TRANSLATIONAL PHYSIOLOGY

Regulation of human epithelial tight junction proteins by *Lactobacillus plantarum* in vivo and protective effects on the epithelial barrier

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1TI Food & Nutrition, Nieuwe Kanaal, Wageningen; 2Host-Microbe Interactomics Group, Animal Sciences, University of Wageningen; 3Department of Internal Medicine, Division of Gastroenterology & Hepatology, Nutrition and Toxicology Research Institute Maastricht (NUTRIM), Maastricht University, Maastricht; 4NIZO Food Research, Ede; 5Laboratory of Microbiology, Wageningen University, Wageningen, The Netherlands; 6School of Health and Medical Sciences, Örebro University, Örebro, Sweden

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Karczewski J, Troost FJ, Konings I, Dekker J, Kleerebezem M, Brummer RM, Wells JM. Regulation of human epithelial tight junction proteins by *Lactobacillus plantarum* in vivo and protective effects on the epithelial barrier. *Am J Physiol Gastrointest LiverPhysiol* 298: G851–G859, 2010. First published March 11, 2010; doi:10.1152/ajpgi.00327.2009.—*Lactobacillus plantarum*, a commensal bacterium of humans, has been proposed to enhance the intestinal barrier, which is compromised in a number of intestinal disorders. To study the effect of *L. plantarum* strain WCFS1 on human barrier function, healthy subjects were administrated *L. plantarum* or placebo in the duodenum for 6 h by means of a feeding catheter. The scaffold protein zonula occludens (ZO)-1 and transmembrane protein occludin were found to be significantly increased in the vicinity of the tight-junction (TJ) structures, which form the paracellular seal between cells of the epithelium. In an in vitro model of the human epithelium, *L. plantarum* induced translocation of ZO-1 to the TJ region; however, the effects on occludin were minor compared with those seen in vivo. *L. plantarum* was shown to activate Toll-like receptor 2 (TLR2) signaling, and treatment of Caco-2 monolayers with the TLR2 agonist Pam-Cys-SK4(PCSK) significantly increased fluorescent staining of occludin in the TJ. Pretreatment of Caco-2 monolayers with *L. plantarum* or PCSK significantly attenuated the effects of phorbol ester-induced dislocation of ZO-1 and occludin and the associated increase in epithelial permeability. Our results identifying commensal bacterial stimulation of TLR2 in the gut epithelium as a regulator of epithelial integrity have important implications for understanding probiotic mechanisms and the control of intestinal homeostasis.

epithelium; intestine

TRANSLATIONAL HIGHLIGHTS

This study demonstrates that, in the small intestine of healthy subjects, administration of *Lactobacillus plantarum* induced changes in the epithelial tight junctions, resulting in increased staining of the scaffold protein zonula occludens-1 and the transmembrane protein occludin. *Lactobacillus plantarum*-induced translocation of zonula occludens-1 to the tight-junction region was also seen in an in vitro model of the human epithelium, and this significantly protected against chemically induced disruption of the tight junction and the associated increase in epithelial permeability. The mechanism was shown to be dependent on Toll-like receptor 2 signaling and highlights the homeostatic role of innate signaling pathways in maintaining human intestinal epithelial barrier functions.

Increased permeability of the intestinal epithelium or “leaky gut” is now recognized as having a role in the pathophysiology of a variety of gastrointestinal disorders and is observed in inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), celiac disease, and the early stages of colon cancer development in mice (6). In IBD, altered permeability increases the infiltration of proinflammatory stimuli to the underlying immune cells, triggering further cytokine-induced changes to the tight junction (TJ) and a vicious cycle of mucosal barrier dysfunction and inflammation (2, 21). Similarly in the diarrhea-predominant form of IBS, altered permeability of the epithelium is thought to increase the load of bacterial and dietary antigens in the lamina propria, leading to the activation of mucosal immune responses involved in the generation of diarrhea and visceral hypersensitivity (1). Altered intestinal epithelial permeability is also associated with diabetes, celiac disease, diarrhea in HIV-infected patients, atopic eczema, and altered sensitivity to food allergens (8, 9, 30, 31, 44). Consequently, modulation of epithelial permeability is a highly relevant target for novel therapeutic or prophylactic treatments against a range of diseases.

