIs) and use of selective cyclooxygenase-2 (COX-2) inhibitors can reduce these gastrointestinal toxicities of NSAIDs.(14, 28, 33) However recent clinical studies using video capsule endoscopy have demonstrated that NSAIDs frequently injure the small bowel as well as the upper gastrointestinal tract, and that PPIs have no inhibitory effect on NSAID-induced enteropathy.(8, 16, 32) Although selective COX-2 inhibitors cause less small bowel injury than traditional NSAIDs(8, 9), it has been reported that the beneficial effects of selective COX-2 inhibitors on the small bowel may be lost with long-term use of them.(15) Novel means of treatment of NSAID-induced enteropathy are thus urgently needed.

Toll-like receptor 4 (TLR4), a receptor of lipopolysaccharide (LPS) of Gram-negative bacteria,(22) plays a key role in the pathogenesis of a variety of tissue injuries via stimulation of multiple signaling events including the nuclear factor- κ B (NF- κ B) and mitogen-activated protein kinases pathways.(12) LPS recognition and

signaling by TLR4 requires the participation of an accessory molecule, MD2(19), which is essential for translocation of functional TLR4 from the cytoplasm to the cell surface.(20) We recently found that NSAIDs trigger inflammatory responses and injure the small intestine through activation of the LPS/TLR4 signaling pathway.(31) Agents which possess anti-microbial activity or inhibit the TLR4 pathway might thus be useful for the treatment of NSAID-induced enteropathy.

It has recently been reported that use of probiotic bacteria may have therapeutic effects in gastrointestinal disorders such as inflammatory bowel disease(5, 23) and irritable bowel syndrome.(21) The *Lactobacillus casei* strain Shirota (LcS) is a lactic acid bacterium originally isolated from humans, and is a probiotic.(18) LcS exerted anti-microbial activity against *Escherichia coli* in a murine model of urinary tract infection.(2) Furthermore, LcS improved dextran sodium sulfate-induced colitis by inhibiting expression of pro-inflammatory cytokines in lamina propria mononuclear cells.(17) In this study, we investigated whether LcS has prophylactic effects on indomethacin-induced small intestinal injury in rats. We also examined the effects of LcS and related products such as L-lactic acid on small intestinal inflammation and LPS/TLR4-triggered inflammatory responses.

MATERIALS AND METHODS

Animals and induction of small intestinal injury. Specific-pathogen-free male Wistar rats (Japan SLC, Hamamatsu, Japan) weighing approximately 200 g were used. They were housed in polycarbonate cages with paper chip bedding in an air-conditioned biohazard room with a 12-h light-dark cycle, and had free access to food and water. All experimental procedures were approved by the Animal Care Committee of the Osaka City University Graduate School of Medicine.

To induce small intestinal injury, non-fasted animals were administered 10 mg/kg indomethacin by gavage and were sacrificed 3 or 24 h later. To determine the extent of injury, 1% Evans blue was injected i.v. 30 min before sacrifice, and the small intestine was opened along the anti-mesenteric attachment and examined for injury under a dissecting microscope with square grids ($\times 10$). The area (mm^2) of macroscopically visible lesions was measured, summed per small intestine, and used as the lesion score.

In vivo experiments. Viable LcS (Yakult Honsha Co., Ltd., Tokyo, Japan) at doses of 10^7 - 10^9 CFU was orally administered once or once daily for a week, while heat-killed LcS at a dose of 1 g (corresponding to 10^9 CFU of LcS) was orally administered once daily for a week. Distilled water (1 ml/rat) was orally administered as a vehicle for a week to control rats. Rats were given 10 mg/kg indomethacin 1 h after final administration of viable or heat-killed LcS, or the vehicle. In a separate experiment,

rats were given L-lactic acid (1-15 mM, Wako Pure Chemical Industries, Ltd., Osaka, Japan), 2.5 mM HCl (the pH of which is similar to that of 15 mM L-lactic acid (pH 2.7)), or distilled water in drinking water for 3 days. They were then challenged with 10 mg/kg indomethacin and sacrificed 24 h later. Animals were given L-lactic acid or HCl throughout the experiment.

Examination of small intestinal bacterial flora and measurement of lactic acid concentration. Rats pretreated with 10^9 viable CFU of LcS once or once daily for a week, or pretreated with vehicle (1 ml of distilled water) for a week, were sacrificed 3 h or 24 h after indomethacin administration, and small intestinal contents were obtained. The methods of isolation and identification of each bacterium have been described elsewhere in detail.(1, 34) Numbers of viable bacteria were expressed as log CFUs per gram small intestinal content. The lower limit of bacterial detection with this procedure was 100 CFU per gram intestinal content.

For assay of concentration of lactic acid, intestinal contents were homogenized in 1 mL of distilled water. The homogenate was placed in an Eppendorf tube and centrifuged at 10,000 rpm at 4°C for 10 minutes. A mixture of 0.9 mL of the resulting supernatant and 0.1 mL of 1.5 M perchloric acid was mixed well in a glass tube and allowed to stand at 4°C for 12 hours. The suspension was then passed through a filter with a pore

size of 0.45 μm (KURABO INDUSTRIES Ltd., Osaka, Japan). The HPLC was performed with a Waters system (Waters 432 Conductivity Detector; Waters Co., Milford, MA) equipped with 2 columns (Shodex Rspack KC-811; Showa Denko Co. Ltd., Tokyo). The concentrations of lactic acid were calculated using a lactate standard curve and expressed as μM per gram small intestinal content.

Preparation of viable LcS, heat-killed LcS, and LcS culture supernatants. For preparation of viable bacteria, freeze-dried powders containing 10^9 CFU of LcS/g were suspended in sterile distilled water, while for preparation of heat-killed bacteria the harvested cells of LcS suspended in distilled water at a concentration of 10^9 CFU/ml were heated at 100°C for 30 min and then cooled on ice.(2)

LcS were grown aerobically in 10 ml of Bacto peptone (Becton, Dickinson and Company, Tokyo, Japan) with glucose at a concentration of 10 mg/ml for 48 h at 37°C . Cell-free culture supernatants were obtained by centrifugation at 10,000 r.p.m. for 10 min at 4°C . Centrifuged culture supernatants were passed through a sterile filter with a pore size of 0.45 μm (KURABO INDUSTRIES Ltd.). Then the concentration of lactic acid in culture supernatants was determined by HPLC as described above, and the supernatants were adjusted to 10 mM lactic acid with culture medium just before use in *in vitro* experiments.

In vitro experiments. The human monocytic cell line THP-1 (Riken Cell Bank, Tsukuba, Japan) was maintained in RPMI1640 medium supplemented with 10% heat-inactivated FBS. THP-1 cells were plated overnight at 5×10^5 /well in 24-well plates, and then treated with 10 ng LPS 055: B5 (Sigma-Aldrich) in the presence or absence of L-lactic acid (0-15 mM), hydrochloric acid (4.2 mM, pH 6.8), or LcS culture supernatant containing 10 mM lactic acid for 1 or 2 h. Culture supernatants of THP-1 cells were collected for TNF- α assay, and THP-1 cells were collected for real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR), western blotting, or assay for quantification of NF- κ B activation.

