

Specific vaginal lactobacilli suppress the inflammation induced by lipopolysaccharide stimulation through downregulation of toll-like receptor 4 expression in human embryonic intestinal epithelial cells

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Vaginal lactobacilli (VLB) spread from the mother to the infant during vaginal delivery. However, the effects of VLB on infant intestinal function remain unclear. We investigated the probiotic function and immune effects of VLB on the human embryonic intestinal epithelial cell line INT-407. VLB survived artificial gastric juice and adhered to INT-407 cells. Exposure of INT-407 cells to VLB attenuated both the lipopolysaccharide (LPS)-induced stimulation of interleukin-8 and tumor necrosis factor alpha production and the LPS-stimulated upregulation of TLR4 expression. These results suggest that specific VLB suppresses the inflammation induced by LPS stimulation through downregulation of TLR4 expression in human embryonic intestinal epithelial cells.

Key words: vaginal lactobacilli, Toll-like receptor, infant, INT-407 cell

Lactobacilli are predominant in the normal vaginal microbiota [1]. *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus jensenii*, and *Lactobacillus vaginalis* are frequently isolated from the vaginal region of healthy women [2–4]. These vaginal lactobacilli (VLB) suppress the growth of pathogenic microorganisms by lowering the environmental pH through production of lactic acid or antimicrobial substances such as hydrogen peroxide [4]. Moreover, Rose *et al.* [5] have reported that VLB modulate the secretion of inflammatory cytokines by vaginal epithelial cell cultures stimulated by bacterial components. Thus, VLB play an important role in preventing pathogenic bacterial infections through modulation of immune systems in the vaginal tract.

In the human newborn infant, colonization by microorganisms begins around birth. It is usually considered that the newborn infant acquires initial intestinal microbiota from the VLB and fecal bifidobacteria of the mother during delivery [6–8].

Interestingly, Matsumiya *et al.* reported that VLB form the intestinal microbiota in the early stage of newborn infants [6]. The gastrointestinal mucosal immune system is quite immature at birth, which puts the newborn infant at risk for a variety of infections [9]. Therefore, VLB spread from the mother's vagina to the newborn infant may play an important role in the immature immune systems of newborn infants such as those of vaginal tracts. However, the effects of VLB on infant intestinal function remain unclear. In the current study, we investigated the probiotic function of VLB and the immune effect of VLB on human embryonic intestinal epithelial cells.

To evaluate the probiotic effects of VLB, we investigated the viability of VLB in artificial gastric juice and the adhesion of VLB to human embryonic intestinal epithelial cells. *Lactobacillus acidophilus* JCM2124, *L. crispatus* JCM2009, *L. gasseri* JCM5344, *L. jensenii* JCM11034, and *L. vaginalis* JCM9505, which were used as VLB, were obtained from the Japan Collection of Microorganisms (JCM; Ibaraki, Japan). The sources from which these lactobacilli were separated are shown in Table 1. *Lactobacillus rhamnosus*, a GG strain that is a known probiotic *Lactobacillus* [10], was obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). INT-407 cells were purchased from the European Collection of Authenticated Cell Culture (ECACC; Salisbury, UK). Survival of VLB in

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Table 1. Vaginal lactobacilli used in this study

Vaginal lactobacilli		Source
<i>L. acidophilus</i>	JCM2124	Vagina
<i>L. crispatus</i>	JCM2009	Urine
<i>L. gasseri</i>	JCM5344	Vaginal tract
<i>L. jensenii</i>	JCM11034	Vaginal swab
<i>L. vaginalis</i>	JCM9505	Vagina

