Mechanism of protection of transepithelial barrier function by \textit{Lactobacillus salivarius}: strain dependence and attenuation by bacteriocin production

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The intestinal epithelium consists of a single layer of cells separating the internal milieu from the external microenvironment of the gut lumen. Paracellular transepithelial ingress of antigenic and microbial contents from the lumen is strictly regulated by the apical junctional complexes composed of the tight junction and the adherens junction (62). The tight junction, at the most apical region of the junctional complexes, is a multiprotein assembly composed of transmembrane and cytoplasmic linker proteins. The transmembrane proteins such as occludin (15), junctional adhesion molecule (JAM) (30), and claudin family members (14) interact with cytoplasmic linker proteins such as zonula occludens-1 (ZO-1) that affiliate with the underlying actin cytoskeleton. These associations play a critical role in maintaining tight junction integrity (12, 19).

Increased intestinal permeability and impaired tight junction integrity have been observed in several gastrointestinal disorders including inflammatory bowel disease (IBD) (17, 24, 56). Although the pathogenesis of IBD is not fully understood, recent genomewide association studies as well as animal models have suggested that tight junction dysfunction would contribute to the pathogenesis of IBD (23, 44). Indeed, the increased intestinal permeability in IBD patients is related to their disease activity and is predictive of relapse after pharmacological and surgical relief from inflammation (31). Excessive production of proinflammatory factors such as reactive oxidative species (50) and cytokines (25, 43) is characteristic of IBD patients, the latter impairing tight junction integrity (1, 21, 34, 47, 60). These factors modulate the assembly, location, and expression of tight junction proteins by activating various intracellular signaling pathways such as MAPK, phosphoinositide 3-kinase, or NF-κB pathways (1, 28, 41, 60). Because disturbance in the intestinal tight junction barrier allows increased antigen penetration followed by an inflammatory response, the protection of tight junctions from inflammatory factors is a potential therapeutic target for IBD (55, 62).

Accumulating evidence suggests that some probiotic bacteria ameliorate inflammation in IBD patients (5, 35, 63). This effect is currently attributed to several mechanisms including modulation of the mucosal immune system, antimicrobial activity, and inhibition of epithelial apoptosis (65). Furthermore, certain probiotic and commensal bacteria suppress tight junction dysfunction in vitro and in vivo (33, 38, 49, 57). However, the precise mechanisms by which these bacteria protect tight junction integrity, and the bacterial component(s) and/or bacterial metabolite(s) responsible for the protecting effects, have not been fully clarified.

The properties of different strains of the same probiotic species vary, and important probiotic traits are increasingly recognized as being strain specific (4). For example, Meijerink and colleagues (32) demonstrated that different strains of \textit{Lactobacillus plantarum} differentially induced proinflammatory and anti-inflammatory cytokines from dendritic cells, in a manner related to bacteriocin production and bile salt hydrolase gene expression. The interaction and effects of \textit{L. casei} on macrophages and monocytes was modulated by varying pro-
tion of exopolysaccharide (69). Our laboratory’s previous study (45) also showed that the level of exopolysaccharide production, which is credited with health-promoting properties, is different among different strains of L. salivarius. Identification of traits related to beneficial effects upon the host may therefore be facilitated by comparative analyses and gene-trait matching in large panels of strains.

L. salivarius strains are widely used as probiotics (40). Their beneficial mechanisms include direct antagonism against pathogens such as Listeria monocytogenes (7), modulation of inflammation in response to pathogens (54), and anticinocceptive activity (52). We have developed a genomics-based platform for identifying host interaction molecules in L. salivarius (6, 64). The aim of the present study was to elucidate whether the barrier-protecting effect of L. salivarius varied at a strain level and to investigate the mechanisms underlying the different effects of these strains. The 33 individual L. salivarius strains, including the genetically well-characterized probiotic strain UCC118 (6, 45), were tested for their effects on hydrogen peroxide (H2O2)-induced tight junction dysfunction. The global transcriptome analysis of L. salivarius allowed us to identify a responsible gene and molecule in L. salivarius that modulates tight junction integrity.

MATeRIALs aNd METhODS

Cell culture. Human intestinal epithelial Caco-2 cells purchased from American Type Cell Culture (Rockville, MD) were grown under standard cell culture conditions as described previously (37, 38). Cells were seeded on polycarbonate membranes in Transwell inserts (6.5, 12, or 75 mm; Corning, Corning, NY) at a density of 1 × 10⁵ cells/cm². At 12–16 days postseeding, transepithelial electrical resistance (TER) was used as an indicator of ion permeability and tight junction integrity and was measured with a Milllicell-ERS instrument (Millipore, Bedford, MA). Caco-2 cell monolayers were used when their TER values were in the range of 400–600 Ω·cm². In some experiments, the flux of FITC-conjugated dextran (molecular weight 4,000; Life Technologies, Carlsbad, CA) across the monolayer was measured as an indicator of macromolecule permeability. FITC-dextran (100 µM) was added to the apical side and the fluorescence in the basal side was determined by using a fluorescence plate reader (ARVO X4, Perkin Elmer).

Antibodies. The following antibodies were used in this study: rabbit polyclonal anti-ZO-1, anti-JAM-1, anti-claudin-1, and mouse polyclonal anti-occludin (Life Technologies); anti-rabbit polyclonal anti-phospho-ERK1/2, anti-phospho-Akt, anti-actin, anti-phospho-p38, anti-p38, anti-phospho-JNK, and rabbit monoclonal anti-ERK1/2, anti-JNK, and mouse monoclonal anti-β-actin, anti-phospho-IκBα, anti-IκBα (Cell Signaling Technology, Danvers, MA); horseradish peroxidase (HRP)-conjugated goat anti-rabbit and anti-mouse IgG (DAKO, Glostrup, Denmark); Alexa Fluor 488-conjugated goat anti-rabbit and anti-mouse IgG (Life Technologies).

