GASTROINTESTINAL, HEPATOBILIARY, AND PANCREATIC PATHOLOGY

***Lactobacillus acidophilus* Induces a Strain-specific and Toll-Like Receptor 2—Dependent Enhancement of Intestinal Epithelial Tight Junction Barrier and Protection Against Intestinal Inflammation**

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Defective intestinal tight junction (TJ) barrier is an important pathogenic factor of inflammatory bowel disease. To date, no effective therapies that specifically target the intestinal TJ barrier are available. The purpose of this study was to identify probiotic bacterial species or strains that induce a rapid and sustained enhancement of intestinal TJ barrier and protect against the development of intestinal inflammation by targeting the TJ barrier. After high-throughput screening of >20 *Lactobacillus* and other probiotic bacterial species or strains, a specific strain of *Lactobacillus acidophilus*, referred to as LA1, uniquely produced a marked enhancement of the intestinal TJ barrier. LA1 attached to the apical membrane surface of intestinal epithelial cells in a Toll-like receptor (TLR)-2—dependent manner and caused a rapid increase in enterocyte TLR-2 membrane expression and TLR-2/TLR-1 and TLR-2/TLR-6 hetero-complex—dependent enhancement in intestinal TJ barrier function. Oral administration of LA1 caused a rapid enhancement in mouse intestinal TJ barrier, protected against a dextran sodium sulfate (DSS) increase in intestinal permeability, and prevented the DSS-induced colitis in a TLR-2— and intestinal TJ barrier—dependent manner. In conclusion, we report for the first time that a specific strain of LA causes a strain-specific enhancement of intestinal TJ barrier through a novel mechanism that involves the TLR-2 receptor complex and protects against the DSS-induced colitis by targeting the intestinal TJ barrier. (*Am J Pathol* 2021, 191: 872–884; <https://doi.org/10.1016/j.ajpath.2021.02.003>)

Intestinal epithelial tight junctions (TJs) are the apical-most junctional complexes and act as a functional and structural barrier against the paracellular permeation of harmful luminal antigens, which promote intestinal inflammation.¹ The increased intestinal permeability caused by defective intestinal epithelial TJ barrier or a leaky gut is an important pathogenic factor that contributes to the development of intestinal inflammation in inflammatory bowel disease (IBD) and other inflammatory conditions of the gut, including necrotizing enterocolitis and celiac disease.^{2,3} Clinical studies in patients with IBD have found that a persistent increase in intestinal permeability after clinical remission is predictive of poor clinical outcome and early recurrence of the disease, whereas normalization of intestinal permeability correlates with a sustained long-term

clinical remission.^{4–6} Accumulating evidence has found that a defective intestinal TJ barrier plays an important role in exacerbation and prolongation of intestinal inflammation in IBD. Currently, no effective therapies that specifically target the tightening of the intestinal TJ barrier are available.

Intestinal microbiota play an important role in modulating the immune system and in the pathogenesis of

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intestinal inflammation.⁷ Patients with IBD have bacterial dysbiosis in the gut, characterized by a decrease in bacterial diversity and an aberrant increase in some commensal bacteria, which are an important factor in the pathogenesis of intestinal inflammation.^{8,9} Normal microbial flora of the gastrointestinal tract consists both of bacteria that are known to have beneficial effects (probiotic bacteria) on intestinal homeostasis and bacteria that could potentially have detrimental effects on gut health (pathogenic bacteria).¹⁰ The modulation of intestinal microflora affects the physiologic and pathologic states in humans and animals. For example, fecal transplantation from healthy, unaffected individuals to patients with refractory *Clostridium difficile* colitis is curative in up to 94% of the treated patients, and transfer of stool microbiome from obese mice induces obesity in previous lean mice, whereas transfer of microbiome from lean mice preserves the lean phenotype.^{11–13} The beneficial effects of gut microbiota are host and bacterial species-specific.¹⁴ Although multiple studies indicate that some commensal bacteria play a beneficial role in gut homeostasis by preserving or promoting the intestinal barrier function, because of conflicting reports, it remains unclear which probiotic species cause a persistent predictable enhancement in the TJ barrier and could be used to treat intestinal inflammation by targeting the TJ barrier. For example, some studies suggest that *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus plantarum*, or *Lactobacillus rhamnosus* cause a modest enhancement in the intestinal epithelial TJ barrier, whereas others have found minimal or no effect of these probiotic species on the intestinal TJ barrier.^{15–25} The major aim the current study was to perform a high-throughput screening of *Lactobacillus* and other bacterial species to identify probiotic species that induce a rapid, predictable, and marked increase in the intestinal epithelial TJ barrier and protect against the development of intestinal inflammation by preserving the intestinal TJ barrier.

In the studies described herein, most of the probiotic species tested (>20 species or strains) had a modest or minimal effect on intestinal TJ barrier function. *L. acidophilus* uniquely caused a rapid and marked increase in intestinal TJ barrier function. Further analysis indicated that the effect of *L. acidophilus* was strain-specific, limited to a specific strain of *L. acidophilus*, and did not extend to other *L. acidophilus* strains. The *L. acidophilus* enhancement of the intestinal TJ barrier was mediated by live bacterial-enterocyte interaction that involved Toll-like receptor (TLR)-2 heterodimeric complexes on the apical membrane surface of intestinal epithelial cells. Our animal studies also found that *L. acidophilus* causes a marked enhancement in mouse intestinal barrier function and protects against the dextran sodium sulfate (DSS)-induced colitis by preserving and augmenting the mouse intestinal barrier function in a strain-specific manner.

