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Lactobacillus delbrueckii ssp. *lactis* R4 Prevents *Salmonella typhimurium* SL1344-Induced Damage to Tight Junctions and Adherens Junctions

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Cell junctions are the gatekeepers of the paracellular route and defend the mucosal barrier. Several enteropathogenic bacteria can invade intestinal epithelial cells by targeting and damaging cell junctions. It is not well understood how *Salmonella typhimurium* is able to overcome the intestinal barrier and gain access to the circulation, nor is it understood how *Lactobacillus* prevents the invasion of *S. typhimurium*. Therefore, we sought to determine whether infection with *S. typhimurium* SL1344 could regulate the molecular composition of cell junctions and whether *Lactobacillus delbrueckii* ssp. *lactis* R4 could affect this modification. Our data demonstrated that infection of Caco-2 cells with *S. typhimurium* over 2 h resulted in a redistribution of claudin-1, ZO-1, occluding, and E-cadherin. Western blot analysis of epithelial cell lysates demonstrated that *S. typhimurium* could decrease the expression of cell junction proteins. However, *L. delbrueckii* ssp. *lactis* R4 ameliorated this destruction and induced increased expression of ZO-1, occludin, and E-cadherin relative to the levels in the control group. The results of these experiments implied that *S. typhimurium* may facilitate its uptake and distribution within the host by regulating the molecular composition of cell junctions. Furthermore, *Lactobacillus* may prevent the adhesion and invasion of pathogenic bacteria by maintaining cell junctions and the mucosal barrier.

Keywords: *Lactobacillus*, *S. typhimurium*, tight junction, adherens junction

Introduction

The intestinal mucosal barrier, which is composed of mucosal epithelium, a slime layer, the mucosal immune system and the normal micropopulation, plays an important role in defending against the invasion and diffusion of enteropathogenic microorganisms (Deitch, 2002). The cell junctions encircling gastrointestinal epithelial cells are also a critical component of the mucosal barrier. Tight junctions and adherens junctions are located between the lateral membranes of epithelial cells. The intracellular space between epithelial cells forms the paracellular route between adjacent cells, and the tight junctions and adherens junctions control the diffusion of fluid, electrolytes, macromolecules and pathogens along the paracellular route (Gumbiner, 1993). Tight junctions are composed of several proteins including transmembrane proteins (occludin and the claudins) and intracellular proteins (ZOs) (Furuse *et al*., 1993). E-cadherin is a transmembrane protein that is part of the adherens junction and controls the correct assembly of tight junctions. However, it has become increasingly apparent that several bacterial pathogens are able to overcome the barrier presented by epithelial cell-cell junctions and enter the host, thereby introducing toxins into the host through the paracellular route (Nusrat *et al*., 2001; Wroblewski *et al*., 2009). *Salmonella* species are a major cause of food poisoning, which induces diarrhea. Like other enteropathogens, *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) has developed several methods to breach the mucosal epithelial barrier and gain access to deeper tissues; these methods include the direct internalization by epithelial cells (Bruno *et al*., 2009) and capture by dendritic cells or microfold cells. Recent findings suggest that *S. typhimurium* can disrupt cell junctions, influencing paracellular permeability (Kohler *et al*., 2007). There is accumulating evidence that *Lactobacillus* is able to effectively preventing intestinal infection in both humans and animals due to its ability to inhibit the adhesion of pathogens, such as *S. typhimurium* and *Helicobacter*, to the intestinal mucosa (Lehto and Salminen, 1997; Sgouras *et al*., 2004; De Keersmaecker *et al*., 2006; Yu *et al*., 2011). We still do not know whether *Lactobacillus* can protect cell junctions from the damage caused by *S. typhimurium* and maintain the integrity of the paracellular route. The data in this area are relatively sparse and often controversial.

In this study, we hypothesized that epithelial cells treated with *Lactobacillus* may be protected from the deleterious effects of subsequent infection with *S. typhimurium* SL1344 and that *Lactobacillus* may sustain the cell junctions. The goal of the present study was to further elucidate the mechanisms underlying the effects of *L. delbrueckii* ssp. *lactis* R4 and *S. typhimurium* SL1344 on tight junctions and adherens junctions.