The crucial structures that hold epithelial cells together and control epithelial paracellular permeability are the TJ that encircle the apical parts of the lateral surfaces of the adjacent epithelial cells to create “kisses” in the plasma membrane. The large submembrane component of the TJ comprises a complex of integral membrane proteins including occludin, claudins, and junction adhesion molecule that interact with the zonulins zonula occludens (ZO)-1, ZO-2, and ZO-3 that are in turn bound to the perijunctional ring of cytoskeletal actin (29, 37). Alterations to TJ are associated with changes in epithelial permeability as confirmed by studies on the overexpression of different claudins and RNA interference experiments in polarized cell lines coupled with measurements of transepithelial electrical resistance (TER) (12). A number of protein kinase C (PKC) isoform signaling pathways have been implicated in the...
regulation of TJs. These pathways can dramatically increase transepithelial flux of macromolecules without cell death or loss of barrier integrity (39).

Several species of *Lactobacillus* are naturally present in the human intestinal tract, and several species and strains have been evaluated for their probiotic activity. Certain probiotic strains have given significant and promising results in human clinical trials and experimental animal models of gastrointestinal disease. The enhancement of epithelial barrier function is one of the proposed mechanisms by which certain probiotic organisms may confer beneficial activities (7). Some probiotic studies in humans have reported a decrease in intestinal permeability (36, 38), whereas others have been negative or inconclusive (11, 24), suggesting that this activity may depend on the probiotic strain and species as well as the target population and its resilience capacity of the intestinal mucosa. Evidence for probiotic effects on barrier function has also been demonstrated in rat models of chronic stress, hemorrhagic shock, and sepsis although the mechanisms have not been elucidated (32, 47). Recently, the probiotic *Escherichia coli* (*E. coli*) Nissle 1917 was shown to inhibit a leaky gut by upregulation of ZO-1 expression in murine intestinal epithelial cells and to confer protection from dextran sodium sulfate (DSS)-induced colitis (41, 48). In addition, there is evidence from in vitro studies that particular strains of *Lactobacillus* spp can protect against intestinal barrier dysfunctions caused by invasive pathogens or proinflammatory cytokines (13, 28, 34, 35).

Several animal studies have shown that probiotic strains of *Lactobacillus plantarum* (*L. plantarum*) can reduce the hyperpermeability associated with experimentalenterocolitis (23), biliary obstruction (46), and infection with an enteropathogen (20). In vitro studies using polarized Caco-2 cell monolayers have also shown protective effects of *L. plantarum* strains on the reduction of TER induced by tumor necrosis factor-α (TNF-α) and *Listeria monocytogenes* (*L. monocytogenes*) (17, 18). In the latter study, changes in the total amount of cellular ZO-1 were implicated in the opposing effects of *L. plantarum* and *L. monocytogenes*, but effects on TJ composition itself were not studied.

The mechanisms by which *L. plantarum* enhances epithelial integrity have not been demonstrated, but they might conceivably involve activation of the Toll-like receptor 2 (TLR2) signaling pathway, as synthetic ligands for TLR2 have been shown to transiently enhance epithelial resistance in vitro (5). Furthermore, oral treatment of DSS-induced colitis in the mouse with the synthetic TLR2 ligand Pam3Cys-SK4 (PCSK) was demonstrated to significantly suppress mucosal inflammation and apoptosis by restoring the TJ-associated integrity of the intestinal epithelium (4).

Given the existing evidence for the protective effects of selected probiotics on epithelial barrier function and the potential role of ZO-1, we set out to investigate the effects of administering *L. plantarum* strain WCFS1 (16) on the levels of TJ-associated ZO-1 and occludin in the duodenal epithelium in humans. In parallel, a polarized Caco-2 cell model of the intestinal epithelium was employed to investigate the physiological effects of exposure to *L. plantarum* on TJ protein distribution, paracellular permeability, and protection from barrier disruption. The potential role of TLR2 signaling in barrier modification by *L. plantarum* was also investigated.

