**Lactobacillus brevis KB290 Enhances IL-8 Secretion by Vibrio parahaemolyticus-Infected Caco-2 Cells**

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**Vibrio parahaemolyticus** in uncooked seafood causes acute gastroenteritis. The microorganism has two sets of type III secretion systems and two hemolysins. When it injects its effector proteins into a host cell via type III secretion system 1, one of the type III secretion systems induces secretion of interleukin (IL)-8, a proinflammatory chemokine, through the phosphorylation of ERK 1/2 and p38 MAPK. Although probiotics have beneficial effects on hosts and can help control some infectious diseases, there is little research on the efficacy of probiotics in *V. parahaemolyticus* infection. Here we pretreated *V. parahaemolyticus*-infected human intestinal epithelial cells with heat-killed *Lactobacillus brevis* KB290, a probiotic isolated from fermented vegetables (traditional Japanese pickles) and utilized as an ingredient of beverages and supplementary foods, and demonstrated its efficacy in enhancing IL-8 secretion from *V. parahaemolyticus*-infected cells. Among the three heat-killed lactic acid bacterial strains we tested, *L. brevis* KB290 induced the highest level of IL-8 secretions in the infected cells. Relative to control cells (Caco-2 cells pretreated with PBS), *V. parahaemolyticus*-infected Caco-2 cells pretreated with heat-killed *L. brevis* KB290 secreted IL-8 earlier, although concentrations were similar 450 min after infection. Heat-killed *L. brevis* KB290 pretreatment also induced earlier ERK 1/2 phosphorylation, greater p38 MAPK phosphorylation, and enhanced IL-8 mRNA expression. Heat-killed *L. brevis* KB290 accelerated IL-8 secretion, a host cell immune response, in *V. parahaemolyticus*-infected cells. We consider this to be beneficial because IL-8 plays an important defensive role against infection, and would contribute to the repair of injured epithelial cells.

**Key words:** *Vibrio parahaemolyticus*, *Lactobacillus brevis*, probiotics, Caco-2 cell, interleukin-8, infection

*Vibrio parahaemolyticus*, a pathogenic microorganism found in raw and undercooked seafood, was discovered in 1950 following a deadly outbreak of seafood poisoning in Japan [7]. The organism causes acute gastroenteritis with symptoms that include watery diarrhea, headache, vomiting, nausea, abdominal cramps, and low-grade fever [29]. *V. parahaemolyticus* poisoning occurs frequently in Japan, where the consumption of raw seafood is common, but it also occurs worldwide [19]. The Codex Alimentarius Commission developed guidelines for the hygienic control of pathogenic *Vibrio* species in seafood in 2010 [5], and FAO/WHO published a risk assessment of *V. parahaemolyticus* in seafood in an effort to manage the risks posed by food-borne pathogens [4]. Since probiotic organisms have been shown to have beneficial effects on hosts with intestinal infections [6], our aim was to test whether probiotic organisms could enhance the immune response in intestinal epithelial cells infected with *V. parahaemolyticus*.

The pathogenicity of *V. parahaemolyticus* derives from two sets of type III secretion systems, T3SS1 and T3SS2 [17]. Previous findings from our laboratory suggest that VPI680, an effector protein of T3SS1, plays an important role in the secretion of the proinflammatory chemokine IL-8 by Caco-2 cells during *V. parahaemolyticus* infection, and that it induces IL-8 mRNA expression through ERK (extracellular signal-regulated kinase) 1/2 phosphorylation and contributes to the stabilization of IL-8 mRNA through p38 mitogen-activated protein kinase (MAPK) phosphorylation [27].