Materials and Methods

Bacterial growth

S. typhimurium SL1344 was grown in Luria-Bertani (LB) broth, pH 7.0. After an overnight incubation at 37°C with

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vigorous shaking, the bacteria were diluted 1:100 in fresh LB and grown for 2 h until the mid-log phase for all experiments. *L. delbrueckii* ssp. *lactis* R4 was grown in DeMan Rogosa Sharp (MRS) medium at 37°C under anaerobic conditions. After an overnight incubation, strain R4 was diluted 1:10 in fresh MRS and grown for 4 h until the mid-log phase. The bacterial concentrations were confirmed by serial dilution followed by CFU counting. The counting of *S. typhimurium* SL1344 was performed on LB agar after a 16 h incubation, and the counting of lactobacillus was performed on MRS agar after a 48 h incubation under anaerobic conditions. For each experiment, the bacteria were harvested by centrifugation at 3,000×g for 10 min and then resuspended in antibiotic-free DMEM medium.

Cell culture

Caco-2 cells, a model of mature enterocytes of the small intestine, were obtained from the American Type Culture Collection (ATCC, USA). Caco-2 cells were maintained in DMEM supplemented with 10% fetal bovine serum. The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. The confluent monolayers of Caco-2 cells were trypsinized, washed and resuspended and then seeded on Millicell filter inserts (1 μm, Millipore, USA) at a seeding density of 10^4 cells/cm^2. The cells reached confluence in 7–8 days and were used for experimentation between days 14 and 21. The medium was changed every other day. The cell culture medium was replaced with fresh medium without antibiotics prior to treatment of the cells with bacteria.

The influences of *Lactobacillus* and *S. typhimurium* on the distribution of cell junction proteins

When the Caco-2 cells reached confluence on the Millicell filter inserts, *Lactobacillus* (10^9 CFU/L) and/or enteropathogenic bacteria (ETEC K88 or SL1344, 10^8 CFU/L) diluted in DMEM without antibiotics were added to the apical compartments of the Millicell filter inserts either alone or simultaneously for 2 h. The immunofluorescence staining protocol was adapted from the protocol of Zareie *et al.* (2005). Briefly, confluent Caco-2 cell monolayers were rinsed in PBS, followed by fixation and permeabilization in 5% formaldehyde for 15 min. Then, cells were incubated in 5% normal goat serum in PBS for 1 h at room temperature and then incubated with primary cell junction protein antibodies (Zymed, USA) for 1 h at 37°C. After the unbound primary antibodies were rinsed away with PBS, the cells were incubated with the secondary Cy3-conjugated goat anti-rabbit IgG (1:200 dilution; Zymed) or FITC-conjugated goat anti-mouse IgG (1:500 dilution; Boster) for 1 h at room temperature. The cells were thoroughly rinsed with PBS and examined with a fluorescence microscope (Olympus BX5, Japan).

The influences of *Lactobacillus* and *S. typhimurium* on the expression of cell junction proteins

After the Caco-2 cells were treated with bacteria, the cells were homogenized in chilled RIPA buffer (150 mM NaCl, 50 mM Tris-HCl; pH 7.4, 0.5% sodium deoxycholate, 1% Triton X-100, 1 mM EDTA), including protease and phosphatase inhibitors (1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF and 5 g/ml of each of aprotinin, leupeptin, and pepstatin). After centrifugation at 10,000×g for 10 min at 4°C, the supernatant was recovered and assayed for protein content. The protein concentrations were determined by the Bradford method.