Materials and Methods

Determination of Caco-2 Epithelial Monolayer Resistance and Paracellular Permeability

Caco-2 cells (passage 18) were purchased from ATCC (Manassas, VA) and maintained at 37°C in a culture medium as previously described.²⁶ For growth on filters, Caco-2 cells were plated on Transwell for 3 to 4 weeks until the transepithelial resistance (TER) reached 400 to 550 $\Omega \cdot \text{cm}$.² The Caco-2 monolayer paracellular permeability was determined by using an established paracellular marker inulin (¹⁴C-250 μCi -radioactive tracer).²⁷

Preparation of Bacterial Culture and Cell-Free Culture Supernatant

The following *Lactobacillus* species and strains were purchased from ATCC: *L. acidophilus*, *Lactobacillus johnsoni*, *L. rhamnosus*, *L. plantarum*, *Lactobacillus brevis*, *Lactobacillus helveticus*, and *L. casei*. These bacteria were grown overnight in MRS broth (Difco, Detroit, MI) at 37°C with shaking. Live bacteria were spun down by centrifuging at $12,000 \times g$ for 10 minutes and pellets were stored at -80°C for additional experiments. Supernatants were separated from spun-down bacteria, filtered, and diluted in cell culture media Dulbecco's modified Eagle's medium (with no fetal bovine serum and no antibiotics) for further use. Viable lactobacilli were heat-killed by incubations in a water bath at 60°C for 1 hour. Viable count was performed to make sure no viable bacteria survived. Bacteria were stored at -80°C as heat-killed lactobacilli. In the treatment of Caco-2 monolayers, the bacterial pellet was suspended in Dulbecco's modified Eagle's medium and diluted to $\text{OD}_{600\text{nm}} = 0.135$ (1×10^8 CFU) in the same media and applied to the apical surface of cell monolayers. Other probiotic species studied include *Bifidobacterium bifidum*, *Bifidobacterium longum*, *Bifidobacterium breve*, and *Escherichia coli* Nissle, which were grown in an anaerobic environment in MRS broth.

Determination of Mouse Small Intestinal Permeability *In Vivo* and Measurement of TER

Studies were approved by the Penn State University and University of New Mexico Institutional Animal Care and Use Committee. TLR-2 null (TLR-2^{-/-}) and wild-type (WT) mice (both of C57BL/6 background) 9 to 12 weeks of age were obtained from The Jackson Laboratory (Bar Harbor, ME). The effect of *L. acidophilus* on intestinal permeability in an *in vivo* mouse model system was determined using a recycling intestinal perfusion method. For *in vivo* studies, 1×10^9 CFU of *L. acidophilus* in 200 μL of phosphate-buffered saline was administered daily by oral-gastric gavage, and mouse intestinal permeability was measured at different treatment periods. A 10-cm segment

of mouse small intestine was isolated and cannulated with a small-diameter plastic tube (in an anesthetized mouse maintained in 1% isoflurane in oxygen) and continuously perfused with 5 mL of Krebs phosphate saline buffer for a 2-hour perfusion period. An external recirculating pump was used to recirculate the perfusate at a constant flow rate (0.75 mL/minute). Intestinal permeability was assessed by measuring luminal-to-serosal flux rate of paracellular probe, fluorescein isothiocyanate-labeled dextran (molecular weight, 10,000 g/mol), as described previously.²⁸ For measurement of TER, intestinal tissues were harvested immediately after euthanasia, cut longitudinally, and placed on 0.11-cm²-aperture Ussing chambers. TER ($\Omega \cdot \text{cm}^2$) was calculated from the spontaneous potential difference and short-circuit current.²⁹ Mice were euthanized by inhalation of carbon dioxide, as recommended by the American Veterinary Medical Association. Mice were placed in a chamber and exposed to carbon dioxide until all signs of respiration ceased.

Induction of DSS-Induced Colitis and Determination of Mouse Colonic Permeability *in Vivo*

Mice received 3% (w/v) DSS (molecular weight, 36,000 to 50,000; MP Biomedicals, Santa Ana, CA) in autoclaved drinking water for 7 days. The body weights of mice were monitored daily, and histologic grading of colitis lesions was performed as described previously.^{29,30} To quantify the extent of mucosal damage, a segment from the distal colon was fixed in 4% paraformaldehyde, paraffin embedded, sectioned (5 μm), and stained with hematoxylin and eosin. For prevention studies, *L. acidophilus* was gavaged starting 2 days before DSS treatment and continued throughout DSS administration. For active treatment studies, *L. acidophilus* was gavaged once daily starting at day 4 after DSS administration and continued up to 9 days. Colonic permeability was measured by recycling colonic perfusion method at the end of 7 or 9 days of DSS treatment, as previously described.^{29,30} The colonic permeability was assessed by measuring the luminal-to-serosal flux rate of a paracellular probe, fluorescein isothiocyanate-labeled dextran (molecular weight, 10,000 g/mol).