We report here that heat-killed *Lactobacillus brevis* KB290, a plant-derived probiotic lactic acid bacterium...
(LAB) [8, 21, 31, 32], enhanced the secretion of IL-8, which is an immune response, by human intestinal epithelial cells infected in vitro with V. parahaemolyticus. We investigated the virulence factors involved, and found that T3SS1, which contributes to the translocation of effector proteins, was essential for the accelerated IL-8 secretion during V. parahaemolyticus infection. We further showed that in heat-killed L. brevis KB290, those changes followed from induction of time-dependent changes in the phosphorylation of ERK 1/2 and enhanced phosphorylation of p38 MAPK in Caco-2 cells. Heat-killed L. brevis KB290 also enhanced the synthesis of IL-8 mRNA in Caco-2 cells.

**Materials and Methods**

**Bacterial Strains, Growth Conditions, and LAB Preparation**

We used V. parahaemolyticus RIMD2210633 (Kanagawa phenomonopositive, serotype O3:K6) as the wild-type (WT) strain [17] and ΔVPE1680, a T3SS1 effector protein deletion mutant, and AT3SS1, a T3SS1 deletion mutant, as previously described [23]. We cultured the strains at 37°C for 12 h, with shaking, in 2 ml of Luria–Bertani medium (Difco) with 3% (w/v) NaCl. We inoculated L. brevis KB290 (deposited in the RIKEN BioResource Center as JCM17312, L. brevis JCM10159<sup>®</sup> (KB176), and L. rhamnosus GG (ATCC53103, KB377) into 10 ml of Man Rogosa Sharp (MRS) medium (Difco), cultured them without shaking at 30°C (L. brevis) or 37°C (L. rhamnosus) for 18 h, inoculated the cultured LAB at 1% (v/v) into 10 ml of MRS medium, and cultured them under the same conditions. We then inoculated the LAB [1% (v/v)] into 500 ml of MRS medium and cultured them under the same conditions. We harvested the cells by centrifugation at 5,000 × g for 10 min at 4°C, washed them with PBS, suspended them in sterile distilled water, heated them at 100°C for 30 min, lyophilized the suspension, and then resuspended the cells in PBS at 2.0 or 20.0 mg/ml.

**Cell Culture**

We cultured Caco-2 human intestinal epithelial cells in DMEM (Sigma-Aldrich) containing 10% fetal bovine serum (Tissue Culture Biologicals) and 100 µg/ml gentamicin (Sigma-Aldrich), incubated them at 37°C in 5% CO<sub>2</sub> in a humidified environment, seeded them in 6- or 24-well culture dishes at 5 × 10<sup>4</sup> cells/cm<sup>2</sup>, and used them within 4–5 days.

**Pretreatment of Caco-2 Cells with Heat-Killed LAB and Infection with V. parahaemolyticus**

To pretreat Caco-2 cells with heat-killed LAB, we replaced the Caco-2 cell medium with DMEM (without fetal bovine serum and gentamicin) containing 5% (v/v) heat-killed LAB suspension or PBS (vehicle control) and incubated them for 4 h at 37°C in 5% CO<sub>2</sub> before infecting them with V. parahaemolyticus. We cultured V. parahaemolyticus cells in Luria–Bertani medium with 3% NaCl, harvested them by centrifugation at 7,740 × g for 3 min, washed them three times with PBS, and diluted the suspension with PBS to an OD of 1.0 at 600 nm (10<sup>8</sup> colony forming units/ml). We diluted the suspension further with PBS and added them to the wells containing pretreated Caco-2 cells at 2 × 10<sup>5</sup> colony forming units/cm<sup>2</sup>, co-culturing the cells at 37°C in 5% CO<sub>2</sub> for various times up to 450 min.

**IL-8 Measurement**

We measured IL-8 secreted into the culture medium by using a colorimetric ELISA kit for human IL-8 (Thermo Scientific Pierce) according to the manufacturer’s instructions.

**Cell Viability Assay**

Cell viability after heat-killed LAB pretreatment was evaluated by LDH released from Caco-2 cells during incubation with LAB. The LDH released into the culture medium was assayed using a Cytotox96 Non-Radioactive Cytotoxicity Assay kit (Promega) according to the manufacturer’s instructions.