Determination of mRNA levels by RT-PCR. Total RNA was extracted from small intestinal tissue or THP-1 cells stimulated with 10 ng LPS for 2 h in the presence or absence of L-lactic acid or culture supernatant of LcS using an ISOGEN kit (Nippon Gene Co., Ltd. Tokyo, Japan). Real-time quantitative RT-PCR analyses were performed as previously described.(30) Expression of mRNAs for inflammatory mediators including TNF- α , cytokine-induced neutrophil chemoattractant-2 α (CINC-2 α) (rat interleukin-8 homolog), inducible nitric oxide synthase (iNOS), and TLR4 was normalized for expression with GAPDH. The sequences of PCR primers and TaqMan probes are shown in Table 1.

Measurement of TNF- α production by THP-1 cells. THP-1 cells were stimulated with 10 ng LPS for 2 h as described above. Cells supernatants were collected and assayed for TNF- α production by standard sandwich ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Western blot analysis. THP-1 cells stimulated with 10 ng LPS in the presence or absence of 10 mM L-lactic acid or LcS culture supernatant containing 10 mM lactic acid for 1 h were lysed on ice in a buffer containing 0.5% NP-40, 40 mM Tris HCl pH 8.0, 120 mM NaCl, 1 mM PMSF, and 10 μ g of leupeptin per ml. Protein in the lysate was measured with a modified bicinchoninic acid method (BCA protein assay reagent kit, Pierce, Rockford, IL). Proteins were denatured with SDS sample buffer, subjected to 10% SDS-polyacrylamide gel electrophoresis, and transferred to a polyvinylidene difluoride membrane. Membranes were blocked in Tris-buffered saline containing 5% BSA and incubated overnight at 4 °C with one of the following antibodies: anti-TLR4 (Cell Signaling Technology, Danvers, MA), anti-MD2 (Stressgen, Victoria, Canada), total inhibitory- κ B (I- κ B)- α and phospho-I- κ B- α (both from Cell Signaling Technology, USA), anti-phospho-ERK, anti-total ERK (both from Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or anti-actin (Sigma) antibody. Subsequently, the membranes were washed in TBS and incubated with the appropriate secondary antibodies coupled to HRP,

washed in TBS again, and bands were visualized using enhanced chemiluminescence (Amersham, Arlington Heights, IL) in accordance with the manufacturer's instructions. Relevant bands were quantified by laser-scanning densitometry (Bio-Rad, Hercules, CA).

Nuclear extract and assay for NF- κ B activation. THP-1 cells were stimulated with 10 ng LPS in the presence or absence of 10 mM L-lactic acid or LcS culture supernatant containing 10 mM lactic acid for 1 h, and nuclear extract was prepared using commercially available kit (Pierce). To detect and quantify NF- κ B activation in THP-1 cells, translocation of p65, the subunit of NF- κ B, from cytoplasm to nucleus was assayed using the ELISA-based TransAm NF κ B p65 kit (Active Motif, Carlsbad, CA). In brief, nuclear extracts were incubated for 1 h on a 96-well plate coated with an oligonucleotide containing the NF- κ B binding consensus sequence. After incubation with an antibody to p65, a horseradish peroxidase-conjugated secondary antibody was added, followed by developing solution. Absorbance was read at 450 nm with a reference wavelength of 655 nm on a spectrophotometer.

Measurement of myeloperoxidase (MPO) activity. MPO activity in small intestinal tissue, a marker of neutrophil infiltration, was assayed as previously described.(30) One unit of MPO activity was defined as that degrading 1 μ mol of peroxide per min at 25 °C.

Proteins were measured with a modified bicinchoninic acid method. Results are expressed as units per gram tissue protein.

Measurement of concentrations of TNF- α and CINC-2 α in intestinal tissue. Small intestinal tissue from rats given indomethacin with or without LcS treatment were homogenized in ice-cold lysis buffer (50 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl₂, 1 mM EGTA, 1 mM sodium vanadate, 10 mM sodium pyrophosphate, 10 mM NaF, 300 μ M p-nitrophenyl phosphate, 1 mM PMSF, 10 μ g/ml leupeptin, and 10 μ g/ml aprotinin) containing 1 mM protease inhibitor (Sigma). Homogenates were centrifuged at 14,000 g for 15 min, supernatants were collected, and immunoreactive TNF- α and CINC2- α were assayed using commercially available ELISA kits (R&D System and Immuno-Biological Laboratories Co., Ltd., Takasaki, Japan, respectively) according to the manufacturer's instructions.

Statistical Analysis. Values are the means \pm SEM. One-way analysis of variance was used to test for significance of differences among treatment group means, and results were examined by Fisher's protected least-significant-difference test.

Differences with *P* values less than 0.05 were considered significant.

RESULTS

Viable LcS, but not heat-killed LcS, prevented indomethacin-induced small intestinal

injury. Indomethacin induced multiple deep ulcerations in the small bowel by 24 h. One-week treatment with viable LcS inhibited this injury in dose-dependent fashion, with 73% inhibition by 10^9 CFU of LcS, while one-week treatment with heat-killed LcS had no inhibitory effect on injury (Figure 1). A single dose of 10^9 CFU of viable LcS tended to inhibit injury ($p=0.21$), though not to a significant extent. Histologically, indomethacin caused intestinal ulceration with destruction and necrosis of the epithelium and infiltration by inflammatory cells in controls (Figure 2A) by 24 h, while less severe injury with mild to moderate inflammation was observed in rats treated with viable LcS for a week (Figure 2B).

Inhibition of indomethacin-induced intestinal inflammation by LcS. Indomethacin increased MPO activity by 4.2-fold and mRNA level for iNOS by 3.1-fold, and also increased expression of TNF- α and CINC-2 α at both mRNA and protein levels by 24 h (Table 2). Treatment with viable LcS at a dose of 10^9 CFU for a week inhibited these increases. Indomethacin also significantly increased the level of expression of TLR4 mRNA. Repeated administration of LcS had no inhibitory effect on TLR4 expression.

Analysis of bacteria flora and lactic acid concentration in small intestine. As shown in Table 3, numbers of *Enterobacteriaceae* and *Enterococci*, which are

Gram-negative and Gram-positive bacteria, respectively, were increased by 24 h after indomethacin challenge. Treatment with viable LcS at a dose of 10^9 CFU once or once daily for a week had no inhibitory effect on the increase in numbers of these bacteria, while it decreased the number of *Bacilli* by 24 h. LcS was detected in the small intestinal content with both single and repeated administration of LcS by 3 h after indomethacin challenge. In the single-administration group, LcS was not detected by 24 h, while a comparatively large number of LcS remained detectable by 24 h in the repeat-administration group.

We next measured concentrations of lactic acid in the small intestine.

Indomethacin challenge increased the concentration of lactic acid by 24 h. Single administration of viable LcS at a dose of 10^9 CFU had no effect on lactic acid concentration, while repeated administration of LcS significantly increased the concentration of lactic acid, with prevention of increase in pH of small intestinal content (Table 4).