### MATERIALS AND METHODS

**Human study.** The study was approved by the University Maastricht Ethics Committee and conducted in full concordance with the principles of the Declaration of Helsinki (52nd WMA General Assembly, Edinburgh, Scotland, October, 2000). *L. plantarum* WCFS1 was grown on de Man, Rogosa and Sharpe (MRS) medium under anaerobic conditions, using standard laboratory procedures. Fifteen minutes before each experiment, 10^12 freshly prepared *L. plantarum* WCFS1 were resuspended in 2,400 ml saline and 10 g/l glucose at 37°C. Seven healthy nonsmoking subjects (28 ± 6 yr), without a history of gastrointestinal complaints and free of any medication, participated in a double-blind randomized crossover study. After an overnight fast, an intraduodenal feeding catheter (nasointestinal tube, Flocare Bengmark; Nutricia Healthcare, Chatel-St.Denis, Switzerland) was placed oroagastically following the manufacturers’ instructions. Briefly, after nasogastric positioning of the tube into the stomach, it emptied into the small intestine by normal peristaltic movements. After positioning of the catheter in the small intestine (tube tip positioned ~5 cm distal to the pylorus), a test solution containing a total of 10^12 *L. plantarum* WCFS1 and 10 g/l glucose in saline or, randomly on another test day, only 10 g/l glucose in saline, was injected continuously at 6.7 ml/min for 6 h. Subjects remained in the supine position until the end of the experiment. Food or beverage consumption was not allowed during the experiment. After this 6-h period, tissue samples were obtained from the horizontal part of the duodenum by standard flexible gastroduodenoscopy, at ~15 cm distal to the pylorus. After sampling, tissue samples were snap frozen in liquid nitrogen and stored at −80°C until analysis.

**Cell culture.** Caco-2 BBE cells (CRL 2102) were purchased from the American Type Culture Center (Manassas, VA) at passage 48 and grown in DMEM (Invitrogen, Paisley, UK) containing Glutamax and supplemented with 10% FBS (PAA Laboratories, Colbe, Germany) and 100 U/mll penicillin/100 μg streptomycin (Sigma, St. Louis, MO) in an atmosphere of 5% CO2-95% O2 at 37°C. Cells were trypsinized weekly and only used for 20 passages. For microscopy and the dextran flux experiment, 1.6 × 10^6 cells were seeded in transparent six-well filter inserts ( Falcon, BD Biosciences, Oxford, UK) and cultured for 2 wk. For electrophysiology experiments, 2 × 10^4 cells were seeded in transparent 24-well filter inserts (Falcon, BD) and cultured for 2 wk. Cell monolayers typically reached a density of ~2.6 × 10^5 cells/cm² with a TER of 800 Ohm/cm². The resistance of an empty filter insert filled with DMEM medium was subtracted from the resistance values. Unless indicated, untreated Caco-2 BBE cells were used as controls. The conditioned media was prepared by spinning down bacteria in a Eppendorf centrifuge at 10,000 rpm for 10 min at 4°C and carefully aspirating supernatant.

**Confocal laser scanning microscopy.** Biopsies were mounted in TissueTek optimal cutting temperature compound (Bayer, Pittsburgh, PA: catalog no. 4583), and 40-μm sections were made at −18°C. The sections were collected on Superfrost object glasses (Menzel-Glaser, Braunschweig, Germany), and the material was fixed with 4% (wt/vol) paraformaldehyde and permeabilized using PBS containing 0.2% (vol/vol) Triton X-100, 1% (vol/vol) normal goat serum and 0.1% (wt/vol) sodium azide. Occlusion was stained overnight at 4°C using polyclonal antibodies from Zymed and the Cy3-conjugated secondary antibody (Jackson Laboratories, West Grove, PA). ZO-1 was stained using mAb 33-9-00 from Zymed (Invitrogen) and Alexa 488-labeled secondary antibodies from Molecular Probes (Invitrogen). Nuclei were stained for 10 min using 4’6-diamidino-2-phenylindole (DAPI, Molecular Probes, Invitrogen). Stacks of confocal images were obtained using a Zeiss LSM 510 system consisting of a Zeiss Axioskop microscope (Zeiss, Jena, Germany) equipped with an argon laser and a 488-nm and 543-nm filter set. The images were captured using a Zeiss AxioCam camera (Zeiss, Jena, Germany). The superimposed images were analyzed using ImageJ software.
obtained in the ZO-1 and occludin channel and plotted in GraphPad Prism 3.0 software (GraphPad, San Diego, CA).

Fluorometric analysis of permeability. Caco-2 BBE cells were cultured for 2 wk on 4.2 cm² filter inserts in 2 ml of HBSS ringer solution containing 2 mg/ml FITC-conjugated dextran with a molecular size of 4.4 kDa (FD4, Sigma) were applied to the mucosal side of the monolayer. Samples were taken from the basolateral compartment and analyzed using a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA).