Gel Electrophoresis and Western Blotting

The lysates of Caco-2 monolayers and mouse intestinal tissues were prepared and processed for SDS-PAGE as described previously.²⁶ Equal amounts of protein were loaded in individual wells on the SDS-PAGE gel. After protein transfer to membrane, the membranes were probed using anti-TLR-1, -2, -4, -5, and -6 (Abcam, Cambridge, MA) and β -actin (Santa Cruz Biotechnology, Dallas, TX) antibodies.

Confocal Immunofluorescence

Immunolocalization of *L. acidophilus* and TLR-2 was assessed by confocal immunofluorescence. *L. acidophilus* was labeled with Vybrant DiO cell-labeling solution (Thermo Fisher Scientific, Waltham, MA) before treatment. The labeled *L. acidophilus* was added to Caco-2 at the apical bathing buffer solution for 2 hours; monolayers were then rinsed twice in cold phosphate-buffered saline, and fixed with methanol for 10 minutes. The cell monolayers were then blocked in normal serum and labeled with TLR-2 primary antibody (Abcam; catalog number 191,458) in blocking solution overnight at 4°C. After being washed with phosphate-buffered saline, the cells were incubated in Cy3-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). All the primary and secondary antibodies were used at the concentrations suggested by the manufacturers. ProLong Gold antifade reagent (Invitrogen, Carlsbad, CA), containing DAPI as a nuclear stain, was used to mount the cell filters on glass slides. The slides were examined using a confocal fluorescence microscope (Leica SP8). Images were processed with LAS X software (Leica Microsystems, Wetzlar, Germany).

siRNA Cell Transfections

Caco-2 monolayers were transiently transfected with TLR-1, -2, -4, -5, and -6 siRNA using DharmaFect transfection reagent (Dharmacon, Lafayette, CO), as described previously.²⁷ In brief, filter-grown Caco-2 monolayers were treated with 5 ng (0.5 nmol) siRNA and DharmaFect transfection reagent in Accell media (Thermo Fisher Scientific). The efficiency of silencing was confirmed by Western blot analysis after 72 hours of treatment.

RNA Isolation, Reverse Transcription, and Quantification of Gene Expression Using Real-Time PCR

Total RNA was isolated using the miRNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Reverse transcription was performed using the QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's protocol. Real-time PCR was performed using the PikoReal 96 Real-Time PCR system (Thermo Scientific). TaqMan Gene expression master mix kit was used for gene expression studies, and glyceraldehyde-3-phosphate dehydrogenase was used as an internal control. A pre-developed TaqMan Gene expression assay for TLR-2 and glyceraldehyde-3-phosphate dehydrogenase was used to determine the real-time gene expression (Qiagen). Primer sets for TLR-2 were purchased from Applied Biosystems (Foster City, CA) (5'-TCTGGGCAGTCTTGAACATTT-3' 5'-AGAGTCAGGTGATGGATGTCG-3').

Statistical Analysis

The values of experimental data are expressed as the means $\pm$ SE and analyzed using analysis of variance (≥ 2 groups or treatments) or unpaired *t*-test (2 groups or treatments) (Graph Pad Prism version 6.00 for Windows; GraphPad Software, San Diego, CA). All experiments were repeated at least three times to ensure reproducibility. *P* < 0.05 was considered significant.

Results

Effect of Probiotic Bacterial Species on Caco-2 Intestinal Epithelial TJ Barrier Function

To determine the intestinal TJ barrier—modulating effects of probiotic bacterial species, the effects of >20 commercially available probiotic species within *Lactobacillus* and *Bifidobacterium* genus as well as other bacterial species were examined. *Lactobacillus* species, including *L. acidophilus*, *L. rhamnosus*, *L. plantarum*, *L. johnsonii*, *L. brevis*, *L. casei*, and *L. helveticus*, were evaluated for their effects on intestinal epithelial TJ barrier function by measuring TER in filter-grown Caco-2 monolayers. Bacterial concentrations were measured by bacterial absorbance (600 nm).³¹ On the basis of previous studies that found the beneficial concentrations of probiotic bacterial species to range from OD₆₀₀ 0.1 to 0.2 or 1×10^7 to 1×10^9 CFU/mL,^{32–34} bacterial concentration of 1×10^8 CFU/mL corresponding to an OD₆₀₀ of 0.135 was used in the current studies. Probiotic bacteria were applied to the apical compartment of filter-grown Caco-2 monolayers for up to 7 days in the experimental period. Bacterial concentration of 1×10^8 CFU/mL was maintained throughout the entire experimental period by daily rinsing off of the bacteria and recharging the apical compartment with 1×10^8 CFU/mL bacteria. The effects of various species of *Lactobacillus* on Caco-2 TER measurement were determined. *L. johnsonii* and *L. rhamnosus* (1×10^8 CFU/mL) caused a modest time-dependent enhancement (10% to 20% increase) in Caco-2 TER, whereas *L. plantarum*, *L. brevis*, *L. helveticus*, and *L. casei* did not have a significant effect on Caco-2 TER (data not shown). In contrast, *L. acidophilus* caused a marked increase (80% to 100%) in Caco-2 TER by 24 hours (Figure 1A), which persisted throughout the 7-day experimental period. *L. acidophilus* also caused a corresponding decrease in apical-to-basolateral flux of paracellular marker inulin (Figure 1B). The graph plot of the *L. acidophilus* effect on inulin flux versus TER indicated a direct linear correlation between increasing TER and decreasing trans-epithelial flux of inulin with a correlation coefficient of *r* = 0.911 (Figure 1C), confirming that the increase in Caco-2 TER correlates with a decrease in paracellular permeability. Other probiotic species studied had a modest or no effect on Caco-2 TER (data not shown).