Inhibition of LPS/TLR4 signaling pathway by L-lactic acid and LcS culture

supernatant. Treatment with 10 ng LPS markedly increased both TNF- α production and TNF- α expression in THP-1 cells (Figure 3A and 3B). L-lactic acid inhibited TNF- α production and TNF- α mRNA expression in dose-dependent fashion, while 4.2

mM HCl, the pH of which is similar to that of 15 mM L-lactic acid, had no inhibitory effect on LPS-induced TNF- α overexpression. Culture supernatant of LcS containing 10 mM lactic acid exerted a stronger inhibitory effect on TNF- α expression at both protein and mRNA levels than L-lactic acid alone.

Western blotting showed that LPS induced phosphorylation of I- κ B- α and ERK as well as I- κ B- α degradation in THP-1 cells, while these changes were inhibited by LcS culture supernatant (figure 4A and 4B). L-lactic acid also inhibited phosphorylation of I- κ B- α , but did not inhibit the phosphorylation of ERK. Neither LcS culture supernatant nor L-lactic acid affected the expression of TLR4 or MD2.

LPS treatment for 1 h markedly activated NF- κ B in THP-1 cells. L-lactic acid at a concentration of 10 mM and culture supernatant of LcS containing 10 mM lactic acid strongly inhibited this activation. (Figure 4C)

In vivo effects of L-lactic acid. Finally, we examined the anti-ulcerogenic effects of L-lactic acid. Since by 3 h after indomethacin challenge the mean concentration of lactic acid in the small intestinal contents in rats given LcS at a dose of 10^9 CFU for a week was 13.8 ± 2.4 μ M/g (= mM/kg, Table 4), we used L-lactic acid at concentrations of 1 - 15 mM. L-lactic acid supplied in drinking water inhibited indomethacin-induced small intestinal injury in dose-dependent fashion, whereas 2.5 mM HCl, the pH of

which is similar to that of 15 mM L-lactic acid (pH 2.7), did not prevent this injury (Figure 5). Neither amount of food intake, amount of drinking, nor body weight differed among the groups.

DISCUSSION

In this study, we demonstrated that LcS had a strong inhibitory effect on indomethacin-induced small intestinal injury. Since a single dose of viable LcS, which resulted in transitory colonization of LcS in the small intestine as did heat-killed LcS, failed to inhibit injury, sustained colonization of LcS during development of the injury may be needed for exertion of a probiotic effect on NSAID-induced small intestinal injury.

Gram-negative bacteria play a key role in the pathogenesis of NSAID-induced enteropathy(31). Probiotic *Lactobacillus* strains including LcS have been reported to possess anti-microbial activity (2, 27) exerted via various mechanisms.(26) In particular, by producing metabolites such as lactic acid and thus lowering pH, *Lactobacillus* strains inhibit the growth of bacterial pathogens and sometimes even kill them.(10) However, we found that repeated administration of LcS, which elevated lactic acid concentration in the small intestine, failed to inhibit the increase in number of *Enterobacteriaceae* (a Gram-negative bacteria) as well as *Enterococci* (Gram-positive

bacteria), although it prevented elevation of pH of intestinal content. Prevention of injury by LcS may thus be independent of anti-bacterial activity.

Studies of the effects of lactic acid on inflammation have yielded conflicting results. Douvdevani et al. demonstrated a decrease in LPS-induced TNF- α mRNA expression and NF- κ B DNA binding activity in human blood-derived macrophages incubated with lactated dialysis solution.(6) Kellum et al. also demonstrated that lactic acid inhibited mRNA expression of iNOS, IL-6, and IL-10 as well as NF- κ B DNA binding activity in LPS-stimulated RAW 264.7 cells, a mouse macrophage cell line, in dose-dependent fashion.(13) In contrast, Jensen et al. reported an increase in TNF- α expression in macrophages cultured with lactic acid.(11) In this study, we demonstrated that L-lactic acid, an isomeric form of lactic acid produced by LcS, prevented NF- κ B activation, phosphorylation of I- κ B- α , and TNF- α expression in LPS-stimulated THP-1 cells. This anti-inflammatory effect of L-lactic acid may not be due to its pH-lowering effect, since 4.2 mM hydrochloric acid, the pH of which is similar to 15 mM L-lactic acid (pH6.8), failed to inhibit overexpression of TNF- α in LPS-stimulated THP-1 cells.

I- κ B has been recognized as an inhibitor of NF- κ B (3), a major transcription factor that regulates the expression of a large number of genes that code for inflammatory

mediators such as cytokines.(4) NF- κ B molecules are present in specific heterodimeric complexes present in inactive form in the cytoplasm of resting cells, with each molecule bound to one of the I- κ B proteins. Upon stimulation, I- κ B is phosphorylated, ubiquitinated, and degraded by the proteasome, allowing NF- κ B to translocate to the nucleus.(7) L-lactic acid may thus prevent NF- κ B activation through inhibition of phosphorylation of I- κ B- α , leading to suppression of LPS-induced TNF- α expression in THP-1 cells.

Recently, Lien et al demonstrated that Caco-2 cells coincubated with *L. casei* exhibited stabilization of I- κ B even after subsequent stimulation by *Shigella* or TNF- α , and that *L. casei* induced down-regulation of genes involved in ubiquitination/degradation processes which are responsible for the degradation of I- κ B in Caco-2 cells.(29) The effects of lactic acid and culture supernatant of LcS on NF- κ B activation may thus be mediated by effects on the ubiquitin/proteasome system.

Repeated administration of viable LcS elevated lactic acid concentrations in small intestinal content and inhibited indomethacin-induced small intestinal damage with decrease in inflammatory responses such as neutrophil infiltration and cytokine expression, whereas single administration of viable LcS did not affect lactic acid concentration and did not inhibit injury. We also found that L-lactic acid given in

drinking water markedly inhibited injury independent of its pH-lowering effect.

Furthermore, we previously reported that neutralizing antibodies to TNF- α prevented such injury in rats.(31) Collectively, these findings suggest that the anti-inflammatory effects of L-lactic acid produced by LcS may be a key mechanism by which LcS exerts probiotic effects on NSAID-induced small intestinal injury.

Interestingly, Schurr et al. also reported a protective effect of lactic acid on neuronal injuries such as those resulting from hypoxia upon reoxygenation (24, 25), suggesting that lactic acid may have preventive effects on a variety of tissue injuries.

Indomethacin challenge increased the concentration of lactic acid in small intestinal content by 24 h, although the mechanism by which it did so is unclear. However, indomethacin did not increase lactic acid concentration by 3 h. Since treatment with viable LcS for a week significantly increased the concentration of lactic acid by 3 h as well as 24 h after indomethacin challenge, elevation of lactic acid concentration in the early phase of injury may play an important role in inhibition of inflammatory responses and injury induced by indomethacin.

Notably, LcS culture supernatant containing 10 mM lactic acid had stronger anti-inflammatory effects on THP-1 cells stimulated with LPS than 10 mM L-lactic acid alone: culture supernatant and L-lactic acid inhibited production of TNF- α

protein by 85% and 54%, respectively, and inhibited the level of expression of TNF- α mRNA by 83% and 37%, respectively. Furthermore, culture supernatant inhibited LPS-induced phosphorylation of ERK as well as I- κ B- α without affecting expression of TLR4 or MD2. These findings suggest that in addition to lactic acid, LcS may secrete metabolites which act as negative regulators of the ERK activation pathway. The anti-inflammatory effects of LcS may thus be mediated via synergistic effects of L-lactic acid and secreted molecules other than lactic acid.