Bacterial strains and culture conditions. The L. salivarius strains used in this study are listed in Table 1. The relatedness and genomic composition of the strains has been previously described (45). The strains were routinely cultured at 37°C under microaerophilic conditions (5% CO2) in de Man-Rogosa-Sharpe (MRS) medium (Oxoid, Hampshire, UK). Bacterial strains were maintained as frozen stocks at −80°C in 25% glycerol. For the murine colitis model, L. salivarius strains (rifampicin-resistant derivatives of strains UCC118, UCC119 Bac− and AH43324) were inoculated in MRS supplemented with rifampicin (100 µg/ml) at 37°C and 5% CO₂ for 16 h until reaching the required optical density. Bacteria were washed once and resuspended in sterile PBS, adjusted to a final cell density of ~1 × 10⁸ viable organisms/0.2 ml.

Table 1. Lactobacillus salivarius strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Origin</th>
<th>Source or Reference</th>
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<tbody>
<tr>
<td>UCC118</td>
<td>Human ileal-cecal region</td>
<td>6, 61</td>
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<tr>
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<td>LGM</td>
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<td>Parakeet with sepsis</td>
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<td>L21</td>
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</table>

CGU, Culture Collection University Göteborg; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen; JCM, Japan Collection of Microorganisms; LMG, Laboratorium für Mikrobiologie, Universiteit Gent; NCIMB, National Collections of Industrial Food and Marine Bacteria. L21 was provided by Professor Gerald Tannock, University of Otago, Otago, New Zealand.

Cell treatment. The Caco-2 cell monolayers were washed and serum-deprived in Dulbecco’s modified Eagle’s medium containing 0.5% fetal bovine serum for 18 h. H2O2 (0.5–50 mM) was added to the apical and/or basal side of the monolayers. L. salivarius strains were cultured in MRS medium at 37°C for 16 h, and the bacterial cells were washed with PBS and added to the apical side [multiplicity of infection (MOI) of 10:1] 1 h prior to H2O2 administration (10 mM, to the basal side). In some experiments, the monolayers were pretreated with the ERK-inhibitor U0126 (10 µM) 30 min prior to L. salivarius administration.

Cytotoxicity assay. Cell death was assessed by measuring lactate dehydrogenase (LDH) release. LDH activity was determined according to the method described by Rao et al. (46) with some modifications. Briefly, the Caco-2 monolayers were incubated with H2O2 (10 mM, the basal side) for 3 h or 0.1% Triton X-100 for 30 min as a positive control, and medium from the apical side was harvested for assessment of LDH release (Promega, Madison, WI). Total cellular LDH activity was determined by solubilizing the cells with 1% Triton X-100.

Preparation of whole cell extracts and detergent-insoluble and -soluble fractions. For preparation of whole cell extract, the Caco-2 cell monolayers were washed with ice-cold PBS three times and then lysed in RIPA buffer [1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaOH, and 1 mM EDTA in 25 mM Tris, containing protease and phosphatase inhibitor cocktail (Pierce Bio-technology, Rockford, IL), pH 7.5]. Detergent-insoluble and -soluble fractions were prepared according to the method described by Suzuki.
and H. pylori L. salivarius strains and H2O2 for 3 h, and the medium from the apical were frozen at 37°C. Following incubation with Tris-buffered saline containing 2% and 0.1% Tween, the membranes were probed with specific primary antibodies followed by anti-mouse or anti-rabbit secondary antibodies conjugated with HRP. The blots were developed by use of Immobilon Western Chemiluminescent HRP substrate (Millipore). Band intensities were quantified with ImageJ software.

**Measurement of H2O2.** The concentration of H2O2 in the culture medium was measured according to the method described by Pick and Mizez (42), which is based on the HRP catalysis of the phenol red oxidation by H2O2. The Caco-2 monolayers were incubated with L. salivarius strains and H2O2 for 3 h, and the medium from the apical or basal side was harvested for the assay. The absorbance was measured at 600 nm to evaluate H2O2 concentration (compared with a standard curve).

**Immunofluorescence microscopy.** The Caco-2 cell monolayers were washed with ice-cold PBS three times and fixed with methanol for 10 min at −20°C. After blocking with 2% BSA in PBS, the cell monolayers were incubated with specific primary antibodies followed by incubation with anti-mouse or anti-rabbit secondary antibodies conjugated with Alexa Fluor 488. The fluorescence was visualized via a Zeiss LSM 510 confocal microscope (Carl Zeiss MicroImaging, Thornwood, NY). Images from x-y sections (1 μm) were collected and stacked by use of ImageJ software.

**Microarray analysis of bacterial transcription.** For the analysis of bacterial gene transcription, the Caco-2 cells were seeded in 100-mm Transwells and grown for 12–14 days to allow complete differentiation. The medium was changed twice to remove all traces of antibiotics. Briefly, the cell monolayers were washed with ice-cold PBS three times and incubated on ice between mechanical disruption in a bead beater with incubation on ice for 5 min at 4°C to sediment the high-density actin-rich fraction. The pellet was suspended in RIPA reagents (Tropix, Bedford, MA) and Hara (59), and the former corresponds to the proteins associated with the actin cytoskeleton. The latter was added to the lysis buffer together with 0.5 ml of acid-washed glass beads. The mixture was subjected to 3 cycles of freezing and thawing, followed by centrifugation at 15,600 g for 5 min at 4°C to sediment the high-density actin-rich fraction. The pellet was suspended in RIPA buffer. Protein concentrations in the different fractions were determined by BCA protein assay (Pierce Biotechnology).