Whether the observed *L. acidophilus* effect on the Caco-2 TJ barrier was a species-wide or a strain-specific effect was examined. In these studies, the effects of three different *L. acidophilus* strains (referred to as LA1, LA2, or LA3) (obtained from ATCC) on Caco-2 TER were studied. LA1 (1×10^8 CFU/mL) caused a near-doubling of Caco-2 TER (90% to 100% increase), whereas LA2 caused a moderate (50%) increase, and LA3 did not affect the TER in the 24-hour experimental period (Figure 1D). Together, these data suggest that the *L. acidophilus* enhancement of Caco-2 TJ barrier function is strain specific. Next, the effects of *L. acidophilus* bacterial supernatant or heat-killed *L. acidophilus* on Caco-2 TER was determined. Neither the supernatant nor heat-killed *L. acidophilus* affected the Caco-2 TER (Figure 1E), suggesting that live bacterial-epithelial cell interaction was required for the *L. acidophilus* increase in Caco-2 TER. Similar to LA1, neither the supernatant from LA2 or LA3 culture nor the heat-killed LA2 or LA3 affected the Caco-2 TER (Figure 1, F and G). None of the supernatant from other lactobacilli bacterial culture or heat-killed bacteria affected the Caco-2 TER, except for the supernatant from *L. casei* culture, which caused a small but significant increase in Caco-2 TER (data not shown).

Each of the three strains was authenticated to be *L. acidophilus* species by 16S rRNA gene sequencing and by total genome sequencing.

L. acidophilus Enhancement of Caco-2 TJ Barrier Function Is Mediated by TLR-2

TLRs are pathogen recognition receptors and play a critical role in innate immune response of intestinal epithelial cells.³⁵ Previous studies have found that bacterial/host cell interactions are mediated by plasma membrane-localized TLRs and activation of various TLR signal transduction cascades, including TLR-2, TLR-4, and TLR-5.^{36,37} In the following studies, the effects of LA1 on expression of TLR-2, TLR-4, or TLR-5 on filter-grown Caco-2 monolayers were determined. LA1 (1×10^8 CFU/mL) caused an increase in TLR-2 protein expression by day 1 (Figure 2A) but not in TLR-4 or TLR-5 protein expression (Figure 2A) in Caco-2 monolayers. LA1 also caused an early increase in TLR-2 mRNA expression in Caco-2 monolayers by 6 hours of treatment (Figure 2B). To determine the requirement of TLR-2 in LA1 enhancement of Caco-2 TER, the effect of siRNA-induced knockdown of TLR-2 was examined on LA1-induced increase in Caco-2 TER. The TLR-2 siRNA transfection caused a near-complete depletion of TLR-2 in filter-grown Caco-2 monolayers (Figure 2C) and inhibited the LA1-induced increase in Caco-2 TLR-2 expression and TER (Figure 2D). The siRNA-induced knockdown of TLR-4 or TLR-5 did not inhibit the LA1-induced increase in Caco-2 TER (Figure 2E). To further investigate the role of TLR-2 in LA1 enhancement of Caco-2 TJ barrier, the expression of TLR-2 was examined in response to the *L. acidophilus* strain (LA3) that did not affect the Caco-2 TER. In contrast to LA1 and LA2, LA3 did not cause an

Figure 1 Effect of *Lactobacillus* species and strains (1×10^8 CFU/mL) on filter-grown Caco-2 tight junction (TJ) permeability. **A:** Time-course of the effect of *Lactobacillus acidophilus* on Caco-2 transepithelial resistance (TER). **B:** Time-course of the effect of *L. acidophilus* on Caco-2 mucosal-to-serosal flux of paracellular marker inulin. **C:** Graph plot of the effect of *L. acidophilus* on increasing TER and decreasing transepithelial flux of inulin with a correlation coefficient of $r = 0.911$. **D:** Caco-2 TER in the presence of strains LA1, LA2, and LA3 during the 24-hour experimental period. **E:** Filter-grown Caco-2 TER in the presence of live LA1, LA1 bacterial supernatant, or heat-killed LA1. **F:** Caco-2 TER in the presence of live LA2, LA2 bacterial supernatant, or heat-killed LA2. **G:** Caco-2 TER in the presence of live LA3, LA3 bacterial supernatant, or heat-killed LA3. Each experiment was performed at least three times. $n = 4$. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus control; † $P < 0.05$, †† $P < 0.01$, and ††† $P < 0.001$ versus live *L. acidophilus* (LA1 or LA2).

increase in TLR-2 expression (Figure 2F), and knocking down TLR-3 did not have a significant effect on Caco-2 TER after LA3 treatment (Figure 2G). Together, these studies suggest that the LA1-induced increase in Caco-2 TJ TER requires an increase in TLR-2 expression.