In conclusion, our findings suggest that LcS protects against indomethacin-induced small intestinal injury in rats, and that its probiotic effects may be mediated through anti-inflammatory effects of bacterial metabolites including L-lactic acid via interference with the signaling pathways triggered by LPS. Our findings clearly provide a rational basis for the clinical use of the probiotic LcS for prevention of NSAID-induced enteropathy.

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FIGURE LEGENDS

Figure 1. Effects of viable and heat-killed *L. casei* strain Shirota on indomethacin-induced small intestinal injury. Rats were orally administered viable or heat-killed *L. casei* strain Shirota once or once daily for a week, and were given 10 mg/kg indomethacin. They were sacrificed 24 h later, and lesion score for each rat was calculated. Each column represents the mean \pm SEM. N = 6. *p < 0.01 vs. vehicle-treated controls. LcS, *L. casei* strain Shirota, HK, heat-killed.

Figure 2. Histological findings in the small intestine 24 h after indomethacin administration. Indomethacin caused intestinal ulceration with destruction and necrosis of the epithelium and infiltration by inflammatory cells in controls (A) by 24 h, while less severe injury with mild inflammation was observed in rats treated with viable *L. casei* strain Shirota for a week (B).

Figure 3. Effects of L-lactic acid and culture supernatant of *L. casei* strain Shirota on LPS-induced TNF- α expression in THP-1 cells. THP-1 cells were treated with 10 ng LPS for 2 h in the presence or absence of L-lactic acid (0-15 mM), hydrochloric acid (pH 6.8), or culture supernatant of *L. casei* strain Shirota containing 10 mM lactic acid. Culture supernatant of THP-1 cells was collected for TNF- α assay, and THP-1 cells were collected for quantification of TNF- α mRNA expression by real-time RT-PCR.

Each column represents the mean \pm SEM. N = 4-6. *p < 0.01 vs. LPS-stimulated, vehicle-treated group. DW, distilled water; CM, culture medium; LcS-CS, culture supernatant of *L. casei* strain Shirota.

Figure 4. Effects of L-lactic acid and culture supernatant of *L. casei* strain Shirota on phosphorylation of I- κ B- α and ERK and activation of NF- κ B induced by LPS, and expression of TLR4 or MD2 in THP-1 cells. THP-1 cells were treated with 10 ng LPS for 1 h in the presence of 10 mM L-lactic acid or culture supernatant of *L. casei* strain Shirota containing 10 mM lactic acid. The cells were subjected to Western blot analysis or assay for quantification of NF- κ B activation. (A) A representative Western blot for phospho-I- κ B- α , total I- κ B- α , phospho-ERK, total ERK, TLR4, MD2, and β -actin. (B) Levels of expression of phospho-I- κ B- α , total I- κ B- α , TLR4, and MD2 were normalized to β -actin, while level of expression of phospho-ERK was normalized to that of total ERK. Each column represents the mean \pm SEM. N = 3. *p < 0.05, **p < 0.01 vs. LPS-non-stimulated group (control group), #p < 0.05, ##p < 0.01 vs vehicle-treated group. (C) Translocation of p65, the subunit of NF- κ B, from cytoplasm to nucleus was assayed using the ELISA-based Trans-Am NF κ B p65 kit. Each column represents the mean \pm SEM. N = 3. **p < 0.01 vs. LPS-non-stimulated group (control group), ##p < 0.01 vs. vehicle-treated group. DW, distilled water; LA, lactic

acid; CM, culture medium; CS-LcS, culture supernatant of *L. casei* strain Shirota.

Figure 5. Effect of L-lactic acid on indomethacin-induced small intestinal injury.

Rats were given L-lactic acid at concentrations of 1-15 mM, 2.5 mM HCl, or distilled water in drinking water for 3 days. Animals were then challenged with 10 mg/kg indomethacin and sacrificed 24 h later. Each column represents the mean \pm SEM. N = 4-5. * $p < 0.01$ vs. vehicle-treated group.

Table 1. *The PCR Primers and TaqMan Probes*

Gene	Primer and probe
Rat	
TNF- α	Primer (forward) 5'-CCAGGAGAAAGTCAGCCTCCT-3'
	Primer (reverse) 5'-TCATACCAGGGCTTGAGCTCA-3'
	Probe 5'-FAM-AGAGCCCTTGCCCTAAGGACACCCCT-TAMRA-3'
CINC-2 α	Primer (forward) 5'-AACATCCAGAGCTTGACGGTG-3'
	Primer (reverse) 5'-TCTTGACCATCCTTGAGAGTGG-3'
	Probe 5'-FAM-ACCGCACTGCACCCAGACAGAAGTCATA-TAMRA-3'
iNOS	Primer (forward) 5'-AGCGGCTCCATGACTCTCA-3'
	Primer (reverse) 5'-TGCACCCAAACACCAAGGT-3'
	Probe 5'-FAM-AGAGGGCTCAAAGGAGGCCGCAT-TAMRA-3'
TLR4	Primer (forward) 5'-ATTCCTGGTGTAGCCATTGCT-3'
	Primer (reverse) 5'-ACCACCACAATAACTTTCCGG-3'
	Probe 5'-FAM-CCAACATCATCCAGGAAGGCTTCC-TAMRA-3'
Human	
TNF- α	Primer (forward) 5'-TCTCACATACTGACCCACGGCT-3'
	Primer (reverse) 5'-GTCCCGGATCATGCTTTCA-3'
	Probe 5'-FAM-TCTCTCCCCTGGAAAGGACACCATGA-TAMRA-3'

Table 2. *Effects of indomethacin on neutrophil infiltration and expression of mRNAs for inflammatory mediators in rat small intestine*

	Normal	Vehicle	LcS
MPO activity (U/g tissue protein)	5.0 ± 0.8	20.1 ± 1.4**	3.0 ± 0.3#
Fold increase in mRNA			
TNF- α	1.0 ± 0.2	3.0 ± 1.0*	0.4 ± 1.0#
CINC-2 α	1.0 ± 0.1	10.0 ± 3.0**	1.0 ± 0.2#
iNOS	1.0 ± 0.2	3.1 ± 0.4**	0.5 ± 0.4#
TLR4	1.0 ± 0.1	1.5 ± 0.1*	1.5 ± 0.2*
Protein concentration (pg/mg tissue protein)			
TNF- α	1.2 ± 0.2	3.1 ± 0.4**	2.3 ± 0.2*#
CINC-2 α	6.5 ± 0.9	16.8 ± 3.04**	8.9 ± 0.7*#

Rats were orally administered *L. casei* strain Shirota (LcS) at a dose of 10^9 CFU or vehicle for a week, and were given 10 mg/kg indomethacin. The animals were sacrificed 24 h later, and small intestinal tissue was subjected to measurement of MPO activity and quantification of mRNA levels and protein levels for inflammatory mediators. mRNA levels are expressed as ratios to the mean value for normal small intestinal tissue. Results are means \pm SEM.

N = 5 - 6. **p < 0.01, *p < 0.05 vs. normal rats. #p < 0.05 vs. vehicle-treated rats.

Table 3. Changes in small intestinal bacterial flora after indomethacin challenge.