Equal amounts of total protein were separated on 10% SDS-polyacrylamide gels and then transferred to PVDF (polyvinylidene fluoride) membranes. After blocking over-

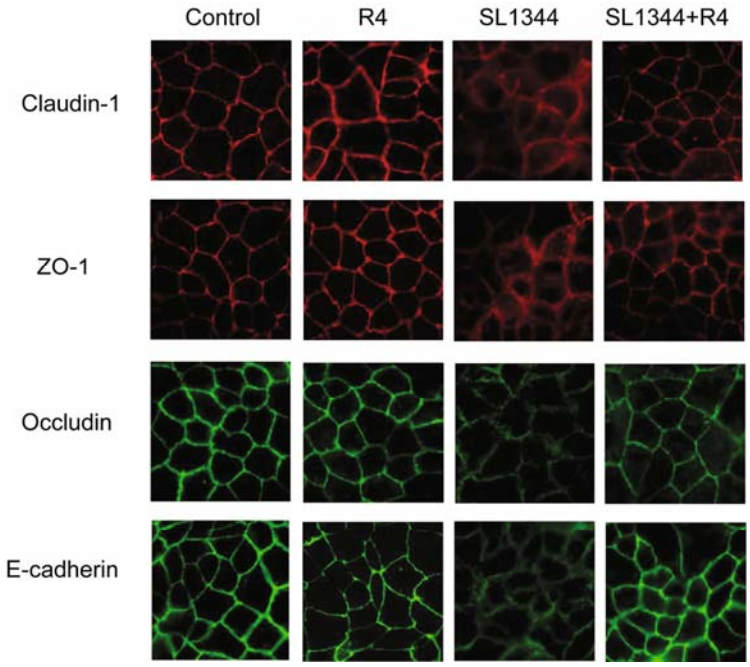

Fig. 1. *Lactobacillus* and *S. typhimurium* modulate tight junctions and adherens junctions in Caco-2 cells. Immunostaining for tight junction proteins (claudin-1, ZO-1 and occludin) and an adherens junction protein (E-cadherin) after apical stimulation with *S. typhimurium* SL1344 and/or *Lactobacillus* for 2 h. Original magnification, 100×.

night in Tris-buffered saline (TBS) containing 0.05% Tween (TBST) and 5% dry powdered milk, the membranes were washed three times for 5 min each with TBS-T and incubated overnight at 4°C with the primary antibody (from Zymed). After three washes with TBST, the membranes were incubated for 1 h with the horseradish peroxidase-conjugated secondary antibody. Following two washes with TBST and one wash with TBS, the membranes were developed for visualization of the protein by the addition of an enhanced chemiluminescence reagent (Pierce, USA).

Statistics

The results were expressed as the means±standard errors of the means. Significant differences between multiple groups were calculated using analysis of variance (ANOVA).

Results

Lactobacillus and *S. typhimurium* modulate the distribution of cell junction proteins in polarized intestinal epithelia

In this experiment, we explored the impact of *L. delbrueckii* ssp. *lactis* R4 and *S. typhimurium* SL1344 on the distributions of claudin-1, ZO-1, occluding, and E-cadherin (Fig. 1). In the control group, the four proteins visualized using fluorescence were distributed at the intercellular borders, encircling the cells and delineating the cellular borders. The results also demonstrated that *L. delbrueckii* ssp. *lactis* R4 did not affect the distribution of the four cell junction proteins relative to the distribution observed in the control group. The epithelial cells still possessed intact cell boundaries, and the fluorescence was spread out in a continuous manner. However, when the cells were infected with *S. typhimurium* SL1344, the fluorescence levels of claudin-1, ZO-1, occluding, and E-cadherin became weak, and these proteins were dispersed in the cytoplasm. Furthermore, the cell border was discontinuous, indicating that the cell junction structures had been broken down. Interestingly, when the Caco-2 cells were co-cultured with *L. delbrueckii* ssp. *lactis* R4 and *S. typhimurium* SL1344, the distribution of green or red spots increased, and the fluorescence boundary became clear gradually relative to that in the infection group, indicating that the changes in the distributions of claudin-1, ZO-1, occluding, and E-cadherin caused SL1344 were ameliorated.