**Western blot.** The proteins (5 μg for phospho- and total ERK, and 20 μg for the others) were separated by SDS-PAGE (10%) and transferred onto polyvinylidene difluoride membranes (Millipore). After blocking with Tris-buffered saline containing 2% and 0.1% Tween, the membranes were probed with specific primary antibodies followed by anti-mouse or anti-rabbit secondary antibodies conjugated with HRP. The blots were developed by use of Immobilon Western Chemiluminescent HRP substrate (Millipore). Band intensities were quantified with ImageJ software.

**Quantitative real-time PCR.** The Caco-2 cells were seeded in six-well plates and grown for 12–14 days, then cocultured with bacteria (MOI of 100) for 90 min. The total RNA was extracted as described above, and cDNA was synthesized using Transcriptor Reverse Transcripase (Roche Applied Science, Mannheim, Germany) according to the manufacturer’s instructions. Quantitative real-time PCR was performed with a LightCycler 480 System (Roche Applied Science) and SensiMix II Probe kit (Bioline, London, UK). The primer sequences used for quantitative PCR (qPCR) are shown in Table 2.

**Mice.** Specific pathogen-free female BALB/c OlaHsd mice, 9 wk of age, weighing 17–21 g, were obtained from Harlan, UK. Mice were housed in individually ventilated cages (OptiMICe, Animal Care Systems, Littleton, CO) in groups of four to five mice per cage, with sterile bedding, temperature 21°C, 12:12-h light-darkness, humidity 50% in a dedicated animal holding facility. They were fed a sterilized pellet diet and tap water ad libitum. Mice were allowed at least 2 wk to acclimatize before entering the study. All animal procedures were performed following the protocols recommended by the manufacturer. All hybridization reactions were supplied by Agilent. Following hybridization, the slides were washed according to the protocol recommended by the manufacturer and scanned with an Agilent Microarray Scanner System (G2505B) with Agilent scan control software version 7.0 at a resolution of 5 mm. Agilent Feature Extraction software version 9.1 was used to process the image file from the scanner. The extracted data were further processed by use of an in-house microarray transform platform that performed the following statistical analyses: 1) The replicates for each gene were combined and the mean was calculated. 2) outliers were identified using the Grubbs test as follows, whereby the z-value ( mean − x )/sd, where x is the ratio of a spot and sd is the standard deviation, was calculated, if the z-value was greater than (N − 1)/N, where N is the number of spots analyzed, then the spot is an outlier. 3) The P value for each gene was calculated using a Cyber-t test. The parameters for the Cyber-t test were a Bayes t-test with a betafit of 1, a winsize of 101, and a confidence level of 10. The log-transformed mean from the Cyber-t test was then converted to nonexponential numbers to give a value for up or down fold regulation.

Table 2. Sequences of primers used for quantitative PCR

<table>
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<th>Gene</th>
<th>Sequence</th>
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<td>Isl_1910</td>
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<td>Isl_1916</td>
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<td>Isl_1917</td>
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<td>16S</td>
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</table>

**Transcription of bacterial RNA.** Total RNA from the bacteria was isolated using TRIzol reagent (Life Technologies, Carlsbad, CA). The cDNA was synthesized using the SuperScript First-Strand Synthesis System (Invitrogen) following the manufacturer’s protocol. The cDNAs were labeled with Cy3 and Cy5 dyes for use in microarray experiments as performed with a chemical labeling kit (Kreatech, Diagnostics, Amsterdam, Netherlands), following the manufacturer’s instructions. The efficiency of labeling was determined with a NanoDrop spectrophotometer (NanoDrop Technologies, Rockland, DE). Labeled cDNA was either used immediately or stored at −80°C until required.

We have previously described custom microarrays for L. salivarius based on the reference genome of strain UCC118 (11). The labeled cDNAs were hybridized to the microarray for 16 h at 55°C by using the protocol recommended by the manufacturer. All hybridization reactions were supplied by Agilent. Following hybridization, the slides were washed according to the protocol recommended by the manufacturer and scanned with an Agilent Microarray Scanner System (G2505B) with Agilent scan control software version 7.0 at a resolution of 5 mm. Agilent Feature Extraction software version 9.1 was used to process the image file from the scanner. The extracted data were further processed by use of an in-house microarray transform platform that performed the following statistical analyses: 1) The replicates for each gene were combined and the mean was calculated. 2) outliers were identified using the Grubbs test as follows, whereby the z-value ( mean − x )/sd, where x is the ratio of a spot and sd is the standard deviation, was calculated, if the z-value was greater than (N − 1)/N, where N is the number of spots analyzed, then the spot is an outlier. 3) The P value for each gene was calculated using a Cyber-t test. The parameters for the Cyber-t test were a Bayes t-test with a betafit of 1, a winsize of 101, and a confidence level of 10. The log-transformed mean from the Cyber-t test was then converted to nonexponential numbers to give a value for up or down fold regulation.

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formed according to national ethical guidelines following approval by University College Cork Animal Experimentation Ethics Committee.