Electrical resistance measurements in monolayer cell cultures. Caco-2 cells were grown on 0.4-µm polycarbonate Transwell filters (Corning Costar, Corning, NY) for 14 days. TER was measured using an epithelial Volt/Ohm-meter (World Precision Instruments, Sarasota, FL). All experiments were performed in DMEM as described in the cell culture section. The values of an empty filter insert were subtracted from the measured values. In experiments where the TLR2 receptor was neutralized using 1 µg/ml monoclonal antibody (mAb; mAbThr2, Invivogen), the TER was measured using a CellZscope (Nanoanalytics, Munster, Germany). This device allows the continuous and automated measurement of 24 individual filter inserts under cell culture conditions. As a control, 1 µg/ml monoclonal IgG1 isotype antibody (M5584, Sigma) was included in DMEM medium.

Neutral red uptake assay of cell viability. This cytotoxicity assay is based on the ability of viable cells to incorporate and bind neutral red, as previously described (15). Briefly, after incubation the monolayer was washed once with DMEM. Fresh complete DMEM with 50 µg/ml neutral red was added to the wells. After 30 min of incubation at 37°C cells were washed rapidly with 40% formaldehyde-10% CaCl₂ to remove extraneously adhering, unincorporated dye. Neutral red was extracted from the cells with 500 µl 1% acetic acid-50% ethanol, and 150 µl extract was transferred to a 96-well titer plate. The neutral red content was measured on a SpectraMax M5 microplate reader (Molecular Devices) at 550 nm. The readings were expressed as percentages of the nonexposed cells.

Luciferase assay. Human embryonic kidney (HEK) cell lines were seeded at 5 × 10⁵ cells/cm² into 96-well plates and incubated overnight under standard culture conditions. TNF-α (10 ng/ml; Cal-Biochem, La Jolla, CA), PCSK (5 µg/ml, Invitrogen), LPS (100 ng/ml, Invivogen) or bacteria were added for 6 h. L. plantarum was grown in static culture for 14 to 16 h in MRS at 37°C. Luciferase activity was measured using the Bright-glo luciferase assay (Promega Benelux b.v. NL).

Statistics. Comparison between two groups was made using the Student’s t-test (unpaired) for grouped data. The significance in all tests was derived at the 95% or greater confidence level.

RESULTS

In vivo effects of L. plantarum on epithelial TJs in humans. In a randomized human crossover study, seven healthy subjects were administered either a buffer containing glucose (10 g/l) and saline or a total of 1 × 10¹² L. plantarum cells suspended in the same buffer on two separate occasions. Fluids were infused into the duodenum over a period of 6 h using an intraduodenal feeding catheter. After a 2-wk interval the test sample was reversed for each individual in a randomized crossover fashion. After the 6-h injection period, six duodenal biopsies were collected and processed for gene expression analysis and immunohistochemistry. Immunostaining of the tissue samples with fluorescent antibodies to occludin and ZO-1 showed colocalization of these proteins near the apical surface where the TJs form a connection between the cell membranes of adjacent epithelial cells (Fig. 1A). Whereas occludin was located almost entirely in the TJs, the staining for ZO-1 was also diffusely associated within the cell cytoplasm.

To quantify the fluorescent staining of ZO-1 and occludin in the TJ region, each tissue sample (blindly coded) was subjected to confocal analysis of uniform Z sections perpendicular to the cell surface of the epithelium in 3 different areas per section. The identification of the samples was then decoded and the fluorescence intensities plotted as a function of cell location using the peak fluorescence signal from the TJ region to align each intensity profile. Administration of live L. plantarum significantly increased the fluorescent staining of occludin in the TJ (P < 0.05 for sections indicated in Fig. 1B). Similarly, administration of L. plantarum significantly increased the staining of ZO-1 in the apical part of the cell near the vicinity of the TJ, but not the peak fluorescent intensity or cytoplasmic distribution of ZO-1 (P < 0.05 for all sections indicated in Fig. 1B).