**Western Blotting**

We washed Caco-2 cells pretreated with heat-killed L. brevis KB290 or PBS with ice-cold PBS at various times after adding V. parahaemolyticus or PBS to the culture and then extracted proteins with RIPA buffer (Thermo Scientific Pierce) containing proteinase inhibitor cocktail and phosphatase inhibitor cocktail (Roche). We centrifuged the mixture at 20,400 × g for 10 min at 4°C, collected the supernatant, and measured the total protein concentration using a Micro BCA assay kit (Thermo Scientific Pierce) according to the manufacturer’s instructions. Using 10% SDS-polyacrylamide gel electrophoresis, we separated out 70 µg of total protein from each sample, transferred it onto a polyvinylidene fluoride membrane (Millipore), blocked the membrane with TBST (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20) containing 5% (w/v) non-fat dry milk, and incubated it with the primary antibody against phosphorylated ERK 1/2 [Phospho-p44/42 MAPK (ERK 1/2) (Thr202/Tyr204) rabbit antibody] or p38 MAPK (Phospho-p38 MAPK (Thr180/Tyr182) (12F8) rabbit mAb). We probed the primary antibody binding with a horseradish peroxidase-conjugated donkey anti-rabbit IgG whole Ab (GE Healthcare) as a secondary antibody and used ECL. Prime Western Blotting Detection Reagent (GE Healthcare) for detection. We detected and quantified the chemiluminescence signals using a ChemiDoc XRS Imaging System (Bio-Rad). We incubated the membrane with Restore Western Blot Stripping Buffer (Thermo Scientific Pierce) to be re-probed and then blocked. The membrane was incubated with the primary antibody against total ERK 1/2 or p38 MAPK corresponding to the detected phosphorylated protein. We probed and detected the primary antibody binding in the same way. We calculated the ratio of the chemiluminescence signals from phosphorylated ERK 1/2 or p38 MAPK to those from total ERK 1/2 and p38 MAPK, respectively, for normalization. The primary antibodies were purchased from Cell Signaling Technology, Inc.

**RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (RT-PCR)**

Caco-2 cells pretreated with heat-killed L. brevis KB290 or PBS were added to TRIzol Reagent (Invitrogen) at various times after addition of V. parahaemolyticus or PBS, and total RNA was extracted according to the manufacturer’s instructions. cDNA was synthesized from 1 µg of total RNA using the PrimeScript RT- Reagent Kit (Takara). RT-PCR analysis was conducted using an ABI 7900HT Fast Real Time System (Applied Biosystems) with the SYBER Premix Ex Taq II (Takara) in 96-well plates to a final
volume of 20 µl. The expression levels of IL-8 were normalized with the 18S ribosomal RNA housekeeping gene. The following human-specific primers (Takara) were used: IL-8, forward primer 5'-ACACTGCGCCAACACAGAAATTA-3' and reverse primer 5'-TTTGCTTGAAGTTTCACTGCGATC-3'; 18S ribosomal RNA, forward primer 5'-ACTCAACACCGGAAACCTCAG-3' and reverse primer 5'-AACACGACAATTCCGTCCAC-3'. The melt-curve analysis was carried out to confirm the specificity of the amplified product.

Statistical Analysis
Statistical analyses were based on ANOVA, and the results shown are means from three independent experiments with standard errors. We used the Tukey–Kramer test to compare between-group means and considered p < 0.05 statistically significant. All statistical analyses were carried out with StatView 5 software (SAS Institute).