**Murine DSS-induced colitis model.** At the start of the study, mice were randomized in groups of 4 or 10 mice per treatment. Dextran sodium sulfate (DSS, 40 kDa, TdT) was administered by including in the drinking water at a concentration of 4% (wt/vol) for 6 days, followed by 3 days of water. Mice (DSS-treated or control group) were orally gavaged with L. salivarius strains (UCC118, UCC118 Bac, and AH43324) starting on day 5, and repeated daily until the end of the experiment (day 9). Mice were monitored daily for body weight loss, stool consistency, and fur texture/posture, forming a disease activity index (DAI) as described previously (39). The stool was collected at different time points for bacterial enumeration. Briefly, serial dilutions of a fecal/PBS suspension (neat to $10^6$) were plated on MRS agar supplemented with rifampicin (100 µg/ml) by a spot-plate technique and incubated overnight at 37°C and 5% CO$_2$. At the day of termination, the colon was dissected and its length and weight were recorded. A longitudinal piece of distal colon was frozen in liquid nitrogen for protein analysis and 0.5 cm of the distal colon was frozen in RNAlater (Ambion) for RNA analysis.

**Evaluation of in vivo intestinal permeability.** Intestinal permeability was assessed by a FITC-labeled dextran method, as described (16). Mice were gavaged with FITC-dextran (4,000 kDa; at 60 mg/ml; 0.1% Triton X-100 for 30 min on TER (% decrease in TER; solid bars) and lactate dehydrogenase (LDH) release (% total cellular activity; hatched bars) in Caco-2 cell monolayers. Results are expressed as means ± SE (n = 4). **P < 0.01 vs. Control.

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**Fig. 1.** Effect of H$_2$O$_2$ on transepithelial resistance (TER) in Caco-2 cell monolayers. A: time course of effects of 0 (Control; ○), 0.5 (●), 1 (▲), 5 (■), 10 (●), or 50 (▲) mM H$_2$O$_2$ on TER in Caco-2 cell monolayers. TER was measured at different times after apical and basal application of H$_2$O$_2$ and expressed as % corresponding initial TER (n = 2). B: effect of apical and basal (●) or basal (▲) application of H$_2$O$_2$ (10 mM) on TER in Caco-2 monolayers. Results are expressed as means ± SE (n = 4). **P < 0.01 vs. control (○). C: effect of basal H$_2$O$_2$ (10 mM; ●) on FITC-dextran flux in Caco-2 monolayers. Results are expressed as means ± SE (n = 3). **P < 0.01 vs. control (○). D: effect of basal H$_2$O$_2$ (10 mM) for 3 h and apical Triton X-100 (0.1%) for 30 min on TER (% decrease in TER; solid bars) and lactate dehydrogenase (LDH) release (% total cellular activity; hatched bars) in Caco-2 cell monolayers. Results are expressed as means ± SE (n = 4). **P < 0.01 vs. Control.

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**Fig. 2.** Effect of H$_2$O$_2$ on expression and assembly of tight junction proteins. Western blot analysis of tight junction proteins in whole cell extract and detergent-insoluble and -soluble fractions in Caco-2 cell monolayers treated with H$_2$O$_2$ (10 mM) for 3 h. Each blot is representative of 3 similar experiments. ZO-1, zonula occludens-1.
Sigma-Aldrich). After 4 h, cardiac puncture was performed, blood was spun, and serum was collected and stored at −80°C until analysis.

Fluorescence intensity of each sample was measured by spectrophotofluorometry (excitation 490 nm, emission 520 nm; Synergy 2 high-performance multimode plate reader, BioTek, Winooski, VT). FITC-dextran concentrations were determined from standard curves generated by serial dilution of FITC-dextran in sterile PBS. Permeability was calculated by linear regression of sample fluorescence.

Data analyses. All data are expressed as means ± SE. All experiments were repeated a minimum of two times. Statistical analysis was performed by Student’s t-test or one-way ANOVA followed by Tukey’s post hoc test. A value of P < 0.05 was considered significant. Statistical comparison of measurements from the DSS colitis model were performed by one-way ANOVA followed by Bonferroni’s multiple-comparison test.

RESULTS

H$_2$O$_2$-induced tight junction disruption in Caco-2 cell monolayers. Previous studies on TER restoration by commensal lactobacilli involved prestressing cells with TNF-α (38) or

![Graph A](image1)

![Graph B](image2)

![Graph C](image3)

![Graph D](image4)
hydrogen peroxide (57). To establish an assay involving the latter stimulus, the disruptive effects of H$_2$O$_2$ on tight junction integrity were evaluated by measuring TER. The addition of 50 mM H$_2$O$_2$ to both the apical and basal sides of Caco-2 cell monolayers resulted in the TER decreasing to nearly zero within 0.5 h, indicating that this H$_2$O$_2$ concentration caused cell death (Fig. 1A). A concentration of 10 mM H$_2$O$_2$ reduced the TER in a time-dependent manner (Fig. 1A), whereas concentrations of 5 mM or less did not have any effect on TER. Consistent with a previous report (46), addition of H$_2$O$_2$ to the basal side of the monolayer was sufficient to dissipate the TER (Fig. 1B). The basal addition of 10 mM H$_2$O$_2$ led to an increase in the flux of FITC-dextran 3 h after stimulation (Fig. 1C). Under this stimulatory condition, the release of LDH (a marker for cytoplasmic contents) was not altered, whereas LDH release was significantly increased by the administration of 0.1% Triton X-100, as positive control for cell lysis (Fig. 1D). Therefore, basal stimulation with 10 mM H$_2$O$_2$ was chosen for subsequent experiments as the dissipation of TER obtained at this concentration was due to degradation of barrier function rather than cell death.

