Lactobacillus rhamnosus GR-1 Ameliorates *Escherichia coli*-Induced Inflammation and Cell Damage via Attenuation of ASC-Independent NLRP3 Inflammasome Activation

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Escherichia coli is a major environmental pathogen causing bovine mastitis, which leads to mammary tissue damage and cell death. We explored the effects of the probiotic *Lactobacillus rhamnosus* GR-1 on ameliorating *E. coli*-induced inflammation and cell damage in primary bovine mammary epithelial cells (BMECs). Increased Toll-like receptor 4 (TLR4), NOD1, and NOD2 mRNA expression was observed following *E. coli* challenge, but this increase was attenuated by *L. rhamnosus* GR-1 pretreatment. Immunofluorescence and Western blot analyses revealed that *L. rhamnosus* GR-1 pretreatment decreased the *E. coli*-induced increases in the expression of the NOD-like receptor family member pyrin domain-containing protein 3 (NLRP3) and the serine protease caspase 1. However, expression of the adaptor protein apoptosis-associated speck-like protein (ASC, encoded by the *Pycard* gene) was decreased during *E. coli* infection, even with *L. rhamnosus* GR-1 pretreatment. Pretreatment with *L. rhamnosus* GR-1 counteracted the *E. coli*-induced increases in interleukin-1 β (IL-1 β), -6, -8, and -18 and tumor necrosis factor alpha mRNA expression but upregulated IL-10 mRNA expression. Our data indicate that *L. rhamnosus* GR-1 reduces the adhesion of *E. coli* to BMECs, subsequently ameliorating *E. coli*-induced disruption of cellular morphology and ultrastructure and limiting detrimental inflammatory responses, partly via promoting TLR2 and NOD1 synergism and attenuating ASC-independent NLRP3 inflammasome activation. Although the residual pathogenic activity of *L. rhamnosus*, the dosage regimen, and the means of probiotic supplementation in cattle remain undefined, our data enhance our understanding of the mechanism of action of this candidate probiotic, allowing for development of specific probiotic-based therapies and strategies for preventing pathogenic infection of the bovine mammary gland.

Mastitis affects cows in all regions of the world and can cause a decrease in milk production and quality, resulting in a major economic burden to the dairy industry (1). *Escherichia coli* is by far the most common cause of mastitis, since it is isolated in more than 80% of cases of coliform mastitis (2). Antibiotic therapy frequently leaves residues in milk, potentially facilitating the development of antibiotic resistance. Probiotics represent a novel alternative to antibiotics for controlling pathogen infections.

Lactobacillus rhamnosus GR-1 is a probiotic bacterium isolated from the female urethra, and oral ingestion of *L. rhamnosus* GR-1 and *L. fermentum* RC-14 reduces vaginal colonization by pathogenic bacteria and yeasts and maintains urogenital health in women (3). Pretreatment of pregnant CD-1 mice with *L. rhamnosus* GR-1 culture supernatant decreases lipopolysaccharide (LPS)-induced production of various cytokines and chemokines (4). *Lactobacillus rhamnosus* GR-1 suppresses expression of nuclear factor (NF)- κ B-related inflammatory genes and activates alternate mitogen-activated protein kinase (MAPK) and activator protein 1 pathways to recruit host defense factors in *Candida albicans* infection (5). Although several possible mechanisms underlying the beneficial effects of *L. rhamnosus* have been investigated, including direct antimicrobial action, as well as competitive exclusion of pathogens or inhibition of pathogen adhesion, enhancement of epithelial barrier function, and modulation of both local and systemic host immune responses (6), our previous studies demonstrated that pretreatment with high doses of *L. rhamnosus* may counteract the preventative effects (7, 8).

Recently, probiotics are considered a possible alternative treatment for mastitis. In humans, oral administration of lactobacilli (*L. fermentum* CECT5716 or *L. salivarius* CECT5713) isolated

from breast milk is an efficient alternative to antibiotics for the treatment of infectious mastitis during lactation (9, 10). In cattle, direct feeding of *L. acidophilus* NP51 induces a decrease in the prevalence of *E. coli* O157 infection (11). Lactic acid bacteria isolated from raw milk, as well as the mammary glands of clinically healthy or mastitic cows, are potentially beneficial strains for preventing bovine mastitis (12, 13). Intramammary inoculation of *L. perolens* CRL 1724 into the bovine udder produces a mild inflammatory response, which is characterized by recruitment of neutrophils to the epithelial zone but does not involve epithelial cell necrosis or apoptosis or any morphological modifications in the nucleolus and nuclear membrane (14). Probiotic *Lactobacillus* strains isolated from dairy products are thought to inhibit the adhesion of *E. coli* to Caco-2 cells and also to promote the secretion of pro- and anti-inflammatory cytokines by human peripheral blood mononuclear cells (15). However, the exact mechanism

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underlying probiotic modulation of the mammary inflammatory response has yet to be elucidated.