TLR-2 is known to form a functional heterodimeric receptor complex with TLR-1 and TLR-6.³⁸ In the following

studies, the specific dimeric complex, TLR-2/TLR-1 or TLR-2/TLR-6, responsible for the LA1 enhancement of Caco-2 TJ barrier function was examined. LA1 treatment produced an increase in both TLR-1 and TLR-6 protein expression, but the effect on TLR-6 was more pronounced (Figure 3A). The siRNA-induced knockdown of TLR-1 or TLR-6 resulted in a similar level of inhibition of the LA1-induced increase in

Figure 2 Effect of *Lactobacillus acidophilus* (1×10^8 CFU/mL) on Caco-2 Toll-like receptors (TLRs). **A:** Representative immunoblot indicating Caco-2 TLR-2, TLR-4, or TLR-5 protein expression in the presence of LA1 at days 1, 3, and 5. Relative densitometry of TLR-2 indicated on the right. **B:** TLR-2 mRNA levels in the presence of LA1. **C:** Immunoblot indicating TLR-2 expression in the presence of TLR-2 siRNA transfection. Relative densitometry of TLR-2 indicated below. **D:** LA1-induced increase in Caco-2 transepithelial resistance (TER) (24-hour experimental period) in the presence of TLR-2 siRNA transfection. **E:** LA1-induced increase in Caco-2 TER (24-hour experimental period) after knocking-down TLR-4 or TLR-5 by siRNA transfection. **F:** Immunoblot indicating TLR-2 protein expression in Caco-2 monolayers in the presence of LA1, LA2, or LA3. Relative densitometry of TLR-2 indicated on the right. **G:** Caco-2 TER in LA3-treated monolayers in the presence of TLR-2 knockdown by siRNA transfection. Each experiment was performed three times. $n = 3$. *** $P < 0.001$ versus control; ††† $P < 0.001$ versus LA1.

Caco-2 TER (Figure 3B). The simultaneous knockdown of both TLR-1 and TLR-6 completely inhibited the LA1-induced increase in Caco-2 TER (Figure 3B).

LA1 Attachment to the Caco-2 Apical Membrane Surface Is Dependent on TLR2-Membrane Expression

In the following studies, the mechanistic role of TLR-2 receptor complex in LA1/enterocyte interaction was

studied by examining the requirement of TLR-2 in LA1 attachment to the Caco-2 apical membrane surface. In the untreated Caco-2 monolayers, TLR-2 was diffusely distributed on the apical membrane surface as seen in the *en face* and *x-z* plane views (Figure 3C). The LA1 addition to the filter-grown Caco-2 monolayers caused a rapid redistribution, leading to a focal aggregation of TLR-2 at discrete points of LA1 attachment to the apical membrane surface. The siRNA-induced knockdown of

Figure 3 Effect of *Lactobacillus acidophilus* 1 (LA1) (1×10^8 CFU/mL) on toll-like receptor (TLR)-1 and TLR-6. **A**: Representative immunoblot indicating protein expression of TLR-1 and TLR-6 in Caco-2 monolayers in the presence of LA1. Graph of relative densitometry indicated on the right. **B**: Caco-2 transepithelial resistance (TER) in the presence of siRNA-induced knockdown of TLR-1 or TLR-6. **C**: Representative images indicating LA1 attachment to the Caco-2 apical membrane surface. TLR-2 (red), LA1 (green), and TLR-2 (yellow in merged panel; **middle row**, 2-hour treatment). Nuclei are shown in blue. The x and z stacks show LA1 and TLR-2. The siRNA knockdown of TLR-2 is indicated in the **bottom row**. Each experiment was performed three times. $n = 4$. $**P < 0.01$, $***P < 0.001$ versus control; $†††P < 0.001$, $††††P < 0.0001$ versus LA1. Scale bars = 10 μm .

TLR-2 inhibited the LA1 attachment to the Caco-2 apical membrane surface. The apical membrane localization of TLR-2 along the apical-to-basal axis (x-z plane) is also shown in **Figure 3C**. In the control Caco-2 monolayers, TLR-2 diffusely distributed at the apical membrane surface. LA1 caused an aggregation of TLR-2

along the apical membrane surface (**Figure 3C**), which was absent after TLR-2 knockdown. These immunostaining studies suggest that LA1 attaches to the Caco-2 cell surface at discrete points of TLR-2 aggregation and that TLR-2 expression is required for the LA1 attachment to the cell surface.