artificial gastric juice was tested using the protocol of Takeda *et al.* [11]. Briefly, VLB were inoculated into de Man, Rogosa and Sharpe (MRS) medium and cultivated for 24 hr at 37°C. Fresh cultures were then inoculated into MRS medium (5.0×10^5 cfu/ml) with 0.04% pepsin (pH 3.0 or pH 4.0) and incubated for 3 hr at 37°C with shaking at 140 rpm. The surviving VLB were enumerated by poured-plate cultivation. Adhesion of VLB to INT-407 cells was tested according to Takeda *et al.* [11] with modifications. Briefly, INT-407 cells were suspended in Eagle's minimal essential medium with L-glutamine and phenol red (EMEM) containing 10% defined fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT, USA) without antibiotics, adjusted to a density of 1.0×10^5 viable cells/ml, and were cultured in 48-well cell culture plates at 37°C in a humidified 5% CO₂ incubator for 2 hr. The supernatant was then removed. Fresh lactobacilli were suspended at a density of 1.0×10^6 cfu/ml in EMEM containing 10% FBS without antibiotics, seeded on INT-407 cells, and cultured for 2 hr. After incubation, the INT-407 cells were washed three times with 0.01 M phosphate-buffered saline (PBS; pH 7.2), and 0.25% trypsin solution (Wako Pure Chemical Industries, Osaka, Japan) was added to detach the INT-407 cells. The number of VLB adhered to INT-407 cells was enumerated by poured-plate cultivation. Gastric acid plays an important role as a primary bactericidal barrier [12]. The pH in the stomach of adults is between 1.0 and 2.0, whereas the pH in the stomach of infants is higher than 4.0, because gastric secretion is immature [13]. Figure 1 shows the viability of the VLB in the artificial gastric juice. All VLB strains were viable in MRS medium (pH 4.0) with 0.04% pepsin. These results suggest that these VLB tolerate gastric acid and arrive in a live state at the intestinal tract of infants. INT-407 cells are derived from the human embryonic intestinal jejunum and ileum [14]. Table 2 shows the adhesion of VLB to INT-407 cells. The number of VLB that adhered to the cells ranged from 2.0×10^4 to 7.7×10^4 cfu/ml. These results suggest that these VLB adhere to the intestinal cells of infants. Matsumiya *et al.* [6] reported that VLB spread from the mother to the infant during vaginal delivery and colonized the infant

intestine shortly after birth. They also reported that the acquired VLB did not last long-term in the intestine of the infant. *L. rhamnosus* GG is one of the most studied probiotics [10], and it is well established that *L. rhamnosus* GG adheres to the intestinal epithelium [10]. As shown in Table 2, the number of probiotic *L. rhamnosus* GG bacteria that adhered to INT-407 was higher than the number of adhered VLB. These results suggest that VLB cannot adhere as effectively as probiotics to the intestinal epithelium. However, intestinal mucus contains antimicrobial products and secretory IgA and forms a physical and immunological barrier to microbiota [15]. Therefore, our results will need to be further confirmed in the context of in vivo mucosal environments, which are crucial for microbial interaction with (and adhesion to) the gut.

Exogenous mediators such as pathogenic bacteria interact with the intestinal epithelium via pattern-recognition receptors such as the Toll-like receptor (TLR) family [16]. For example, lipopolysaccharide (LPS) derived from gram-negative bacteria binds to TLR4 [17]. Binding to these TLRs activates signals that trigger the induction of inflammatory responses, including the production of inflammatory cytokines [16]. Therefore, we next examined the immune effect of VLB on the LPS-stimulated INT-407 cells. VLB were cultivated in MRS medium for 24 hr at 37°C, collected by centrifugation, washed three times with sterile saline, and lyophilized. INT-407 cells were suspended in EMEM containing 10% FBS and 1% Penicillin-Streptomycin Solution (Wako Pure Chemical Industries) and adjusted to 1.0×10^5 viable cells/ml. The cells were cultured in 48-well cell culture plates in the presence of LPS from *Salmonella* Typhimurium (Wako Pure Chemical Industries) and VLB (0 or 10 µg/ml) at 37°C in a humidified 5% CO₂ incubator for 48 hr. The cell culture supernatants were then stored at -80°C until they were assayed for measurement of interleukin (IL)-8 and tumor necrosis factor alpha (TNFα) levels as inflammatory cytokines. The viability of INT-407 cells was determined with a Guava easyCyte flow cytometry single sample system (Millipore, Hayward, CA, USA) using a Guava ViaCount assay kit (Millipore) according to the manufacturer's instructions, the IL-8 and TNFα levels in the INT-407 cell culture supernatants were determined using human IL-8 and TNFα ELISA MAX™ standard sets (BioLegend, San Diego, CA, USA) according to the manufacturer's instructions, and the expression of INT-407 cell surface TLR4 was determined by immunofluorescent labeling according to our previous study [18] with modifications. Briefly, cell surface TLR4 was detected by incubation