The effects of H$_2$O$_2$ on the total expression of tight junction proteins and on tight junction proteins associated with the actin cytoskeleton were evaluated by immunoblot analysis using whole cell extracts and detergent-insoluble and -soluble fractions, respectively, after stimulation with H$_2$O$_2$ for 3 h. The total expression levels of cytoplasmic (ZO-1) and transmembrane (occludin, JAM-1, and claudin-1) proteins in tight junctions were not affected by H$_2$O$_2$ (Fig. 2). However, the protein levels of occludin, JAM-1, and claudin-1, but not ZO-1, in detergent-insoluble fractions decreased following treatment with H$_2$O$_2$ (Fig. 2). These changes were accompanied by increased levels of occludin, JAM-1, and claudin-1 in detergent-soluble fractions (Fig. 2), indicating their dissociation from the actin cytoskeleton. The level of β-actin was not altered in any cell fraction by the treatment (Fig. 2).

Suppressive effects of different L. salivarius strains on H$_2$O$_2$-induced reduction in TER. Caco-2 cell monolayers were preincubated with cells from 33 different L. salivarius strains at an MOI of 10:1, then exposed to H$_2$O$_2$, and TER was measured after 3 h. As shown in Fig. 3, the ability of individual strains to prevent the H$_2$O$_2$-induced reduction in TER was extremely variable. Some strains such as NCIMB8816 and CCUG38008 prevented the reduction in TER almost completely, whereas some were not effective (e.g., strains AH43324 and JCM1044) and other strains such as UCC118 and UCC119 showed intermediate effects (~50% suppression). During the assay, no acidification of the medium was observed in any groups. For further TER restoration assays, the L. salivarius strains CCUG38008, UCC118, and AH43324 were selected as being representative of highly effective, moderately effective, and ineffective strains, respectively.

Protective effects of L. salivarius on H$_2$O$_2$-induced disassembly of tight junction proteins from actin cytoskeleton. To investigate whether L. salivarius strains attenuated the H$_2$O$_2$-induced disassembly of tight junction proteins from the actin cytoskeleton, we evaluated tight junction proteins levels in detergent-insoluble and -soluble fractions in Caco-2 monolayers. Preexposure of Caco-2 cells to both UCC118 and CCUG38008 prevented H$_2$O$_2$-induced redistribution of occludin, JAM-1, and claudin-1 from detergent-insoluble fractions to detergent-soluble fractions (Fig. 4C). Although UCC118 and CCUG38008 strains have significantly different potential to prevent the reduction in TER (Fig. 4A), both strains prevented...
H$_2$O$_2$-induced increase in FITC-dextran flux (Fig. 4B) and redistribution of occludin, JAM-1, and claudin-1 almost completely (Fig. 4C). In contrast, the ineffective strain AH43324 did not impair FITC-dextran flux (Fig. 4B) and did not exert any preventive effects on the redistribution of tight junction proteins (Fig. 4C). We next examined the hypothesis that the degradation of H$_2$O$_2$ contributed to the L. salivarius-mediated barrier-protecting effect. As shown in Fig. 4D, none of the tested L. salivarius strains affected the concentration of H$_2$O$_2$ in the medium 3 h after H$_2$O$_2$ treatment.

Fig. 6. Role of bacteriocin production in barrier-protecting phenotype of L. salivarius. A: UCC118 and AH43324 strains were harvested from Caco-2 monolayers after 90 min incubation (MOI of 100:1), and global transcriptome analysis was performed. Relative gene expression levels of bacteriocin genes in AH43324 compared with UCC118 are shown (red arrows represent upregulated genes in AH43324 compared with UCC118). B: mRNA expression of bacteriocin genes was analyzed by quantitative real-time PCR. *P < 0.05 and **P < 0.01 vs. UCC118. N.D., not detected. C: Caco-2 cell monolayers were pretreated with L. salivarius UCC118 wild-type strain or bacteriocin-negative mutant (UCC118 Bac$^{-}\$) mutant) (MOI of 10:1) for 1 h, and the monolayers were exposed to H$_2$O$_2$ (10 mM). TER was measured 3 h after H$_2$O$_2$ treatment. Results are expressed as means ± SE (n = 4). **P < 0.01 vs. nontreated monolayers (Control); ###P < 0.01 vs. H$_2$O$_2$ alone (H$_2$O$_2$). D: FITC-dextran flux was measured 3 h after H$_2$O$_2$ treatment. Results are expressed as means ± SE (n = 3). **P < 0.01 vs. nontreated monolayers (Control); ###P < 0.01 vs. H$_2$O$_2$ alone (H$_2$O$_2$). E: monolayers were fixed and stained for tight junction proteins. Images were collected by confocal microscopy. Scale bar is 10 μm.

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Protective effects of L. salivarius on H₂O₂-induced relocalization of tight junction proteins. Because the association of tight junction proteins with the actin cytoskeleton is required for stable assembly of these proteins in the tight junction (48), we next assessed the effects of L. salivarius exposure on localization of tight junction proteins. Confocal microscopy demonstrated that H₂O₂ disrupted the junctional organization of occludin, JAM-1, and claudin-1 and induced redistribution of these proteins from the intercellular junctions into the intracellular compartments (Fig. 5). This redistribution of the tight junction proteins caused by H₂O₂ was largely attenuated by pretreatment of the monolayers with UCC118 and CCUG38008, but not AH43324.

Differentially expressed genes in L. salivarius UCC118 and AH43324. Previous comparative genomic hybridization (CGH) analysis (45) had already indicated that AH43324 lacks the smallest plasmid pSF118–20 that is present in UCC118, suggesting the possibility that gene products encoded by this plasmid might be responsible for the superior ability of UCC118 to restore TER. However, analysis of the phenotype of a previously constructed cured derivative of UCC118 (called LS201; Ref. 10) that lacks pSF118–20 showed that it had the same ability to restore TER as UCC118 (data not shown). Since the CGH analysis showed that UCC118 and AH43324 were largely identical at the gene complement level, differential transcription appeared the next most plausible explanation for the different TER restoration phenotypes. To identify L. salivarius genes potentially involved in regulatory effects on tight junction integrity, transcription profiles of UCC118 and AH43324 were compared by using a custom microarray designed on the basis of the genome of UCC118. Among the 2,184 genes, 136 genes were significantly downregulated and 102 genes were upregulated in AH43324 (Table 2).