Effect of LA1 on Mouse Intestinal Permeability *in Vivo*

To assess the *in vivo* relevance of the effect of *L. acidophilus* on the intestinal epithelial TJ barrier, the effects of LA1 on mouse intestinal epithelial barrier function *in vivo* were examined. The effects of LA1 administration via oral-gastric gavage on mouse intestinal permeability were determined *in vivo* by recycling perfusion of small intestine in live mice.^{27,39,40} The time-course effect of LA1 (1×10^9 CFU/mL) administered daily via oral-gastric gavage on mouse small intestinal permeability was determined during the 5-day experimental period by measuring intestinal absorption of paracellular marker (dextran, 10 kDa) from the perfusate solution. LA1 caused a rapid decrease in mouse small intestinal permeability by day 1 of LA1 administration. LA1 decrease in intestinal permeability continued for the entire 5-day treatment period (Figure 4A). Of note, LA1 decrease in intestinal permeability persisted up to 6 weeks of administration (data not shown). LA1 treatment also caused

an increase in mouse small intestinal tissue electrical resistance (Figure 4B) compared with that in the control mouse as determined by intestinal tissue mounting in a Ussing chamber. In contrast, LA3 did not cause a decrease in mouse small intestinal permeability (Figure 4C). The regulatory involvement of TLR-2 on LA1 in decreasing mouse intestinal permeability was also determined. LA1 (1×10^9 CFU/mL) administration caused an increase in mouse small intestinal tissue TLR-2 expression by day 1 (Figure 4D). The LA1-induced decrease in small intestinal permeability was prevented in the TLR-2-deficient (TLR-2^{-/-}) mice (Figure 4E), suggesting that the increase in TLR-2 expression was necessary for the LA1 enhancement in mouse small intestinal barrier function.

Next, the therapeutic efficacy of LA1 on DSS-induced increase in colonic permeability and colitis was determined. Oral administration of 3% DSS for 7 days caused a time-dependent increase in colonic permeability, starting from day 1 (Figure 5A). Administration of 3% DSS caused a mild

Figure 4 Effect of *Lactobacillus acidophilus* 1 (LA1) on mouse intestinal permeability *in vivo*. **A:** The time-course effect of LA1 (1×10^9 CFU/mL) administered daily via oral-gastric gavage on mouse small intestinal permeability determined during the 5-day experimental period by measuring intestinal absorption of paracellular marker (dextran, 10 kDa) from the perfusate solution. **B:** Small intestinal epithelial resistance assessed by mounting the mouse intestinal tissue in a Ussing chamber. **C:** Effect of LA3 (1×10^9 CFU/mL) on the mouse small intestinal permeability to 10 kDa of dextran. **D:** Representative immunoblot indicating Toll-like receptor (TLR)-2 protein expression in mouse small intestinal tissue in the presence of LA1. Graph of relative densitometry indicated on the right. **E:** Mouse small intestinal permeability to 10 kDa of dextran in TLR-2^{-/-} mice in the presence of LA1. Each experiment was performed at least three times. $n = 5$ (**A**) and 3 (**B** and **C**). * $P < 0.01$, ** $P < 0.001$ versus control.

Figure 5 The effect of *Lactobacillus acidophilus* (1×10^9 CFU/mL) on dextran sodium sulfate (DSS)—induced increase in mouse colonic permeability and inflammation. **A:** Mouse colonic permeability to 10 kDa of dextran in the presence of DSS (3% w/v). **B:** Mouse colonic permeability to 10 kDa of dextran in the presence of LA1 and LA3. **C:** DSS-induced increase in mouse colonic permeability to 10 kDa of dextran during the 7-day experimental period in the presence of LA1 and LA3 in TLR-2^{-/-} mice. **D:** Hematoxylin and eosin staining of colonic mucosa after DSS administration in the presence of LA1 or LA3. LA1 administration after DSS treatment in TLR-2 knockout (KO) mice (right panel, middle row). **E and F:** Graph representing the effect of LA1 and LA3 on DSS-induced body weight loss or disease activity. **G:** Effect of LA1 treatment 4 days after DSS administration on DSS-induced increase in colonic intestinal permeability during the 9-day experimental period. **H:** Hematoxylin and eosin staining of colonic mucosa with LA1 treatment after DSS administration compared to DSS alone. Each experiment was performed at least three times. $n = 3$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ versus control; † $P < 0.05$ versus LA1; and ‡ $P < 0.001$ versus DSS treatment. Original magnification, $\times 200$ (D and H).

colitis by day 3 to 4 and more severe colitis by day 5 to 7. The increase in colonic permeability precedes the initial development of colitis.⁴¹ Similar to the small intestine, LA1 oral-gastric gavage, but not LA3, caused a rapid decrease in colonic permeability (Figure 5B). The daily LA1 (1×10^9 CFU/mL) treatment starting 2 days before DSS

administration and continuing throughout the 7 days of DSS ingestion inhibited the DSS-induced increase in colonic permeability (Figure 5C) and the development of colitis (Figure 5, D and E). DSS-induced weight loss was also significantly inhibited in the LA1-treated mice (Figure 5F). In contrast, LA3 did not cause a decrease in colonic