RESULTS
Pretreatment of V. parahaemolyticus-infected Caco-2 Cells with Heat-Killed LAB Strains Enhanced IL-8 Secretion
All three heat-killed LAB strains we tested [L. brevis KB176, L. brevis KB290, and L. rhamnosus KB377 (GG)] increased the concentration of IL-8 found in the culture medium of Caco-2 cells whether the cells were infected or not, and the increase was dose-dependent within each strain. At 1.0 mg/ml heat-killed LAB, the IL-8 concentration in the media of all infected Caco-2 cells was similar, but at 0.1 mg/ml heat-killed LAB, the IL-8 concentration was highest in the medium of the cells pretreated with heat-killed L. brevis KB290 (Fig. 1A). LDH release, however, was similar in all culture media regardless of pretreatment (Fig. 1B), indicating that the differences in IL-8 secretion were not related to cell viability.

Heat-Killed L. brevis KB290 Accelerated IL-8 Secretion During V. parahaemolyticus Infection
In the absence of V. parahaemolyticus infection, the concentration of IL-8 secreted by heat-killed L. brevis KB290-pretreated Caco-2 cells increased slightly over that of the control (Caco-2 cells pretreated with PBS) at 270 min and then plateaued. In the presence of V. parahaemolyticus infection, the concentration increased significantly over the control (Caco-2 cells pretreated with PBS) at 370 min, but there was no significant difference at 450 min (Fig. 2).

T3SS1 Was Essential for Heat-Killed L. brevis KB290 Enhancement of IL-8 Secretion by Infected Caco-2 Cells
We observed a similar pattern of time-dependent increases in IL-8 secretion by heat-killed L. brevis KB290-pretreated

Fig. 1. Heat-killed lactic acid bacteria enhanced IL-8 secretion by Caco-2 cells during V. parahaemolyticus infection. (A) Caco-2 cells were pretreated with 0 (PBS), 0.1, or 1.0 mg/ml heat-killed lactic acid bacteria (LAB) for 4 h in a CO2 incubator at 37°C, then infected (closed columns) or not infected (open columns) with V. parahaemolyticus, and the IL-8 secreted into the medium was measured 6 h later by ELISA. (B) Caco-2 cells were treated with 0 (PBS), 0.1, or 1.0 mg/ml of heat-killed LAB for 4 h in a CO2 incubator at 37°C, and lactate dehydrogenase (LDH) activity released in the medium was measured by a colorimetric method to evaluate the cell viability. L. brevis KB176 (JCM1059T), the type strain of L. brevis, and L. rhamnosus KB377 (GG, ATCC53103), a well-known probiotic strain, were used to compare with L. brevis KB290. PBS was used as the control. Results are shown as the means of three independent experiments. Bars represent SE. Different letters represent significant differences at p < 0.05 (Tukey–Kramer test).

Fig. 2. Heat-killed L. brevis KB290 accelerated IL-8 secretion by V. parahaemolyticus-infected Caco-2 cells. IL-8 concentration in the medium 0, 180, 270, 360, and 450 min, after V. parahaemolyticus was (closed markers) or was not (open markers) added, was measured by ELISA following 4 h pretreatment of the cells with 0.1 mg/ml heat-killed L. brevis KB290 (squares) or PBS (circles). Results are shown as the means of three independent experiments. Bars represent SE.
Caco-2 cells infected with WT *V. parahaemolyticus* and those infected with ΔVP1680, although the rate of increase was lower in the latter. IL-8 secretion from Caco-2 cells infected with ΔVP1680 was decreased at the same ratio in heat-killed *L. brevis* KB290 or PBS pretreatment from those infected with WT. On the other hand, we observed little increase in IL-8 secretion when the cells were infected with ΔT3SS1 (Fig. 3). These data demonstrate that T3SS1 was essential and VP1680 was the main effector protein for the enhancement of IL-8 secretion during *V. parahaemolyticus* infection, and pretreatment with heat-killed *L. brevis* KB290 of Caco-2 cells affected cellular responses to the effector proteins.