The differentially expressed loci were dominated by genes encoding proteins involved in “information storage and processing” and “cellular processes,” as well as hypothetical proteins or proteins of unknown function. Beside the gene categories described above, which we deemed unlikely to be responsible for TER-modulating effects, the largest change was observed in a gene corresponding to abp118α (LS_1917; 108.7-fold upregulated in AH43324 relative to UCC118). Interestingly, all genes related to bacteriocin production from L. salivarius UCC118 (13) (except for abpIM, dedicated to production/immunity), were upregulated in AH43324 relative to UCC118 (Fig. 6A), indicating that AH43324 would produce much more bacteriocin than UCC118 in coculture with the polarized Caco-2 cells.

To verify the results from the microarray analysis, qPCR was carried out for four genes in the bacteriocin gene cluster: lsl_1910 (abpT), lsl_1916 (abpβ), lsl_1917 (abpε), and lsl_1919 (membrane-associated protein). In contrast to microarray data, we did not observe the increased expression of these genes in AH43324 relative to UCC118 (Fig. 6B). However, CCUG38008, a high effective strain, expressed little or no detectable levels of these bacteriocin-related genes. These results indicated that bacteriocin production might influence the barrier-protecting effect of these bacteria.

To further investigate this, the barrier-protecting effect of a previously constructed bacteriocin-nonproducing derivative of UCC118 mutant (7) was compared with that of the wild-type strain. Although wild-type UCC118 and the bacteriocin-negative UCC118 mutant attenuated H₂O₂-induced increase in FITC-dextran flux and redistribution of occludin, JAM-1, and claudin-1 (Fig. 6, D and E), the mutant displayed a significantly higher suppressive effect on H₂O₂-induced reduction in TER compared with wild-type UCC118 (Fig. 6C).

Effect of L. salivarius on intracellular signaling pathways. To further investigate the molecular mechanisms underlying the regulatory effects of L. salivarius on tight junction integrity, the phosphorylation status of ERK, Akt, p38, and JNK was examined. L. salivarius strain UCC118, the bacteriocin-negative UCC118 mutant, and CCUG38008 all induced the phosphorylation of ERK, but not of Akt, p38, and JNK. On the other hand, AH43324 induced the phosphorylation of Akt and p38, but not of ERK and JNK (Fig. 7). To examine whether L. salivarius had an effect on NF-κB activation, the phosphorylated...
lation and degradation of IκB following L. salivarius treatment were tested. As shown in Fig. 7, none of the tested L. salivarius strains caused any changes in the phosphorylation and degradation of IκB over a 1-h period, indicating that L. salivarius had no effect on NF-κB activation. To define the role of ERK in L. salivarius-induced effects on tight junction integrity, we used the ERK inhibitor U0126. The L. salivarius-mediated prevention of H2O2-induced decrease in TER and increase in FITC-dextran flux were significantly attenuated by pretreatment of the monolayers with U0126 (Fig. 8).

Effect of L. salivarius on H2O2-induced activation of intracellular signaling pathways. We next investigated whether L. salivarius affected H2O2-induced activation of intracellular signaling pathways. As shown Fig. 9A, H2O2 induced phosphorylation of ERK, Akt, p38, and JNK within 30 min, but not of IκB. The phosphorylation of Akt and p38 was significantly augmented by the pretreatment with UCC118 or AH43324 (Fig. 9, B and C). In contrast to UCC118, the bacteriocin-negative UCC118 mutant had no effects on H2O2-induced phosphorylation of Akt or p38 (Fig. 9, B and C).

L. salivarius reduces intestinal permeability in a murine colitis model. Mice receiving 4% DSS and PBS developed mild colitis as judged by clinical signs of disease, colonic markers, and IL-1β production (Table 3), had elevated levels of FITC-dextran in the serum following oral gavage (Fig. 10) compared with mice not receiving DSS. When the L. salivarius strain UCC118, its bacteriocin-negative mutant, and strain AH43324 were administered to mice, all three strains were detected in feces at cell counts of 106 per gram (data not shown). As expected, bacterial administration to control mice (i.e., not receiving DSS) had no major effect on clinical signs of disease or colon macroscopic markers and in vivo permeability (Table 3). Administration of UCC118 to DSS-treated mice significantly reduced FITC-dextran serum levels compared with vehicle-DSS-treated mice (Fig. 10). More modest reduction was observed in DSS-treated mice that were administered UCC118 Bac− and AH43324 (Fig. 10), with neither treatment resulting in statistically significantly lower FITC-dextran serum levels than those caused by UCC118. However, administration of none of the L. salivarius strains resulted in any significant alterations clinical symptoms, DAI, or colon macroscopic markers (Table 3).

**DISCUSSION**

This study demonstrates that some strains of L. salivarius can attenuate H2O2-induced decrease in barrier function and disruption of tight junctions. Specifically, L. salivarius prevented the H2O2-induced redistribution of occludin, JAM-1, and claudin-1 in Caco-2 cells, and the magnitude of this barrier-protective effect varies among different strains. The strain dependence of this effect reflects differential activation of intracellular signaling pathways. We also showed that bacteriocin production by L. salivarius is a likely modifying influence on the capacity for barrier protection.