permeability (Figure 5B) and did not inhibit the DSS-induced increase in mouse colonic permeability (Figure 5C), colitis (Figure 5, D and E), or weight loss (Figure 5F). Next, the involvement of TLR-2 in LA1 protection against DSS-induced increase in colonic permeability and colitis was determined. The LA1 protection of intestinal TJ barrier and colonic inflammation in DSS-treated mice was abolished in TLR-2 knockout (TLR-2^{-/-}) mice (Figure 5, C and D), confirming the requirement of TLR-2 in LA1 enhancement and protection of mouse colonic barrier. Lastly, short-term therapeutic effects of LA1 on DSS-induced colitis were examined. LA1 treatment was started on day 4 of DSS administration, after the initial onset of colonic inflammation. In these studies, mice were administered DSS for an additional 5 days for a total of 9 days. LA1 treatment was started on day 4 and continued daily to day 9 of DSS administration. LA1 treatment after the initial onset of DSS-induced increase in colonic permeability and colitis resulted in a decrease in colonic permeability or a retightening of the colonic barrier (Figure 5G) and healing of the DSS-induced colitis (reduced inflammatory changes, preservation of colonic crypts, and regeneration of surface epithelium) (Figure 5H).

Discussion

A defective intestinal TJ barrier is an important pathogenic factor that contributes to the development of gut inflammation.^{42,43} Bacterial dysbiosis is also an important factor that contributes to the development of gut inflammation.^{9,10} The major aim of the present study was to identify probiotic bacteria able to produce a rapid and marked enhancement of intestinal TJ barrier function that can treat intestinal inflammation by targeting the TJ barrier. Although a wide variability exists among individuals, the intestinal microflora of full-term, healthy, breastfed infants consists predominantly of *Lactobacillus* species.⁴⁴ The presence of *Lactobacillus* species is associated with healthy gut, and they have been widely used as probiotic agents to improve gut health.⁴⁵ Many of the beneficial effects of probiotic bacteria in ameliorating gut inflammation have been attributed to their effects on preserving the intestinal barrier function.^{15,46–48} High-throughput screening of probiotic bacteria indicated that *L. acidophilus* produced a rapid and marked enhancement in intestinal epithelial TJ barrier function. In prior publications, *L. acidophilus* was reported to have a minimal or modest effect on intestinal epithelial monolayer TER. Lepine et al¹⁹ reported that *L. acidophilus* caused a 15% increase in Caco-2 TER, whereas Guo et al¹⁷ found *L. acidophilus* to cause a 25% increase in Caco-2 TER. VSL#3, which contains nine probiotic bacterial species, including *L. acidophilus*, produced an approximately 20% increase in TER in T84 colonic monolayers.^{49,50} In the studies described herein, *L. acidophilus* (LA1) produced an approximately 80% to 100% increase in Caco-2 TER, which

is by far the highest reported increase in TER by *L. acidophilus*. The *L. acidophilus* effect on intestinal epithelial TJ barrier was strain specific, with LA3 having no effect and LA2 having an intermediate effect.

Most lactobacilli possess acid and bile salt tolerance, allowing them to survive the hostile environment of the acidic gastric content and bile acid-filled proximal small intestine.^{51,52} Several cell surface components of commensal bacteria are recognized by the intestinal and immune cells via pattern recognition receptors, which are key regulators of innate epithelial cell response to microbe-associated molecular patterns.⁵³ In this regard, TLRs present on the enterocyte plasma membrane surface, including TLR-1, TLR-2, TLR-4, TLR-5, and TLR-6, are an important class of pattern recognition receptors that mediate host response to microbe-associated molecular patterns.^{54–58} Data from the current study indicate that TLR-2 is present on the apical membrane surface of the enterocytes and LA1 colonization causes a rapid increase in apical membrane expression of TLR-2 in Caco-2 cells; moreover, LA1 enhancement in Caco-2 TJ barrier function was dependent on the presence of the TLR-2 receptor complex and the activation of the TLR-2 signal transduction pathway. In support of the TJ barrier-protective role of TLR-2, previous studies have found that TLR-2 pathway activation by synthetic lipopeptide Pam3Cys-SK4 causes an enhancement of Caco-2 TJ barrier and protects against stress-induced damage of Caco-2 TJ barrier via the downstream activation of phosphatidylinositol 3-kinase–Akt.⁵⁹

TLR-2 forms a functional heterodimeric complex with TLR-1 or TLR-6 on enterocyte and immune cell membrane surface,⁶⁰ and TLR-1 and TLR-6 subunits play a critical role in the recognition of microbe-associated molecular patterns. The bacterial cell wall components, including triacyl and diacyl lipopeptides, are typically recognized by TLR-2/TLR-1 and TLR-2/TLR-6 heterodimers, respectively.^{61,62} Diacyl lipopeptides are typically associated with gram-positive bacteria and are recognized by TLR-2/TLR-6 heterodimers,⁶³ whereas triacyl lipopeptides are typically associated with gram-negative bacteria but may also be present in gram-positive bacteria, and are recognized by TLR-2/TLR-1 complex.⁶¹ Current studies suggest that both TLR-2/TLR6 and TLR-2/TLR-1 heterodimers are involved in the LA1-induced up-regulation of the Caco-2 TJ barrier. As would be expected, LA1 caused a disproportionately greater increase in expression of TLR-6. Interestingly, targeted knockdown of TLR-1 or TLR-6 caused a partial but significant inhibition of LA1 enhancement of the TJ barrier. The combined knockdown of TLR-1 and TLR-6 inhibited the LA1 effect, suggesting that the LA1 up-regulation of the Caco-2 TJ barrier was entirely regulated through TLR-2/TLR-6 and TLR-2/TLR-1 receptors.