In vitro effects of L. plantarum on epithelial TJs. The intestinal epithelial cell line Caco-2 has been used extensively as a model of the human epithelium, as it can be grown in the Transwell system as a differentiated cell monolayer that has selective paracellular permeability to ions and solutes. Caco-2 monolayers grown in the Transwell system were incubated on the apical side with or without live L. plantarum [100 colony-forming units (CFU) per cell], which was shown to lack any cytotoxic effects on the basis of the neutral red uptake assay (Fig. 2A). As an additional control the same volume of bacterial conditioned growth medium was added in place of L. plantarum to rule out any possible effects on Caco-2 cells. To match the period of infusion in the human study, the filters containing the cell monolayers were harvested after 6 h and immunostained for ZO-1 and occludin. As found in the human study, administration of L. plantarum increased staining of ZO-1 in the vicinity of the TJ (P < 0.05 for sections indicated in Fig. 2B). However, only one of the Z sections showed significantly increased staining of occludin (P < 0.05; Fig. 2B). No effects were measured on TJ proteins using bacterial growth medium that had been conditioned by overnight growth of L. plantarum (Fig. 2C).

Role of TLR2 signaling in modulation of TJ composition. As it had previously been shown that PCSK, an agonist of TLR2, increased translocation of ZO-1 to the TJs in Caco-2 cells (4), we hypothesized that L. plantarum might also modulate TJ via the TLR2 pathway. To test this hypothesis, L. plantarum was incubated with a HEK cell line that was stably expressing human TLR2 (hTLR2) and carrying a reporter plasmid containing firefly luciferase under the control of the human NF-κB promoter. HEK293 cells do not normally produce TRLS, but, when stably transformed with an hTLR2 expression plasmid, they can activate NF-κB upon addition of PCSK (Fig. 3A). Significant activation of NF-κB was observed after incubation of the reporter cells with live intact or antibiotic-killed L. plantarum bacteria at the same bacteria-to-cell ratio used in Caco-2 monolayer experiments (Fig. 3B). This result was highly reproducible and dose dependent (results not shown). HEK cells transiently transfected with the NF-κB reporter plasmid (pNiFTY) were unresponsive to PCSK, demonstrating the requirement of hTLR2 in this signaling pathway (Fig. 3A). As expected, the NF-κB promoter could be activated in both reporter lines via the TNF-α receptor-mediated pathway but not upon addition of ultrapure LPS from E. coli, a known agonist of a different TLR (i.e., TLR4) (Fig. 3A). To confirm previous reports that Caco-2
cells are indeed responsive to TLR2 ligands (25) and that such a mechanism could account for our in vitro results, we showed that treatment of Caco-2 cells with PCSK resulted in IL-8 secretion, a hallmark of NF-κB activation in epithelial cells (results not shown).

The effect of PCSK on TJ proteins ZO-1 and occludin was also compared with *L. plantarum* by confocal microscopy following 6-h incubation with Caco-2 monolayers. PCSK did indeed mediate changes in TJ composition, but in contrast to *L. plantarum* no differences were observed for ZO-1 compared with the sham-treated control. As found in the in human study after administration of *L. plantarum*, occludin was substantially increased in the TJ but also basolateral to the TJ (Fig. 3C).

*L. plantarum* confers a protective effect on the epithelium. In Caco-2 cells the TJ modifications induced by *L. plantarum* appeared not to influence the TER and permeability to fluorescent dextran over a 6-h period of coculture (Fig. 4, A and B). A challenge assay was also developed to assess possible effects on epithelial integrity under compromised conditions that cause disruption of the TJ complex. In this assay the permeability of the TJ barriers was increased using phorbol 12,13-dibutyrate (PDBu), a derivative of TPA, which causes a gradual dislocation of ZO-1 and occludin (14, 45). Addition of 1 μM PDBu to intact Caco-2 monolayers induced a 60% drop in TER over a period of 5 h compared with the control cells or cells pretreated with *L. plantarum* alone for 6 h (Fig. 4C). Addition of live *L. plantarum* for 6 h before the addition of PDBu significantly (*P < 0.05) attenuated the drop in TER, suggesting that the altered TJ composition reinforced the epithelial barrier function (Fig. 4C).

To investigate whether this protective effect could have been mediated by the TLR2 signaling activity of *L. plantarum*, the experiment was repeated using PCSK, a synthetic TLR2 ago-
confirm that the protective effect of PCSK was mediated by the TLR2 receptor, a specific TLR2 neutralizing monoclonal antibody was applied to both compartments of the filter insert 30 min before treatment of PCSK. The antibody remained present throughout the rest of the experiment. The neutralizing antibody did not activate the receptor because no protection was observed when the Caco-2 cells were treated with the antibody (Fig. 4E). An attenuation of the PDBu-induced drop in TER was observed when cells were treated with PCSK, but this was abolished by coinubation with a TLR2-neutralizing antibody, indicating that protection of the TER was indeed conferred via the TLR2 receptor.