Increasing evidence suggests that some probiotic strains prevent inflammatory cytokine- or oxidative stress-induced tight junction dysfunction (8, 38, 49, 57). Our comparative study showed that different strains of L. salivarius differentially prevent H2O2-induced reduction in TER. Western blot and confocal microscopy analysis indicates that H2O2-induced reduction in TER and increase in FITC-dextran flux are attributed to redistribution of transmembrane tight junction proteins occludin, JAM-1, and claudin-1. In addition to expression levels of tight junction proteins, aberrant localization of these proteins is also implicated in barrier dysfunction caused by various stimuli in Caco-2 cells (3, 26, 70). Thus the large variation in the protective effects of L. salivarius strains on TER could be correlated with their varying potential to regulate tight junction structure. Indeed, UCC118 and CCUG38008 (which prevent reduction in TER and increase in FITC-dextran flux) attenuated redistribution of occludin, JAM-1, and claudin-1, whereas AH43324 (which is ineffective on TER and FITC-dextran flux) did not. Administration of the three lactobacillus strains in the DSS colitis model reduced serum dextran levels but did not significantly ameliorate the disease indexes. This suggests that barrier function is a more responsive phenotype than more overt disease measures, but this may be due to the subtleties of lactobacillus inoculum, duration, and environmental factors contributing to the degree of colitis in this model system.

The differential barrier-protecting effect of individual L. salivarius strains raised the possibility of strain-specific genes responsible for the barrier protection. We previously examined the genome diversity of the strains used in the present study, by applying CGH. Data from the TER analysis were first compared...
with the gene presence/absence patterns in the *L. salivarius* strains obtained. However, no genes showed a high correlation with the suppressive effect on H$_2$O$_2$-induced reduction in TER (data not shown), nor was any correlation detected with exopolysaccharide production levels previously measured in our laboratory (45). However, global transcriptome analysis of UCC118 and AH43324 allowed us to identify a candidate gene cluster, which encodes the bacteriocin, ABP-118. The transcription levels of most genes in the bacteriocin gene cluster were significantly higher in AH43324. Although we did not obtain the same results with qPCR analysis (which may be due to the different conditions of Caco-2 cells, polarized in Transwells compared with being cultured in six-well plates for RNA recovery), the expression of bacteriocin-related genes were barely detected or not detected in CCUG38008, which is the most effective strain. Furthermore, a bacteriocin-negative UCC118 mutant displayed a greater suppressive effect on H$_2$O$_2$-induced reduction in TER than did the wild-type strain. These results suggest that the bacteriocin secreted from *L. salivarius* might negatively regulate tight junction integrity. In contrast to results from TER analysis, the wild-type strain and the mutant equally attenuated the increase in FITC-dextran flux and the redistribution of FITC-dextran.

### Table 3. Effect of administering *Lactobacillus salivarius* strains on DSS-induced colitis

<table>
<thead>
<tr>
<th>Groups</th>
<th>Body Weight, %</th>
<th>DDAI</th>
<th>Length, cm</th>
<th>Colon Weight, mg/cm</th>
<th>Colonic IL-1β, pg/g</th>
</tr>
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<tbody>
<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ PBS</td>
<td>−0.5 ± 0.5</td>
<td>0</td>
<td>9.6 ± 0.2</td>
<td>26.6 ± 1.3</td>
<td>52.7 ± 8.2</td>
</tr>
<tr>
<td>+ UCC118</td>
<td>−0.7 ± 1.0</td>
<td>0</td>
<td>10.4 ± 0.4</td>
<td>25.9 ± 1.6</td>
<td>112.1 ± 21.6</td>
</tr>
<tr>
<td>+ UCC118 Bac&quot;</td>
<td>−0.7 ± 0.9</td>
<td>0.1 ± 0.1</td>
<td>9.4 ± 0.6</td>
<td>25.5 ± 2.4</td>
<td>N/A</td>
</tr>
<tr>
<td>+ AH43324</td>
<td>0.1 ± 0.9</td>
<td>0</td>
<td>9.9 ± 0.2</td>
<td>27.8 ± 1.7</td>
<td>141.4 ± 64.4</td>
</tr>
<tr>
<td><strong>DSS-treated</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ PBS</td>
<td>−2.8 ± 1.5</td>
<td>0.9 ± 0.1$^a$</td>
<td>7.8 ± 0.4$^a$</td>
<td>27.4 ± 1.8</td>
<td>355.8 ± 74.0$^a$</td>
</tr>
<tr>
<td>+ UCC118</td>
<td>−5.6 ± 2.1</td>
<td>1.5 ± 0.2$^b$</td>
<td>7.6 ± 0.3$^b$</td>
<td>33.0 ± 2.0</td>
<td>445.8 ± 72.6</td>
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<tr>
<td>+ UCC118 Bac&quot;</td>
<td>−5.7 ± 2.3</td>
<td>1.5 ± 0.2$^b$</td>
<td>7.2 ± 0.2$^b$</td>
<td>35.9 ± 2.6$^c$</td>
<td>N/D</td>
</tr>
<tr>
<td>+ AH43324</td>
<td>−3.1 ± 2.2</td>
<td>1.4 ± 0.1$^b$</td>
<td>7.7 ± 0.3$^b$</td>
<td>36.4 ± 2.0$^{b,c}$</td>
<td>696.1 ± 203.8</td>
</tr>
</tbody>
</table>