	Normal	3h after IND			24h after IND		
		Vehicle	Single-dose of LcS	Repeat-dose of LcS	Vehicle	Single-dose of LcS	Repeat-dose of LcS
Total	9.2 ± 0.1	8.8 ± 0.1	8.8 ± 0.1	8.8 ± 0.3	9.0 ± 0.3	9.4 ± 0.3	8.8 ± 0.2
Gram negative							
<i>Enterobacteriaceae</i>	4.4 ± 0.4	3.8 ± 0.3	4.2 ± 0.2	3.7 ± 0.2	6.0 ± 0.5*	6.4 ± 0.6*	6.3 ± 0.2*
<i>Bacteroidaceae</i>	2.5 ± 0.3	2.3 ± 0.4	< 1.9	2.4 ± 0.4	< 1.9	2.0 ± 0.1	2.6 ± 0.7
Gram positive							
<i>Bacillus</i>	3.5 ± 0.1	3.6 ± 0.2	3.5 ± 0.3	3.5 ± 0.1	3.4 ± 0.4	2.3 ± 0.4*	2.5 ± 0.4*
<i>Enterococcus</i>	4.9 ± 0.2	4.2 ± 0.1	5.0 ± 0.5	4.5 ± 0.1	7.9 ± 0.3*	8.1 ± 0.4*	7.1 ± 0.7*
<i>Staphylococcus</i>	4.1 ± 0.3	3.9 ± 0.1	3.6 ± 0.2	3.4 ± 0.4	4.2 ± 0.6	5.1 ± 0.7	4.9 ± 0.4
<i>Lactobacillus</i>	9.0 ± 0.1	8.8 ± 0.1	8.8 ± 0.1	8.8 ± 0.3	8.9 ± 0.3	9.4 ± 0.3	8.7 ± 0.2
<i>L. casei</i> Shirota			4.1 ± 0.2	3.6 ± 0.3		< 1.9	6.1 ± 0.1

Rats pretreated with viable 10^9 CFU of *L. casei* strain Shirota once or once daily for a week were sacrificed 3 h or 24 h after indomethacin administration. The number of viable bacteria in the small intestinal contents was counted. Data are expressed as log CFUs per gram small intestinal contents. Results are means ± SEM. N = 5.

*p < 0.05 vs. normal rats. IND, indomethacin; LcS, *L. casei* strain Shirota.

Table 4. Effect of administration of viable *L. casei* strain Shirota in the concentration of lactic acid in the small intestinal content

	Normal	3 h after IND			24 h after IND		
		Vehicle	Single-dose of LcS	Repeat-dose of LcS	Vehicle	Single-dose of LcS	Repeat-dose of LcS
pH	7.5 ± 0.1	7.5 ± 0.1	7.1 ± 0.2	7.2 ± 0.1	7.9 ± 0.1*	8.0 ± 0.1*	7.4 ± 0.3
Lactic acid (μM/g)	5.8 ± 0.8	7.6 ± 1.2	5.1 ± 1.0	13.8 ± 2.4*#	11.8 ± 1.3*	11.8 ± 3.0*	21.4 ± 3.9**#

Rats pretreated with viable 10^9 CFU of *L. casei* strain Shirota once or once daily for a week were sacrificed 3 h or 24 h after indomethacin administration. The concentrations of lactic acid in the small intestinal content were calculated. Data are expressed as μM per gram small intestinal contents.

Results are means ± SEM. N = 5. *p < 0.05, **p < 0.01 vs. normal rats. #p < 0.05 vs. vehicle-treated rats.

IND, indomethacin; LcS, *L. casei* strain Shirota.