**Heat-Killed *L. brevis* KB290 Enhancement of IL-8 Secretion by Infected Caco-2 Cells Was Attributed to Acceleration of ERK 1/2 Phosphorylation and Enhancement of p38 MAPK Phosphorylation**

Western blot analysis showed that relative to PBS, heat-killed *L. brevis* KB290 accelerated phosphorylation of ERK 1/2 and p38 MAPK during *V. parahaemolyticus* infection. Caco-2 cells were pretreated with heat-killed *L. brevis* KB290 (squares) or PBS (open markers) for 4 h and lysed 0, 90, 180, 270, or 360 min after the addition of *V. parahaemolyticus* (closed markers) or PBS (open markers). Proteins were extracted from cell lysates, and the ratio of phosphorylated ERK 1/2 to total ERK 1/2 (A) and of phosphorylated p38 MAPK to total p38 MAPK (B) were calculated using Western blotting with anti-phospho-p44/42 MAPK (ERK 1/2), anti-phospho-p38 MAPK, anti-p44/42 MAPK (ERK 1/2), and anti-p38 MAPK-specific antibodies to detect those proteins (C, D). The results are shown as means from three independent experiments. Bars represent SE.
ERK 1/2 in infected Caco-2 cells. Phosphorylation peaked at 270 min and then attenuated (Fig. 4A). Although we observed a similar pattern of time-dependent change in p38 MAPK phosphorylation in both heat-killed *L. brevis* KB290-treated and control cells, phosphorylation of the protein was higher in the former (Fig. 4B). These data show that the pretreatment of *V. parahaemolyticus*-infected Caco-2 cells with heat-killed *L. brevis* KB290 induced time-dependent changes in ERK 1/2 phosphorylation and p38 MAPK phosphorylation.

Heat-Killed *L. brevis* KB290 Pretreatment Was Associated with Enhanced IL-8 mRNA Expression in *V. parahaemolyticus*-Infected Caco-2 Cells

Quantitative RT-PCR showed that infected Caco-2 cells expressed IL-8 mRNA sooner and in greater quantity when they were pretreated with heat-killed *L. brevis* KB290 (Fig. 5).

**DISCUSSION**

Previous studies have examined the effects of living probiotics and their culture medium on the secretion of chemokines and cytokines from cells infected with enteropathogenic microorganisms such as enterotoxigenic *Escherichia coli* [25], enterohemorrhagic *E. coli*, *Salmonella Enterica* [20], *Listeria monocytogenes* [1], and *Shigella flexneri* [30]. The effects depend on the species, strains, alive/dead of probiotics, and the species of enteropathogenic microorganisms for the infection. Here, we reported the effects of probiotics on a human intestinal epithelial cell line, Caco-2, infected with *V. parahaemolyticus*, an enteropathogenic organism that has not previously been studied in this context.

Our observation of an increase of IL-8 secretion in Caco-2 cells pretreated with the tested heat-killed LABs contrasts with the findings in other probiotic lactobacill, such as *L. rhamnosus* GG in *Salmonella*-infection, which attenuate IL-8 secretion by competing for adhesion sites on the surface of the host cell [16, 25]. *V. parahaemolyticus* binds to fibronectin and phosphatidic acid of the host cell via multivalent adhesion molecule 7 (MAM7), which is expressed in a wide range of Gram-negative pathogenic microorganisms [14], but ETEC and Salmonellae also have adhesins, which differs from MAM7 in adhesion binding-specificity [13]. It seems that differences in the adhesion mechanisms of pathogenic microorganisms is one of the reasons for the different influences of LAB in IL-8 secretion by infected host cells.