Lactobacillus and *S. typhimurium* modulate the expression of cell junction proteins in polarized intestinal epithelia

We also analyzed whether *L. delbrueckii* ssp. *lactis* R4 or *S. typhimurium* SL1344 could regulate the expression of tight junction proteins (claudin-1, ZO-1, and occludin) and an adherens junction protein (E-cadherin) by Western blot (Fig. 2). *L. delbrueckii* ssp. *lactis* R4 had little influence on the expression of these cell junction proteins. Relative to the expression in the control group, the expression of the transmembrane tight junction protein claudin-1 was increased significantly when the cells were co-cultured with *L. delbrueckii* ssp. *lactis* R4 ($P<0.05$). However, when Caco-2 cells were infected with *S. typhimurium* 1344, the expression levels of ZO-1, occluding, and E-cadherin were all considerably lower ($P<0.01$). Furthermore, the change in the ZO-1 level caused by *S. typhimurium* SL1344 was the greatest (control band intensity of ZO-1, 1.20±0.07; band intensity of ZO-1 from cells infected with *S. typhimurium* SL1344, 0.085±0.004). Interestingly, *S. typhimurium* SL1344 did not affect the expression of claudin-1, a result that is similar to those reported previously. This finding is in contrast to the results for *S. flexneri* and *Clostridium perfringens*, which are able to specifically target and regulate claudin-1. Furthermore,

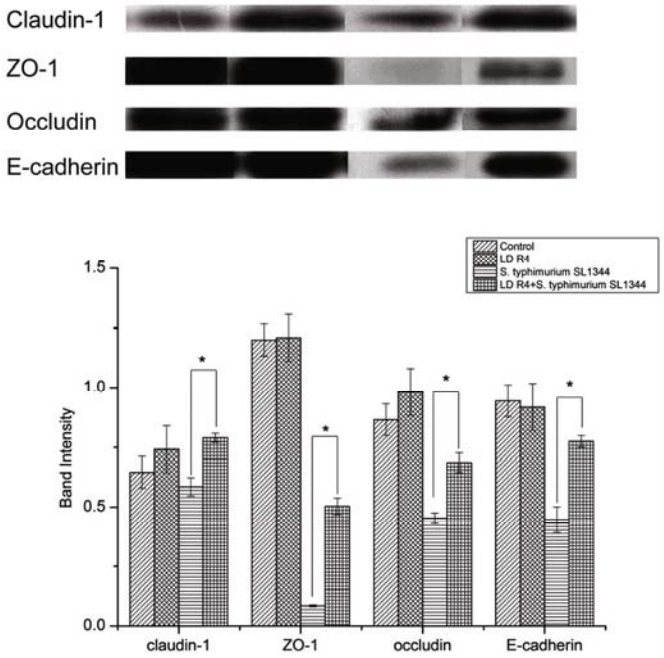

Fig. 2. Ability of *Lactobacillus* and *S. typhimurium* to modulate the epithelial cell tight junctions and adherens junctions. The apical infection of Caco-2 cell monolayers with *S. typhimurium* SL1344 leaded to the rapid negative regulation of ZO-1, occludin and E-cadherin in the epithelial monolayer. *L. delbrueckii* ssp. *lactis* R4 ameliorated this change. Equal amounts of protein were subjected to electrophoresis. The upper panels are representative blots, and the lower panels show the mean±SEM from the densitometric analyses, n=4. *$P<0.01$ (ANOVA)

when *Lactobacillus* and *S. typhimurium* SL1344 were co-cultured with Caco-2 cell monolayers simultaneously, the expression levels of the four cell junction proteins were all increased significantly ($P<0.01$) relative to the levels in the infected group.

Discussion

In this study, we investigated the mechanisms underlying the regulation of cell junctions by *Lactobacillus* and *S. typhimurium*. A number of enteric pathogens are known to disrupt the intestinal epithelial barrier and influence paracellular permeability by altering the distribution and expression of cell junction proteins. For example, enteroinvasive *Escherichia coli* increased the paracellular permeability by reducing the expression of F-actin and tight junction proteins (Resta-Lenert and Barrett, 2009), whereas enterotoxigenic *Bacteroides fragilis* damaged the epithelial barrier by degrading E-cadherin (Obiso *et al*., 1997). It is known that *S. typhimurium* can cause the gross disruption of epithelial cells by disrupting the cytoskeleton. In this study, we demonstrated that *S. typhimurium* SL1344 can not only affect the tight junction proteins claudin-1, occludin and ZO-1 but can also influence the redistribution and expression of the adherens junction protein E-cadherin.