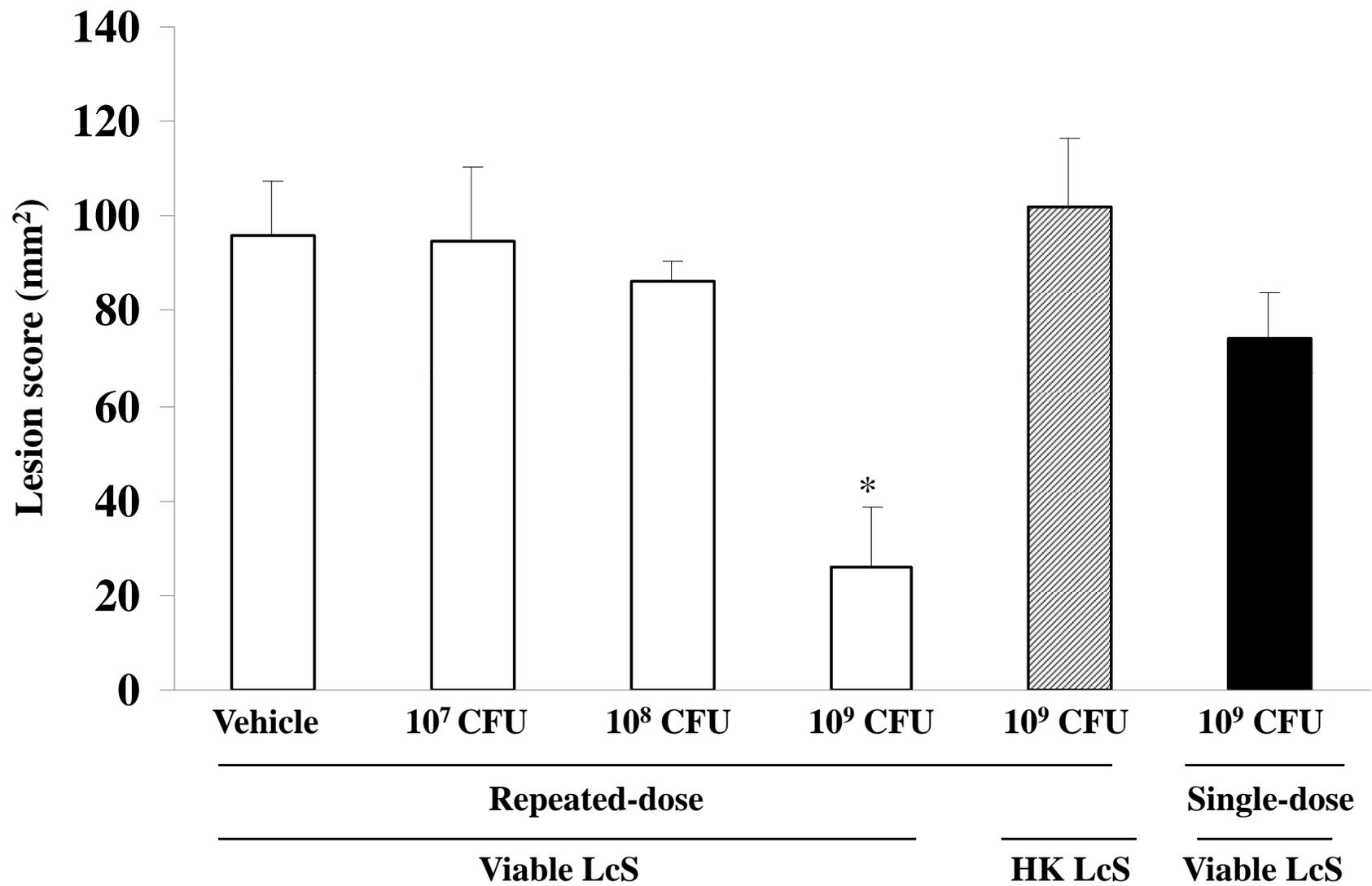


Figure 1

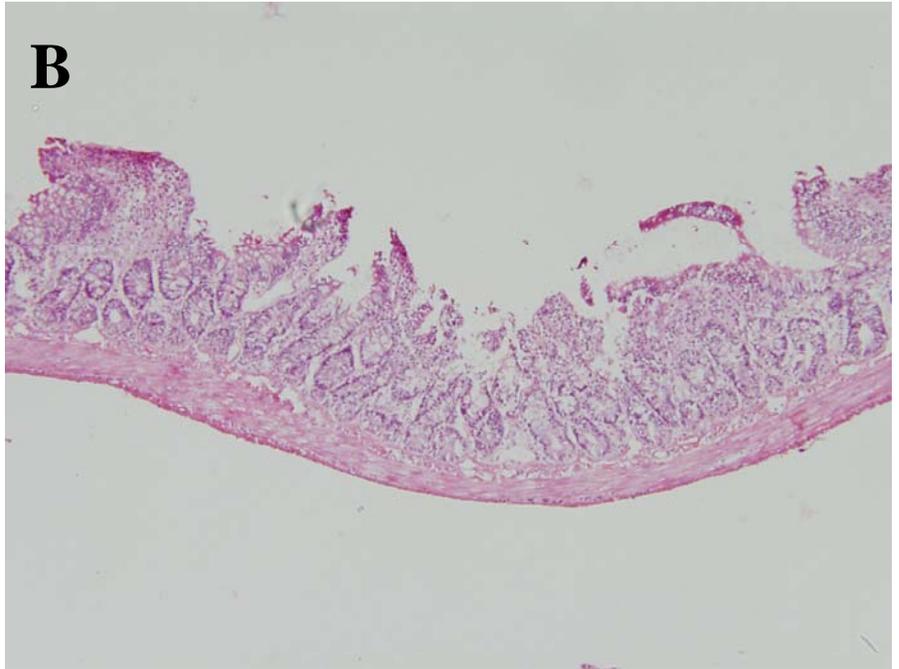