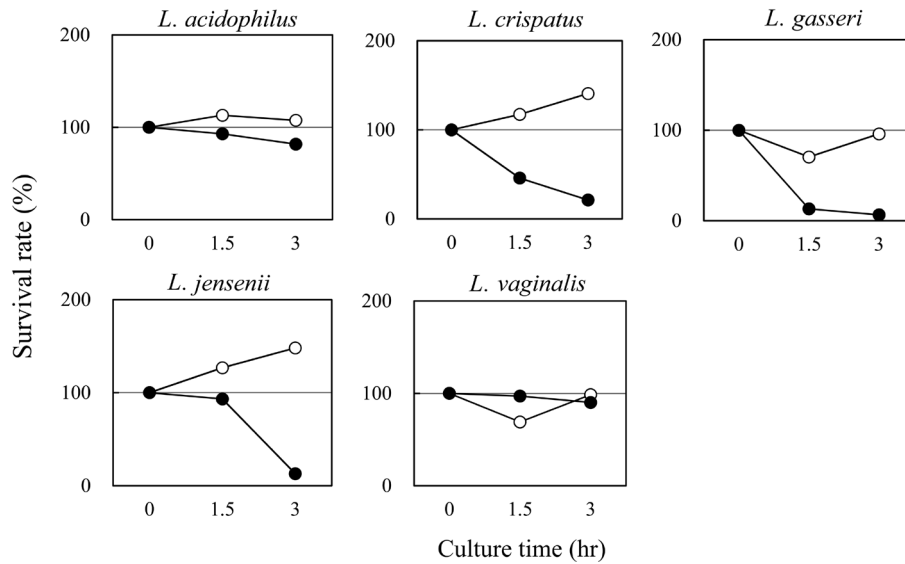


Fig. 1. Survival rate of VLB in artificial gastric juice. The VLB were inoculated into MRS medium with 0.04% pepsin (●, pH 3.0; ○, pH 4.0) and were then incubated for 3 hr at 37°C with shaking at 140 rpm. The surviving VLB were enumerated by poured-plate cultivation.

Table 2. Adhesion of lactobacilli to INT-407 cells

Lactobacilli	Adhesion to INT-407 cells
	($\times 10^4$ cfu/ml)
<i>L. acidophilus</i>	7.2
<i>L. crispatus</i>	2.5
<i>L. gasseri</i>	2.0
<i>L. jensenii</i>	7.7
<i>L. vaginalis</i>	4.7
<i>L. rhamnosus</i> GG	16.0

INT-407 cells were incubated with VLB (1.0×10^6 cfu/ml) for 2 hr. *L. rhamnosus* GG was used as a probiotic strain in this study.

Table 3. Effects of VLB on survival rate and the production of IL-8 and TNF α in cells cultured with LPS

	Survival rate (%)	IL-8 (pg/ml)	TNF α (pg/ml)
Control	56.9 \pm 10.3	285.4 \pm 37.6	1.2 \pm 0.7
LPS	57.9 \pm 0.8	405.9 \pm 59.5*	5.8 \pm 1.5*
LPS + <i>L. acidophilus</i>	49.8 \pm 1.6	400.9 \pm 22.4*	1.9 \pm 1.1
LPS + <i>L. crispatus</i>	67.9 \pm 1.3*	375.1 \pm 28.1	1.9 \pm 1.4
LPS + <i>L. gasseri</i>	63.6 \pm 5.8	293.4 \pm 29.0	0.2 \pm 0.3
LPS + <i>L. jensenii</i>	48.2 \pm 1.7	403.4 \pm 5.2*	0.1 \pm 0.1
LPS + <i>L. vaginalis</i>	47.8 \pm 6.4	461.8 \pm 19.5*	3.7 \pm 1.5*

INT-407 cells were cultured with LPS (0 or 10 μ g/ml) and VLB (0 or 10 μ g/ml) for 48 hr. The IL-8 and TNF α concentrations in the cell cultures were measured using ELISA. The cell survival rate was determined using a Guava easyCyte flow cytometry single sample system. The data are presented as means \pm SD (n=3). *p<0.05 (vs. control).