Claudin-1 and occludin are membrane-spanning proteins that form extracellular loops, ZO-1 is the linkage between transmembrane proteins and the cytoskeleton, and E-cadherin is an essential component of adherens junctions. These four proteins play primary roles in the maintenance of tight junctions and adherens junctions, thereby regulating paracellular transport. We also demonstrated that *S. typhimurium* SL1344 induced only weak modulation of claudin-1 expression, although the influence on the expression levels of ZO-1, occludin, and E-cadherin was tremendous. These were important observations because these data indicated that both the structures of both the tight junctions and adherens junctions were disrupted during the *S. typhimurium* SL1344 invasion process. Furthermore, because tight junctions are the gatekeepers of paracellular route, the permeability of the epithelium may also be increased.

Lactobacillus spp., probiotic bacteria surviving in the gastrointestinal tract, are beneficial for the development of the mucosal immune system. It has also been reported that these bacteria exert their beneficial effects by producing bacteriostatic or bactericidal agents (Takahashi *et al*., 2004; Corr *et al*., 2007), thus competitively excluding pathogenic bacteria (Sherman *et al*., 2005). However, the mechanisms underlying the influence of *Lactobacillus* on mucosal barrier have not been sufficiently elucidated. Our previous study demonstrated that *L. delbrueckii* ssp. *lactis* R4 exerted a stronger antagonistic effect against the invasion of *S. typhimurium* SL1344. When Caco-2 cells were co-cultured with *L. delbrueckii* ssp. *lactis* R4 and *S. typhimurium* SL1344 simultaneously, the invasion rate of *S. typhimurium* SL1344 into Caco-2 cells was significantly lower (Yu *et al*., 2011). In this experiment, we used cultured polarized epithelial cells (Caco-2 monolayers) to investigate whether *L. delbrueckii* ssp. *lactis* R4 partially inhibits *S. typhimurium*-induced damage to cell junctions. Our findings showed that *L. delbrueckii* ssp. *lactis* R4 did not affect the distribution or expression of occludin, ZO-1 and E-cadherin and maintained the integrity of the mucosal barrier. Furthermore, *L. delbrueckii* ssp. *lactis* R4 enhanced the expression of the tight junction protein claudin-1. These results implied that *L. delbrueckii* ssp. *lactis* R4 could maintain the limited permeability of the paracellular route and prevent the transport of pathogenic bacteria and toxins. Furthermore, *L. delbrueckii* ssp. *lactis* R4 ameliorated the *S. typhimurium*-induced redistributions of claudin-1, occludin, ZO-1 and E-cadherin. To support the microscopy observations, we also employed Western blotting techniques to determine the expression levels of claudin-1, occludin, ZO-1, and E-cadherin. The expression levels of the four cell junction proteins were also improved significantly, which implied that *Lactobacillus* prevented the damage to tight junctions and adherens junctions caused by *S. typhimurium* SL1344.

Different hypotheses might explain how *L. delbrueckii* ssp. *lactis* R4 is able to maintain the structure of cell junctions destroyed by *S. typhimurium* SL1344. Several cellular signaling pathways implicated in tight junction and adherens junction regulation could be a target for *Lactobacillus*. For example, *L. reuteri* can enhance activities of mitogen-activated protein kinases (MAPKs), including c-Jun N-terminal kinase and p38 MAPK (Iyer *et al*., 2008). The MAPK signal transduction pathway was activated, the tight junction protein claudin-1 was up-regulated, and the transepithelial electrical resistance was increased (Howe *et al*., 2005). Additionally, *L. delbrueckii* ssp. *lactis* R4 might also modulate cytoskeleton arrangement, which is important in maintaining cell junction structure.

Taken together, the results of this study indicated that *L. delbrueckii* ssp. *lactis* R4 had the ability to protect against the *S. typhimurium* SL1344-induced damage of the epithelial monolayer barrier function by preventing changes in host cell junction morphology. Further work is necessary to elucidate how *Lactobacillus* and *S. typhimurium* modulate signal transduction to affect cell junctions. The influence of bacteria on cell junctions remains an interesting area because the influences of *Lactobacillus* and pathogenic bacteria on the intestinal mucosal barrier have important implications in the prevention of diarrhea and bacterial translocation.

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