Bovine mammary epithelial cells (BMECs) are the predominant cell type in the mammary gland involved in synthesizing milk components that provide immunologic defense and nutritional support to the offspring. BMECs are poised to respond quickly to pathogen invasion by binding to pathogen-associated molecular pattern molecules via the activation of various pattern recognition receptors (PRRs) (16, 17). In general, the binding of a pathogen to a Toll-like receptor (TLR) or nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) activates the MAPK and NF- κ B signaling cascades, leading to upregulate expression of various proinflammatory cytokines, chemokines, and antimicrobial peptides, thereby triggering inflammation and host immune responses (18). The NLR family member pyrin domain-containing protein 3 (NLRP3) forms cytoplasmic multiprotein complexes known as inflammasomes along with the adaptor protein apoptosis-associated speck-like protein (ASC, encoded by the *Pycard* gene) and the serine protease caspase-1 (19). Active caspase-1 specifically mediates the processing of intracellular pro-IL-1 β and pro-IL-18 into their biologically active and secretable forms. LPS-mediated activation of the NLRP3 inflammasome through TLR4 contributes to acute pancreatitis and acute liver injury in mice (20). RNA:DNA hybrids of enterohemorrhagic *E. coli* were shown to activate the NLRP3 inflammasome, a finding that provided new insights into cytosolic immune surveillance mechanisms (21). The NLRP3 inflammasome may be a suitable target for new drugs to treat infectious diseases, including mastitis, in a manner that avoids the risk of antibiotic resistance development (22).

We hypothesized that administration of the probiotic *L. rhamnosus* GR-1 would ameliorate *E. coli*-induced inflammation and cell damage via modulation of ASC- or/and caspase-1-dependent NLRP3 inflammasome activation. The aim of this study, therefore, was to investigate the ability of *L. rhamnosus* GR-1 to prevent *E. coli* adhesion and characterize the effects of *L. rhamnosus* GR-1 on ameliorating *E. coli*-induced morphological and ultrastructural disruption of BMECs and the activation of NLRP3 inflammasomes in BMECs in response to *E. coli* infection.

MATERIALS AND METHODS

Primary BMEC culture. Three clinically healthy Chinese Holstein dairy cows (3 years old; 63 to 83 days postpartum) obtained from a commercial dairy herd in Beijing were selected for the study. The cows were free of pathogens through three consecutive bacteriologic examinations, with each quarter milk somatic cell count being <200,000 cells/ml 10 days before the start of the trial. Each cow was kept in an individual box stall and fed a mixed ration formulated for the dairy herd. Cows were milked three times daily at 03:30, 11:30, and 18:30 h. The animals were treated in strict accordance with the Guidelines for Laboratory Animal Use and Care from the Chinese Center for Disease Control and Prevention and the Rules for Medical Laboratory Animals from the Chinese Ministry of Health. The study protocol (CAU-AEC-2013-369) was approved by the Animal Ethics Committee of the China Agricultural University.

Primary BMECs were prepared from lactating cows as described previously (17), with some modifications. Briefly, mammary tissue pieces (0.5 to 1 mm³) were transferred into 25-cm² cell culture flasks (Corning, Inc., Corning City, NY) and cultured in Dulbecco modified Eagle medium with Ham F-12 nutrient mixture (DMEM/F12; pH 7.2; Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco), 100 U of antibiotic (penicillin and streptomycin; Invitrogen, Carlsbad, CA)/ml, 5 μ g of bovine insulin/ml, 1 μ g of hydrocortisone/ml, 5 μ g of progesterone (I8405, H0888, and P8783, respectively; Sigma-Aldrich, St. Louis, MO)/

ml, and 10 ng of murine epithelial growth factor (PeproTech, Rocky Hill, NJ)/ml. Cells were digested with 0.25% trypsin-EDTA (Gibco, Burlington, Ontario, Canada) to remove fibroblasts. Pure BMECs for use in subsequent experiments were isolated after three passages.

Immunocytochemistry. To verify the epithelial origin and the purity of the isolated BMECs, the cytomatrix and expression of cytokeratin 18 were examined. BMECs (6×10^4 cells/well) were seeded into a 24-well flat-bottom culture plate, with each well containing a glass coverslip. After 24 h of incubation, the coverslips were washed and fixed in 4% paraformaldehyde. BMECs were permeabilized with 0.2% (vol/vol) Triton X-100 (Sigma-Aldrich), blocked with 1% bovine serum albumin, and then incubated with mouse anti-cytokeratin-18 primary monoclonal antibody (MAb) (ab668; Abcam, Cambridge, United Kingdom) at a dilution of 1:200, followed by secondary antibody goat anti-mouse fluorescein isothiocyanate-conjugated IgG (F4143; Sigma-Aldrich). DAPI (4',6'-diamidino-2-phenylindole; Sigma-Aldrich) was used for nuclear staining. BMECs were then visualized and photographed using an FV1000 confocal laser scanning biological microscope (Olympus, Tokyo, Japan).

Bacterial strains and growth conditions. *Lactobacillus rhamnosus* GR-1 (ATCC 55826; American Type Culture Collection, Manassas, VA) was grown in De Man, Rogosa, and Sharpe (MRS) broth (Oxoid, Hampshire, United Kingdom) for 24 h at 37°C under microaerophilic conditions. For all experiments, after overnight incubation, bacteria were diluted 1:100 in fresh MRS broth and grown for about 8 h until reaching mid-log phase (i.e., an optical density at 600 nm [OD₆₀₀] of 0.5), and *L. rhamnosus* GR-1 was plated on MRS agar after serial dilution and quantified by determination of CFU.