of the cells with a mouse anti-human TLR4 monoclonal antibody (clone 76B357.1, Imgenex San Diego, CA, USA) for 15 min at 4°C, followed by incubation with a fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG monoclonal antibody (sc-3699, Santa Cruz Biotechnology, Dallas, TX, USA) for 15 min at 4°C. The mean fluorescence intensity (MFI) of TLR4-positive cells was then determined using the Guava easyCyte flow cytometry single sample system. Data are presented as the mean \pm standard deviation (SD). Statistical analyses were performed using Dunnett’s multiple comparison tests for one-way analysis of variance. Differences were considered significant when p values were less than 0.05. The intestine is exposed to exogenous inflammatory

mediators. Table 3 shows the cell survival rate and the IL-8 and TNF α levels in LPS-stimulated INT-407 cell cultures in the presence or absence of specific VLB. The cell survival rate was significantly higher in the cell culture with LPS and *L. crispatus* than in the control culture (no added LPS and VLB). In addition, there were no significant decreases in cell survival rate between any of the other cell cultures. These results indicate that IL-8 and TNF α production is not influenced by a decrease in the cell survival rate. The IL-8 level was significantly higher in the cell culture with LPS than in the control culture, while the IL-8 level in the cell culture with LPS and *L. crispatus* and that in the cell culture with LPS and *L. gasseri* showed no significant difference

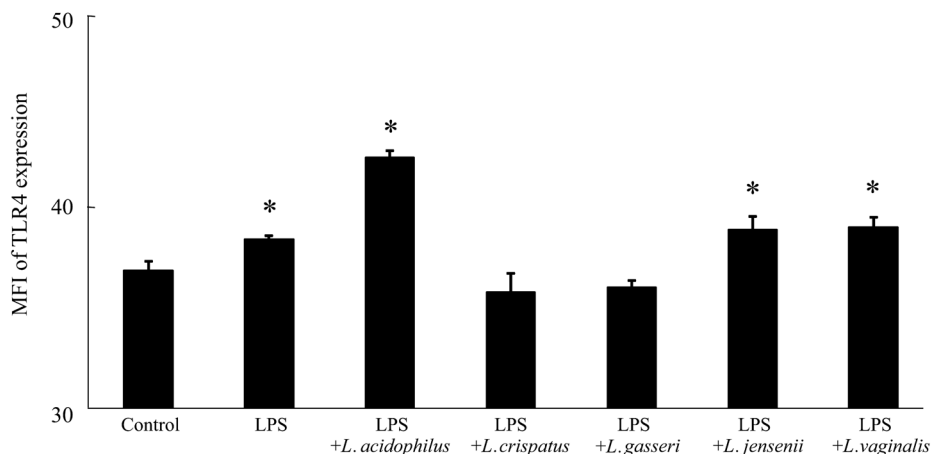


Fig. 2. MFI of TLR4 expression in LPS-stimulated INT-407 cell cultures in the presence of specific VLB. INT-407 cells were cultured with LPS (0 or 10 $\mu\text{g/ml}$) and the indicated VLB (0 or 10 $\mu\text{g/ml}$) for 48 hr. The MFI of TLR4-positive cells was then determined using a Guava easyCyte flow cytometry single sample system. Data are presented as the mean \pm SD (n=3). * $p < 0.05$ (compared with control using Dunnett's multiple comparison test).

compared with the control culture. The TNF α level was significantly higher in the cell culture with LPS than in the control culture, while the TNF α level in the cell culture with LPS and VLB, except *L. vaginalis*, showed no significant difference compared with the control culture. These results suggest that some VLB suppress the IL-8 and TNF α productions that is induced by LPS stimulation in INT-407 cell cultures. Figure 2 shows the MFI of TLR4 expression in INT-407 cells stimulated by LPS in the presence or absence of various VLB. The MFI of TLR4 expression was significantly higher in the cell culture with LPS than in the control culture, while the MFI of TLR4 expression in the cell culture with LPS and *L. crispatus*, and that in the cell culture with LPS and *L. gasseri* showed no significant difference compared with the control culture. These results suggest that some VLB suppress the enhancement of TLR4 protein expression in INT-407 cells that is stimulated by LPS. However, the MFI of TLR4 expression was significantly higher in the cells cultured with LPS in the presence of *L. acidophilus* or *L. jensenii* than in the control culture. These VLB may suppress the production of TNF α through the inhibition of activation of TLR4 signaling rather than through the suppression of TLR4 protein expression in the cells. Therefore, it will be necessary to obtain further evidence on cellular signaling in this model.