**DISCUSSION**

Certain strains of probiotics have been shown to confer health benefits in clinical trials, but identification of the mechanisms that contribute to the in vivo efficacy of a particular strain for a certain health effect is now one of the major challenges facing this field of research. Here we show for the first time that feeding of a live probiotic organism to humans increases localization of occludin and ZO-1 in the TJs of the intestinal epithelium. Microarray analysis of RNA isolated from each human tissue sample revealed that the transcript levels of the TJ proteins were not significantly modulated by administration of *L. plantarum* (40), suggesting that the increased staining of ZO-1 and occludin were attributable to effects on protein dynamics in the TJ.

In a Caco-2 model of intestinal epithelium, coculture with *L. plantarum* for 6 h also significantly increased staining of ZO-1 in the TJ region as in the human study. However, the amount of occludin detected in Caco-2 cells appeared to be less compared with the human tissue, and only one of the Z sections showed significantly increased staining of occludin. The reasons for this are not known, but it may be attributable to the use of a tumor cell line or a characteristic of the Transwell system for growing polarized monolayers.

The effects of *L. plantarum* on ZO-1 might reinforce the TJ, as this scaffold protein is known to bind both cytoskeletal actin and occludin, a transmembrane protein spanning the extracellular junction. Reductions in the amounts of ZO-1 and occludin are known to be associated with epithelial barrier dysfunction and increased epithelial permeability (22, 27). Accumulation of occludin and ZO-1 in the TJ are associated with protection of the epithelial barrier as recently demonstrated for antrum mucosal protein 18 (AMP-18) in the mouse DSS colitis model (43). Furthermore, incubation of cell monolayers with AMP-18 prevented the fall in TER that accompanies oxidative stress, suggesting that this mechanism may protect against barrier dysfunctions caused by a range of physiological insults. Further in vivo evidence for a direct role of ZO-1 in the maintenance of epithelial integrity comes from recent studies with the probiotic *E. coli* strain Nissle (EcN) (41), where administration of EcN to DSS-treated Balb/c mice reduced loss of body weight and colon shortening associated with the development of colitis, ameliorated the leakage of colonic epithelium, and increased ZO-1 expression.

In vitro the TJ protein changes induced in Caco-2 cells by *L. plantarum* did not alter the TER or permeability to low molecular weight dextrans over a 6-h period. This may be due to the fact that paracellular permeability to ions and solutes is...
thought to be mainly controlled by the expression of different claudins rather than scaffold proteins like ZO-1 (19). Nevertheless the changes in the cellular distribution of ZO-1 significantly increased the resistance of the epithelial barrier to disruption by PDBu, a hydrophilic derivative of TPA, which induces leakage of TJ through a mechanism involving PKC activation and dislocation of ZO-1 and occludin (45). These results are compatible with previous studies correlating levels of ZO-1 with protection of barrier disruption attributable to inflammation or oxidative stress (22, 27, 41, 43).

The TLR2 agonist PCSK has previously been shown to regulate ZO-1 in vitro (5) and ameliorate damage to the epithelial barrier in DSS colitis models (4). The mechanism was proposed to involve TLR2-mediated phosphorylation, enzymic activity, and translocation of PKC isoforms PKC-α and PKC-β to the TJ region (5). In this study we showed that addition of PCSK to Caco-2 monolayers for 6 h substantially increased the staining of occludin in the TJ, as found in the human study after administration of L. plantarum. Furthermore, PCSK significantly increased the resistance of the epithelial barrier to disruption by PDBu, and this protective effect was significantly attenuated by coincubation with a TLR2 blocking antibody.