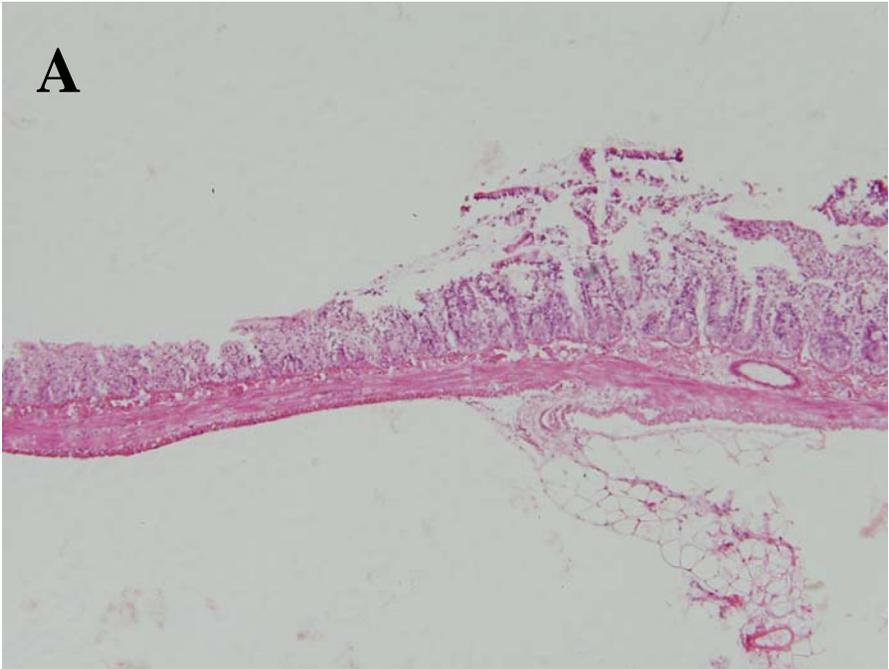
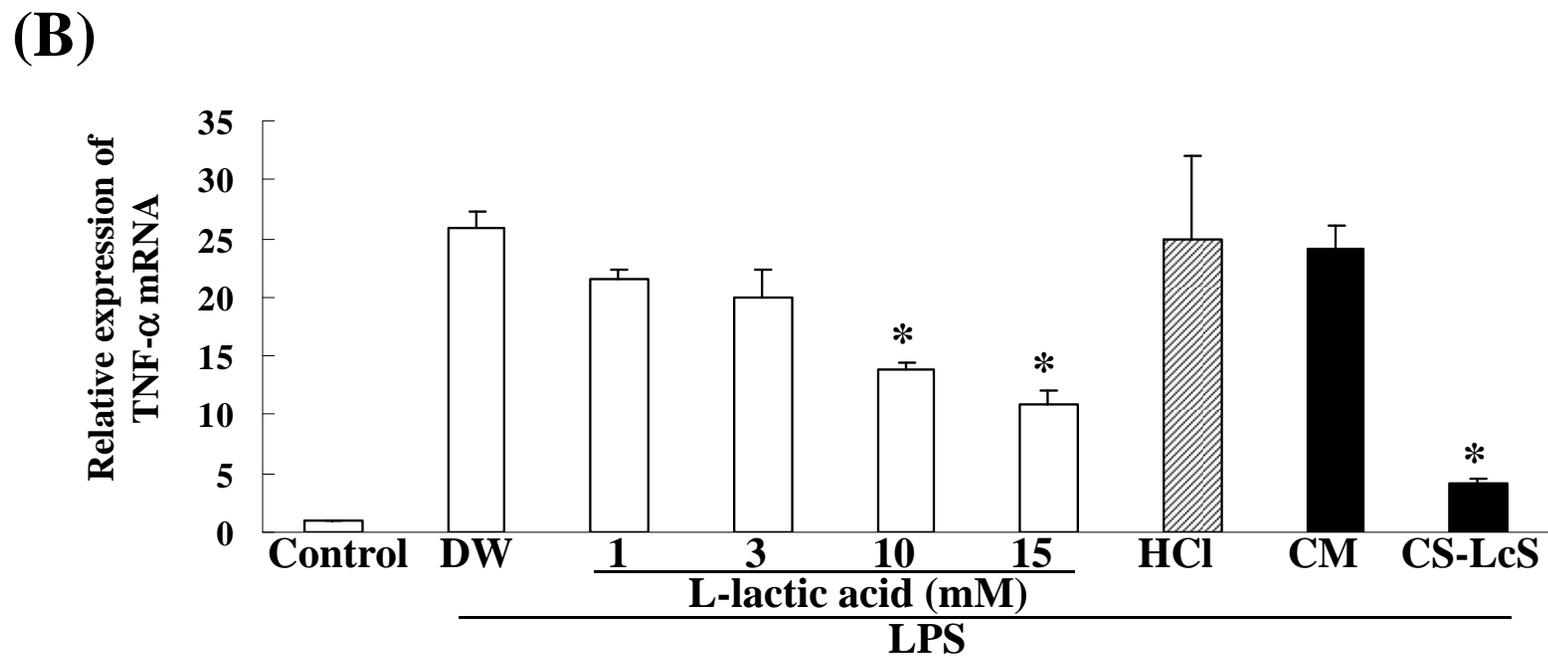
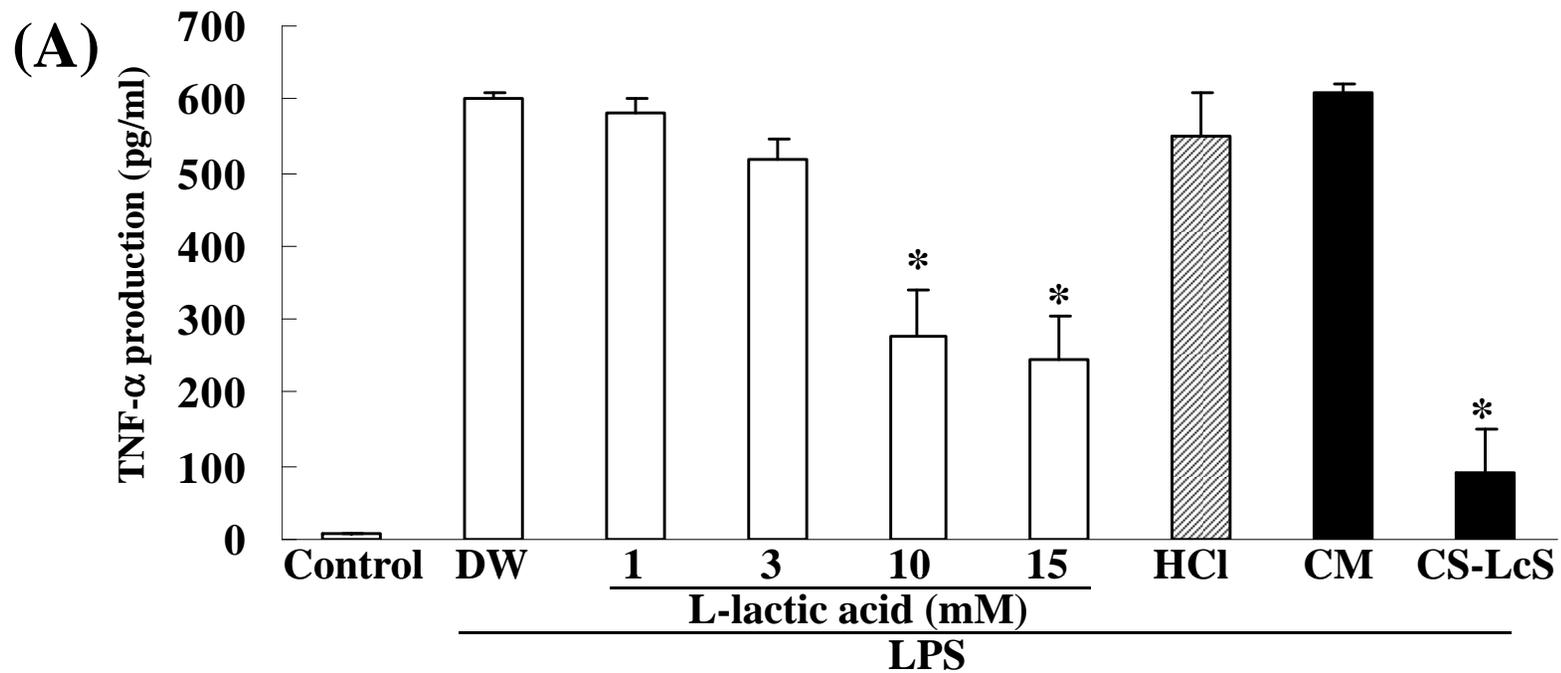