Ren *et al.* [19] reported that some lactobacilli inhibit *S. Typhimurium*-induced IL-8 production through competitive adhesion to intestinal epithelial cells. In the present study, we did not observe immunological

interaction between the anti-inflammatory effects and the adhesion of VLB to the INT-407 cells. It is known that TLR9 recognizes bacterial deoxyribonucleic acid containing unmethylated CpG dinucleotides (CpG-DNA) [20]. Furthermore, Yu *et al.* [21] reported that signal activation of TLR9 inhibited LPS-mediated TLR4 signaling and expression via regulation of multiple cyclin-dependent kinase pathways. Moreover, it is known that *Lactobacillus* strains and the abovementioned bacterial oligodeoxynucleotides bind to TLR9 and induce the innate immune response through TLR9 in human intestinal epithelial cells [22, 23], and Ju *et al.* [24] reported that INT-407 (Henle 407) cells express the majority of the TLRs (with the exception of TLR8) and that CpG-DNA activated signaling in this cell line. In the present study, we investigated the immune effect of CpG-DNA (Hycult Biotech, Uden, Netherlands), a known TLR9 ligand, on LPS-stimulated INT-407 cells. Figure 3 shows the IL-8 and TNF α levels and the MFI of TLR4 expression in LPS-stimulated INT-407 cell cultures in the presence of 1 or 10 μM CpG-DNA. The levels of these cytokines and TLR4 were significantly higher in the cells cultured with LPS than in the control culture (no added LPS or CpG-DNA). In addition, cytokine levels in cells cultured with both LPS and CpG-DNA showed no significant differences compared with those in the control culture. These results suggested that specific VLB suppress the production of inflammatory cytokines and TLR4 expression that are otherwise induced by LPS stimulation of INT-407 cells through TLR9.

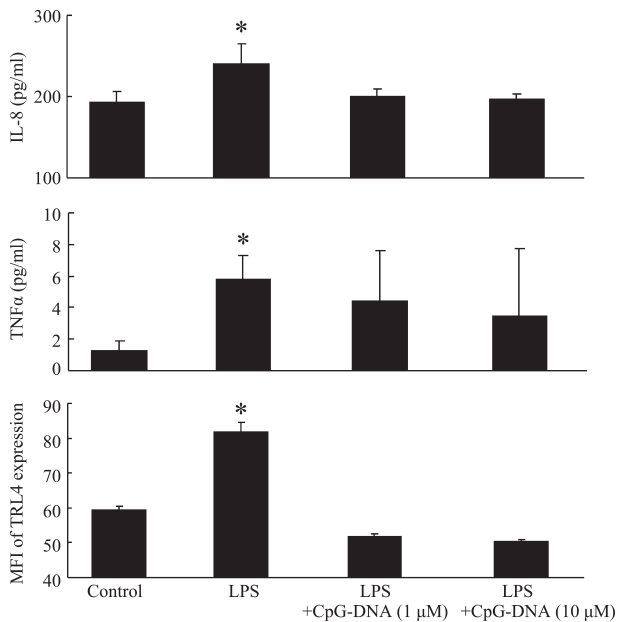


Fig. 3. The IL-8 and TNF α productions and MFI of TLR4 expression in LPS-stimulated INT-407 cell cultures in the presence of CpG-DNA. INT-407 cells were cultured with LPS (10 μ g/ml) and the indicated CpG-DNA (1 or 10 μ M) for 48 hr. The IL-8 and TNF α concentrations in the cell cultures were measured using ELISA. The MFI of TLR4-positive cells was then determined using a Guava easyCyte flow cytometry single sample system. Data are presented as the mean \pm SD (n=3). *p<0.05 (compared with control using Dunnett's multiple comparison test).

Necrotizing enterocolitis (NEC) is one of the most common and severe gastrointestinal conditions in preterm newborn infants, and it has a high mortality rate [25]. Premature birth and abnormal bacterial colonization are the predisposing factors for NEC [25]. The pathogenesis of NEC is associated with an excessive inflammatory IL-8 response [26]. In addition, LPS stimulation through TLR-4 signaling leads to NEC development [27]. The present study suggests that specific VLB suppress the inflammation induced by LPS stimulation through the downregulation of TLR4 expression in human intestinal epithelial cells. Thus, these VLB may prevent NEC in infants.

In conclusion, we established the probiotic function and immune effect of VLB in human embryonic intestinal epithelial cells. Our results suggested that specific VLB suppress the production of inflammatory cytokines that are induced by LPS stimulation through the downregulation of TLR4 expression in human intestinal epithelial cells and further indicated that the TLR9 ligand plays an important role in these effects. Thus, these VLB

may play an important role in the development of the immature immune system in infants.

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