Groups of control mice or mice exposed to 4% dextran sodium sulfate (DSS) were treated with *L. salivarius* strains (UCC118, UCC118Bac$, and AH43324) from day −5 to the end of study at day 9. Body weight denotes percent of body weight loss/gain at day of termination (day 9) in relation to starting body weight at the start of DSS treatment (day 0). Daily Disease Activity Index (DDAI) includes stool consistency, fur texture, and posture. Total colon weight was divided by colon length and expressed as mg/cm. Significant differences between the stated groups as determined by 1-way ANOVA followed by Bonferroni’s multiple comparison test: $^aP < 0.05$ Control + PBS vs. DSS + PBS; $^bP < 0.05$ Control + UCC118 vs. DSS + UCC118; $^cP < 0.05$ Control + UCC118 Bac$^-$ vs. DSS + UCC118 Bac$^-$. $^dP < 0.05$ Control + AH43324 vs. DSS + AH43324; $^eP < 0.05$ DSS + PBS vs. DSS + UCC118Bac$^-$. $^fP < 0.05$ DSS + PBS vs. DSS + AH43324. N/D, not determined.
occludin, JAM-1, and claudin-1. It is possible that the bacteriocin affects other tight junction proteins, such as claudin-2, which was not examined in this study. Claudin-2 forms a cation-selective channel (2), and the increased expression of this protein leads to decrease in TER without affecting the macromolecular flux (60). Increased phosphorylation of p38 and Akt contributes to the increased expression of claudin-2 (60, 68). Notably, UCC118 enhanced H2O2-induced phosphorylation of p38 and Akt, whereas the bacteriocin-negative mutant did not, implying that the bacteriocin may upregulate claudin-2 under oxidative stress conditions via the p38 and Akt pathway, as shown diagrammatically in Fig. 11.

The phosphorylation of ERK by the *L. salivarius* strains capable of TER restoration, and the sensitivity of this process to an ERK inhibitor, corresponds to the central role of ERK activation in the protective effects of epidermal growth factor, and *L. rhamnosus* GG, on H2O2-induced barrier disruption and redistribution of tight junction proteins (3, 57). In contrast, the noneffective strain, AH43324, evidently augmented Akt and p38 phosphorylation, which has previously been shown to contribute to tight junction dysfunction (29, 58, 66). That ablation of bacteriocin production in a probiotic strain increased its ability to restore TER suggests that this antimicrobial peptide could contribute to barrier disruption or increased proinflammatory activity, of unknown duration in vivo. Although the effects of secreted microbial peptides including bacteriocins on host cells are poorly understood, there is evidence that they interact with epithelial cells and immune cells. Maher and colleagues (29) reported that antibacterial peptides, such as melittin, induce reduction in TER in Caco-2 cells. Meijerink and colleagues (32) demonstrated that bacteriocin-negative *L. plantarum* mutants exhibit different immunomodulatory properties in coculture with dendritic cells compared with wild-type strains, including higher level secretion of the anti-inflammatory cytokine IL-10. These lines of evidence imply that bacteriocins may play a wider-than-hitherto-appreciated role in the interaction between probiotic bacteria and host cells, including proinflammatory or immune-activation effects.

In addition to bacteriocins, other multiple bacterial components may affect intestinal tight junction integrity. Several innate pattern recognition receptors in intestinal epithelial cells recognizing evolutionarily conserved microbe-associated molecular patterns have been shown to be involved in the modulation of tight junction integrity by bacteria. Toll-like receptors (TLR) 2 and 9, which recognize bacterial teichoic acids and unmethylated CpG DNA, respectively, play key roles in barrier-protecting effects of commensal or probiotic bacteria (18, 22, 37), whereas TLR4, which recognizes lipopolysaccharide, is involved in gram-negative bacteria-induced barrier dysfunction (20). These conserved bacterial components were not identified as candidate factors by our analysis, suggesting that other molecular-based studies are needed to fully clarify the bacterial effector components. Other strain-specific factors, such as *Bifidobacterium infantis* or *L. rhamnosus* GG-derived peptides, have also been shown to regulate tight junction integrity (9, 57). We are currently using next-

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**Fig. 10.** Effect of *Lactobacillus salivarius* administration on intestinal permeability. Mice were pretreated with vehicle or *L. salivarius* strain, then administered water (control group) or 4% dextran sodium sulfate (DSS) to induce a mild colitis. Values plotted are serum levels of FITC-dextran following oral gavage. *P* < 0.05 between FITC dextran levels in mice receiving vehicle vs. mice receiving UCC118 wild-type strain.

**Fig. 11.** Schematic representation of the effects of *L. salivarius* on barrier function, including relevant signaling pathways. UCC118 induces ERK phosphorylation and prevents H2O2-induced relocalization of claudin-1, occludin, and JAM-1 and H2O2-mediated increase in permeability. However, bacteriocin produced by UCC118 antagonizes this ERK-dependent restorative effect, in part, by facilitating the increased activation of p38MAPK and Akt. This increased activation of p38MAPK and Akt may result in overexpression of claudin-2.
generation sequencing technologies to generate large numbers of draft genomes from our *L. salivarius* strain collection to further elucidate strain-specific genes and to facilitate gene-trait matching.

In conclusion, this study has provided, for the first time, evidence that the *L. salivarius*-mediated barrier protecting trait varies at a strain level. The effective strains protect tight junction integrity from *H*2*O*2 through activation of the ERK pathway, while an ineffective strain enhances *H*2*O*2-induced activation of the Akt and p38 pathways. This study adds to the emerging evidence that bacteriocin production is relevant not only for the anti-infective properties of some commensal lactobacilli, but also for direct signaling to the innate immune system and tight junction apparatus in epithelia.

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**REFERENCES**


**AUTHOR CONTRIBUTIONS**


**DISCLOSURES**

F. Shanahan declares an association with the following companies: Alimentary Health Ltd., GlaxoSmithKline Ltd, the Procter and Gamble Co. The content of this paper was neither influenced nor constrained by that fact.

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