Since *L. brevis* KB290 enhancement of interferon-alpha producing capacity and activation of NK cells [12] were demonstrated in a human trial, we expected it to have an anti-infectional function, and it showed a greater effect on IL-8 secretion by infected Caco-2 cells than *L. brevis* KB176, the type strain of *L. brevis*, and *L. rhamnosus* GG, which tolerates acid and bile and adheres to mucosal cells of the human intestine [22]. IL-8 and its receptor, activated by pathogenic microorganisms, are essential to a mucosal antimicrobial defence [10, 11]. Additionally, IL-8 enhances the migration of intestinal epithelial cells through a CXCR1-dependent pathway [28]. Therefore, *L. brevis* KB290-induced acceleration of IL-8 secretion would be a highly efficient defence against *V. parahaemolyticus*. In contrast, excessive or unnecessary release of microbiocidal products from neutrophils can cause substantial damage to intestinal epithelial cells [18]. Since the total amount of IL-8 secreted by Caco-2 cells pretreated with heat-killed *L. brevis* KB290 was almost the same as that secreted by control cells 450 min after *V. parahaemolyticus* infection, the possibility of excessive IL-8 secretion by heat-killed *L. brevis* KB290 pretreated host cells would be extremely low. A balance between the host pro-inflammatory response and microbial suppression of that response is important for host innate immune responses to enteropathogenic microorganisms, and the contact between bacterial factors such as T3SS1 and the epithelial cell is a key determinant of these innate responses [3]. Furthermore, acute induction of IL-8 in intestinal epithelial cells is sufficient to trigger neutrophil recruitment to the lamina propria, but additional activation signals are needed for full activation and degranulation of neutrophil, mucosal injury, and complete transepithelial invasion.
migration [15]. Further investigation will be required to clarify whether heat-killed L. brevis KB290 pretreatment exerts a negative effect on host defence.

Our laboratory previously reported that the injection of T3SS1 effector proteins is responsible for triggering IL-8 secretion from Caco-2 cells [27]. Our finding here that Caco-2 cells infected with a T3SS1 deletion mutant (ΔT3SS1) showed no enhanced IL-8 secretion, and that the influence of heat-killed L. brevis pretreatment was negligible, suggest that heat-killed L. brevis KB290 pretreatment would modulate the action of T3SS1. Our laboratory also reported that VP1680 plays a pivotal role in manipulating the host cell signaling that leads to IL-8 secretion [27]. IL-8 secretion from Caco-2 cells infected with a VP1680 deletion mutant (ΔVP1680) was decreased at the same ratio in heat-killed L. brevis KB290 or PBS pretreatment from those infected with WT. These results suggest that heat-killed L. brevis KB290 pretreatment would affect the host cell signaling activated by effector proteins, mainly VP1680.

VP1680 enhancement of IL-8 secretion depends to a large extent on increased IL-8 mRNA expression through the phosphorylation of ERK 1/2 and the stabilization of IL-8 mRNA through phosphorylation of p38 MAPK [27]. We found that heat-killed L. brevis KB290 pretreatment did not affect the time-dependent change in p38 MAPK phosphorylation in Caco-2 cells, but it did accelerate ERK 1/2 phosphorylation. Therefore, heat-killed L. brevis KB290 pretreatment resulted in enhanced (i.e., earlier, greater, or both) expression of IL-8 mRNA. The effects of probiotics on ERK 1/2 and p38 MAPK signaling pathways in intestinal epithelial cells are strain specific [24]; sometimes the pathway is activated [2, 26], and sometimes it is inhibited [9, 33]. Further studies are needed to clarify the mechanism of the L. brevis KB290 effects on MAPK phosphorylation in infected Caco-2 cells.

Pretreatment with heat-killed L. brevis KB290 accelerated IL-8 secretion in a human intestinal epithelial cell line infected in vitro by V. parahaemolyticus. Moreover, the pretreatment accelerated IL-8 secretion via early ERK 1/2 phosphorylation and enhanced p38 MAPK phosphorylation through augmentation of the action of effector proteins, mainly VP1680. The acceleration of IL-8 secretion during infection can be a beneficial effect of L. brevis KB290, a probiotic strain, because IL-8 is expected to play an important role in the defence against infection and repair of injured intestinal epithelial cells through rapid migration of immune cells and intestinal epithelial cells.

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