The studies described herein provide insight into the role of TLR-2 in LA1 and intestinal epithelial cell interaction. Previous studies with *L. acidophilus* suggested that bacterial supernatant or bacterial secreted product from *L.*

acidophilus was sufficient to produce an enhancement in intestinal epithelial TJ barrier function.^{19,20,64} In the current studies, LA1 enhancement of the Caco-2 TJ barrier function required live bacterial-intestinal epithelial cell interaction, and heat-killed bacteria or bacterial supernatant had no effect on the Caco-2 TJ barrier, suggesting a direct cell-to-cell interaction. The LA1 Caco-2 epithelial cell attachment studies found that the LA1 attachment to the Caco-2 apical membrane surface caused a rapid redistribution and aggregation of TLR-2 at the focal points of LA1 contact, and LA1 only attached to the membrane surface at the points of TLR-2 aggregation. Moreover, this LA1 attachment was inhibited in Caco-2 cells that lacked the TLR-2, demonstrating the central role of TLR-2 in LA1 attachment. The LA3 strain was not able to attach to the apical membrane surface and did not cause an enhancement in Caco-2 TJ barrier function. Together, the bacterial attachment and TJ barrier function studies, suggest that the TLR-2 receptor complexes play a crucial role in LA1 up-regulation of the TJ barrier, in part, by facilitating LA1 attachment to the intestinal epithelial apical membrane surface and activation of the TLR-2 signal transduction pathway.

in vivo proof-of-concept studies were performed in live mice to investigate the effects of LA1 and LA3 in the enhancement of mouse intestinal barrier and the therapeutic efficacy of LA1 in maintaining intestinal barrier and protecting against the DSS-induced colitis. The DSS-induced colitis is the most commonly used murine inflammation model to study the linkage between defective intestinal TJ barrier and the development of intestinal inflammation.^{65,66} The live mouse intestinal perfusion studies found that LA1, but not LA3, caused a rapid and marked enhancement of small intestine and colonic epithelial barrier in mice. The oral administration of 3% DSS causes an early increase in intestinal permeability (by day 1), which precedes the development of mild histologic inflammation by day 4, and progressively more severe colitis by day 7. In the present studies, LA1 administration, but not LA3, prevented the DSS-induced increase in intestinal permeability and the development of DSS-induced development of colitis, suggesting that LA1 enhancement of intestinal barrier was required for its colitis-protective effect. Treatment studies after the onset of colitis also found that LA1 was effective in the healing of intestinal barrier and DSS-induced colitis. Thus, our data indicate that LA1, the bacterial strain that targets the intestinal TJ barrier, but not LA3, is able to prevent colonic inflammation formation and promote colitis healing in a TLR-2 receptor complex-dependent manner. To examine the possibility that LA1-induced enhancement of TJ barrier function might be related to an alteration in TJ protein expression, LA1 effect on TJ proteins, including occludin, claudin-1, claudin-2, claudin-3, ZO-1, and MLCK, was determined. In these preliminary studies, LA1 did not affect the protein expression of claudin-1, claudin-2, claudin-3, ZO-1, or MLCK, but caused an increase in occludin expression (data not shown), suggesting that the

increase in occludin protein expression may be a contributing factor to the LA1-induced enhancement of TJ barrier function. Interestingly, LA1 also appeared to prevent DSS-induced down-regulation of occludin in mouse intestinal tissue, suggesting a possible role in intestinal barrier preservation. More detailed studies are being planned to fully elucidate the role of occludin expression in LA1 enhancement and preservation of intestinal TJ barrier.

In conclusion, our studies indicate that LA1 causes a strain-specific, rapid enhancement of intestinal epithelial TJ barrier function. The LA1 enhancement of intestinal TJ barrier was mediated by the TLR-2 heterodimeric complexes TLR-2/TLR-1 and TLR-2/TLR-6. The TLR-2 receptor complexes play a key mechanistic role in LA1 attachment to the intestinal epithelial cells and in the up-regulation of intestinal epithelial TJ barrier. The LA1 enhancement and maintenance of the intestinal epithelial barrier were required for the prevention of DSS-induced colitis and for accelerated healing of the colitis. These studies identify a specific strain of *L. acidophilus* that is uniquely capable of producing a rapid and marked enhancement of intestinal TJ barrier and protect against DSS-induced colitis by targeting the TJ barrier.

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Author Contributions

R.A.-S. and T.Y.M. conceived and designed experiments and wrote the manuscript; P.N., M.N., M.H. and M.R. performed experiments; R.A.-S., P.N. analyzed the data; R.A.-S. and T.Y.M. interpreted results of experiments; R.A.-S. and P.N. prepared figures; R.A.-S. wrote the manuscript; T.Y.M. edited and revised the manuscript; and T.Y.M. approved final version of manuscript.

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