Figure 2



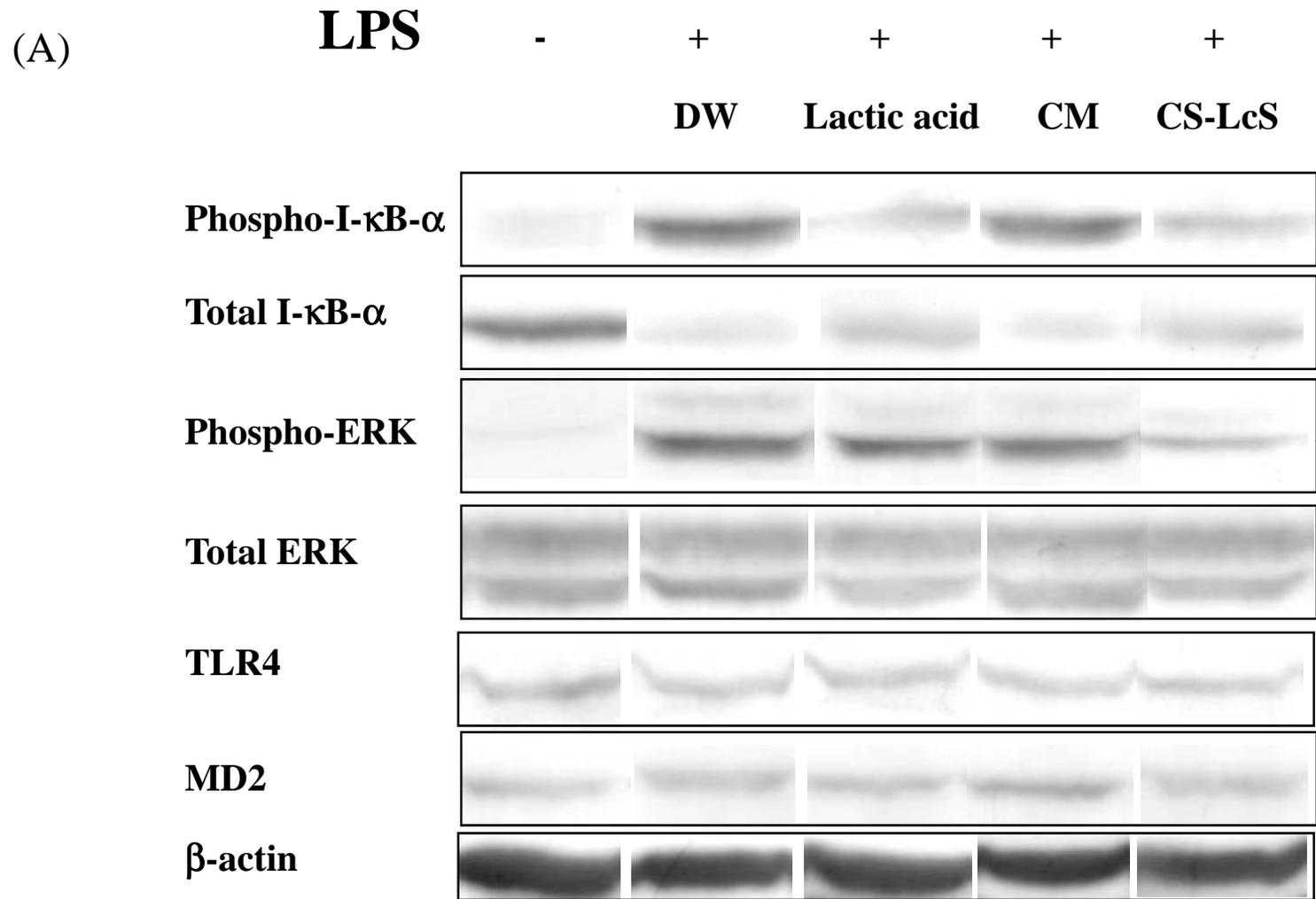


Figure 4

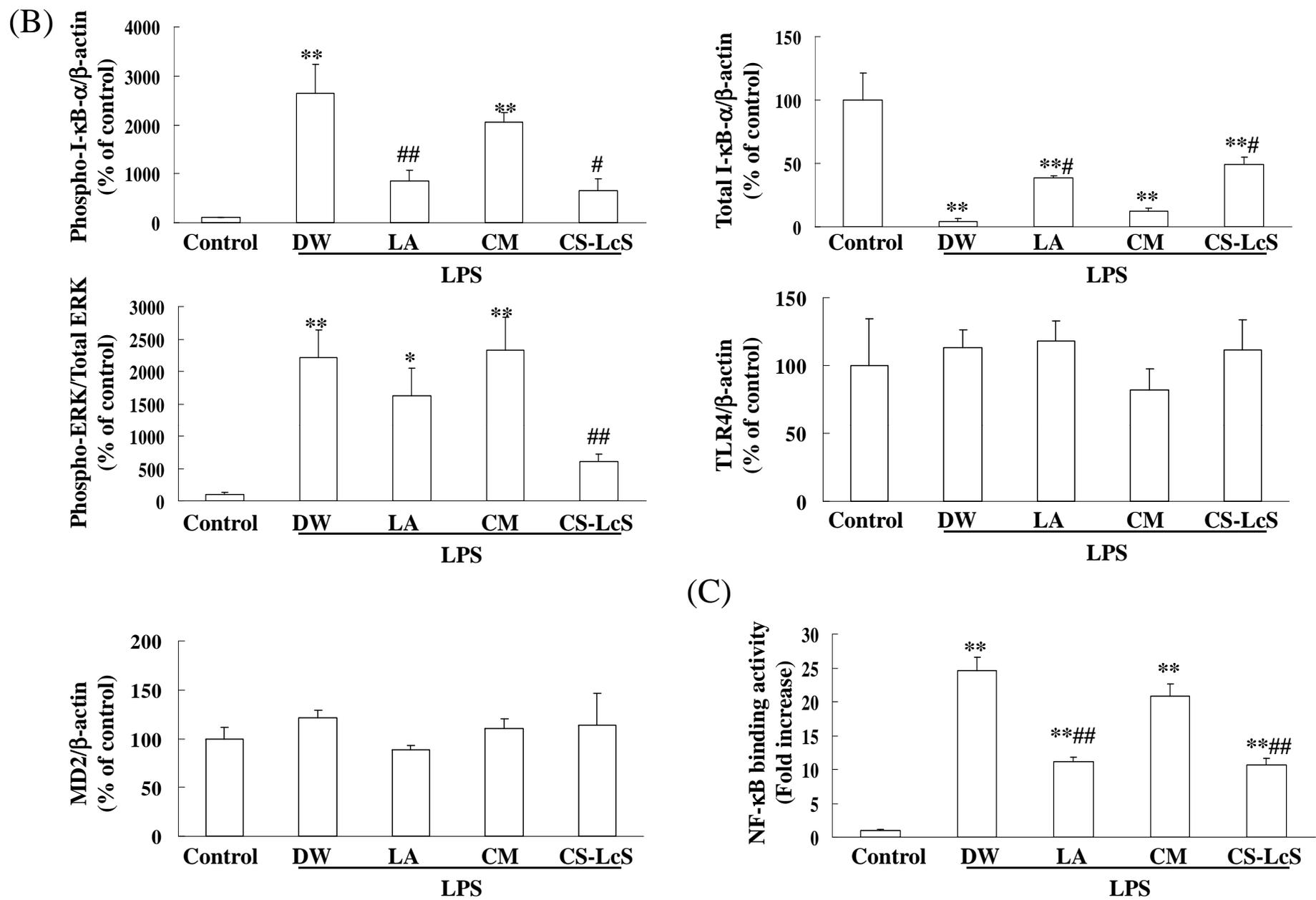


Figure 4

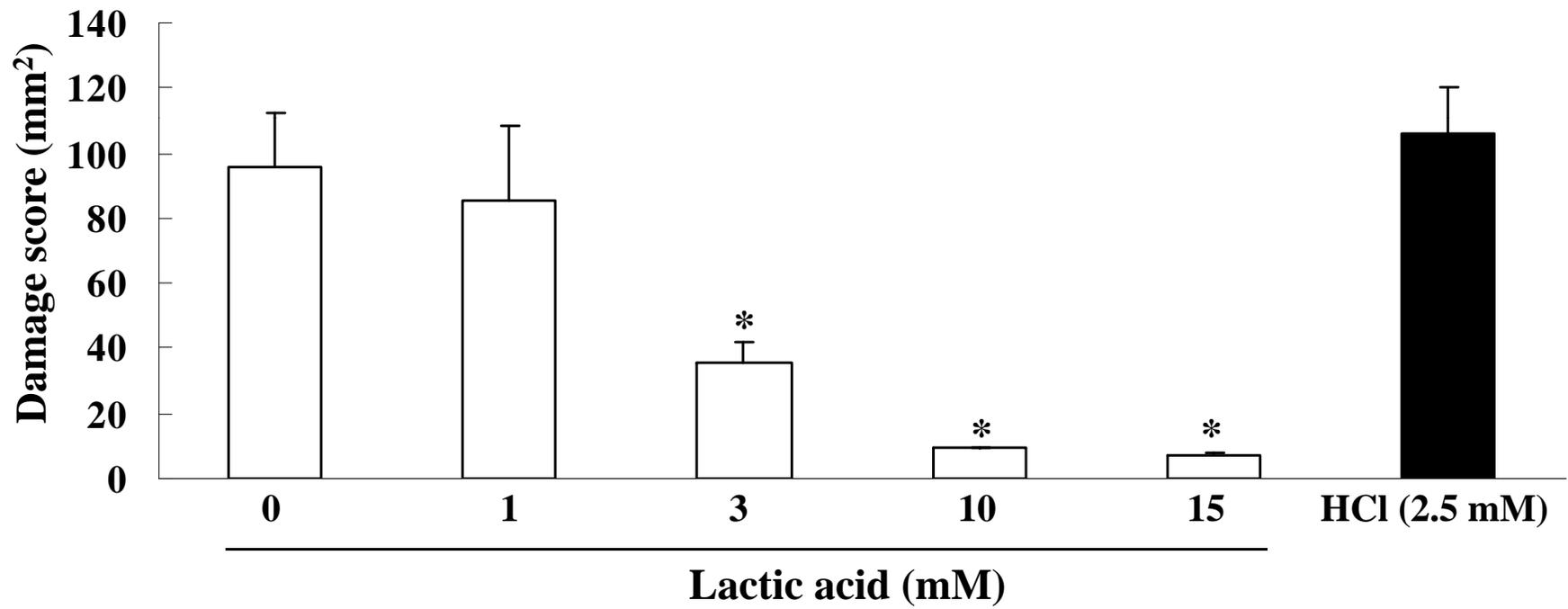


Figure 5