The regulation of TJ proteins by L. plantarum may also involve TLR2 signaling at the apical surface of the epithelium because we showed that this strain activated NF-κB in a reporter cell line in a TLR2-dependent fashion and that IL-8 secretion is induced in Caco-2 monolayers by apical administration of a TLR2 ligand. Furthermore, because live and antibiotic-killed bacteria were able to activate NF-κB in the TLR2 reporter assay to a similar extent, it is most likely that bacterial-associated molecules from L. plantarum are responsible for the TLR2 activation in Caco-2 cells. In vivo this strain has also been shown to modulate NF-κB pathway gene expression in human biopsy samples (42). TLR2 signaling by L. plantarum is most likely mediated by lipoteichoic acids and lipoproteins found in the cell envelope of Gram-positive bacteria through their interaction with TLR2 and heterodimers of TLR2 with TLR1 and TLR6 (3). Microarray hybridization studies with RNA isolated from the human biopsies revealed that TLR2 transcripts are significantly expressed in the duodenal epithelium (42). Taken together, these results suggest that TLR2 signaling mediates the TJ protein changes observed in human tissue following feeding of L. plantarum.

The results of this study provide a highly plausible explanation for the reported protective effects of L. plantarum and other probiotic strains on barrier disruption by inflammatory cytokines, chemicals, and infectious agents (13, 18, 20, 28, 32, 34, 35, 48). However, increased mucin production, immunomodulation, pathogen inhibition, and effects on the resident microbiota could also be contributory factors, particularly in vivo. The mechanism demonstrated here may also contribute to the positive effects seen in probiotic trials in humans with IBS, IBD,
and atopic disease and has important implications for the specific selection and formulation of probiotics for the treatment and prevention of impaired epithelial barrier function seen in many disorders. The capacity of different *Lactobacillus* species and strains to activate TLR2 varies considerably (more than 20-fold), suggesting that the effects demonstrated here will be strain and dose dependent (results not shown). This may also be in part an explanation for the negative studies that have been reported for probiotics in vivo.

Toll-like signaling plays a crucial role in innate immunity and in orchestrating the subsequent adaptive immune responses to invading pathogens. Aside from these well-characterized functions, there is growing evidence from studies in knockout mice that a basal level of TLR activation is necessary for intestinal homeostasis (10, 26, 33). Our results identifying commensal bacterial stimulation of TLR2 in the gut epithelium as a regulator of epithelial integrity have important implications for understanding probiotic mechanisms and the control of intestinal homeostasis.

Fig. 4. Transepithelial resistance (TER) (A) and flux (permeation) of a 4.4-kDa fluorescent dextran (FD4) (B) across Caco-2 cell monolayers were measured at hourly intervals during 6-h apical incubation with *L. plantarum* (100 CFU/cell) or a medium control. Mean TER and flux values are shown for 6 replicate monolayers (± SD). The mean TER and flux values of *L. plantarum*- and control-treated monolayers were not significantly different. *C. L. plantarum* protects against the barrier dysfunction caused by phorbol 12,13-dibutyrate (PDBu). TER measurements on Caco-2 cell monolayers incubated for 300 min in the presence or absence of PDBu (1 μM). Replicate monolayers were pretreated on the apical side with *L. plantarum* (100 CFU/cell) for 6 h before the addition of PDBu or medium control. The values at the beginning of the experiments were set as 100%. Mean TER values (± SD) for 5 experiments are shown for control and PDBu group. 4 experiments for *L. plantarum* and *L. plantarum*/PDBu group. *P < 0.05 for PDBu-treated Caco-2 cells compared with cells treated with *L. plantarum* and PDBu. D: protective effect of *L. plantarum* on barrier dysfunctions caused by PDBu was also observed using the TLR2 agonist PCSK (5 μg/ml). Experiments were performed as described above except that PCSK was used instead of *L. plantarum*. Mean TER values (± SD) for 5 experiments are shown for control and PDBu group. 4 experiments for PCSK and PCSK/PDBu group. *P < 0.05 and **P < 0.01 for effect PDBu-treated Caco-2 cells compared with cells treated with PCSK and PDBu. E: neutralization of the TLR2 receptor reduced the protective effect of PCSK on the PDBu-induced decrease of the TER. A sample (1 μg/ml) of TLR-neutralizing antibody was added 0.5 h before PCSK-treatment and remained present during whole experiment. This part of the experiment was performed using the Cellscope. Mean TER values (± SD) for 4 experiments are shown for control and PDBu group *P < 0.05 for effect PDBu/PCSK-treated Caco-2 cells compared with cells treated with PCSK-PDBu-anti-TLR2.
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DISCLOSURES

The authors are not aware of financial conflict with the subject matter or materials discussed in this article with any of the authors or their academic institutions or employers.

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intestinal mucosa to Lactobacillus plantarum WCFS1 in vivo (Abstract).