Escherichia coli strain O111:K58 (CVCC1450; China Institute of Veterinary Drug Center, Beijing, China) was used for the induction of mastitis and was grown in Luria-Bertani (LB) broth (Oxoid, Basingstoke, England). After overnight incubation at 37°C with constant shaking, bacteria were diluted 1:100 in fresh LB broth and grown for about 3 h until reaching mid-log phase (OD₆₀₀ of 0.5).

Adhesion assay. Bacterial adhesion assays were performed as previously described (23). Briefly, BMECs (5×10^5 cells/well) were seeded onto transwell collagen-coated PTFE filters (pore size, 0.4 μ m; 4.7 cm²; Corning, Inc.). On day 2, confluent monolayers of BMECs were treated under one of six conditions, as follows: preincubation with (i) DMEM alone, (ii) live *L. rhamnosus* GR-1 (3×10^7 CFU), (iii) UV-irradiated *L. rhamnosus* GR-1 (3×10^7 CFU), (iv) heat-killed *L. rhamnosus* GR-1 (3×10^7 CFU), (v) *L. rhamnosus* GR-1 supernatant (pH 7.2), or (vi) DMEM acidified to pH 6.8 with lactic acid. At 3 h after pretreatment, the cells were washed three times with phosphate-buffered saline (PBS) and exposed to *E. coli* (3×10^7 CFU). We chose the bacterial concentration and time of incubation based on the results of lactate dehydrogenase (LDH) release measurements to allow for bacterial adhesion and membrane damage without disruption of the cell monolayer.

At 6 h after *E. coli* challenge, the BMEC monolayer was washed four times with PBS to remove nonadherent bacteria and then harvested by treatment with 0.05% trypsin for 10 min at 37°C. An adhesion assay using *E. coli* alone served as a reference. The adhesion rate was defined as the adhered *E. coli* population on the BMECs pretreated with different conditions relative to the adhered *E. coli* population in the reference experiment.

Internalization assay. Internalization assays were performed as previously described (23). BMECs (5×10^5 cells/well) were seeded onto transwell collagen-coated PTFE filters. On day 2, confluent monolayers of BMECs were treated with *L. rhamnosus* GR-1 (3×10^7 CFU) only or exposed to *E. coli* (3×10^7 CFU) alone. At 2, 4, 6, 12, and 24 h after *L. rhamnosus* GR-1 or *E. coli* challenge, *E. coli* or *L. rhamnosus* GR-1 internalization was measured after an additional 2 h of incubation with DMEM supplemented with gentamicin (100 μ g/ml) to kill bacteria remaining outside the cells.

Scanning electron microscopy. BMECs were treated under four different conditions: (i) DMEM alone, (ii) *E. coli* (3×10^7 CFU) infection

only, (iii) incubation with *L. rhamnosus* GR-1 (3×10^7 CFU) only (LRGR) for 3 h, or (iv) preincubation with *L. rhamnosus* GR-1 (3×10^7 CFU) for 3 h prior to exposure to *E. coli* (3×10^7 CFU). At 6 h after *E. coli* challenge, BMECs were grown on glass coverslips and then fixed with 3% glutaraldehyde (pH 7.4). Subsequently, the fixed cells were incubated with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer before dehydration in graduated ethanol series (30, 50, 70, 90, 95, and 100%), followed by 100% acetone. Samples were critical point dried with liquid carbon dioxide using a CPD 030 critical point dryer (BAL-TEC, Witten, Germany) and then sputter coated with 20-nm gold particles using an SCD 005 (BAL-TEC). The cells were then examined under a Quanta 200 FEG field emission scanning electron microscope (FEI, Eindhoven, The Netherlands).

Transmission electron microscopy (TEM). BMECs were harvested and fixed in 3% glutaraldehyde. The fixed cells were postfixed in 1% osmium tetroxide, dehydrated using a graduated ethanol series (30, 50, 70, 80, 90, and 100%), embedded in Epon (Energy Beam Sciences, Agawam, MA), sliced into ultrathin sections (50 to 60 nm) using a Leica EM UC6 ultramicrotome (Leica Microsystems, Wetzlar, Germany), and stained with 3% uranylacetate and lead citrate. The ultrathin sections were observed under an H7500 transmission electron microscope (Hitachi, Tokyo, Japan).

Morphometric analysis. BMECs were treated as described above and then visualized and photographed in 10 randomly selected fields containing more than 20 cells using a Nikon Eclipse Ti-U inverted microscope (Nikon, Tokyo, Japan).

Cell death assay. To assay cell death under the different conditions examined, LDH levels were measured using a Cytotox96 cytotoxicity assay (Promega, Madison, WI) according to the manufacturer's instructions. To normalize for spontaneous lysis, the percentage of LDH released was calculated using the following equation: $[(\text{LDH infected} - \text{LDH uninfected}) / (\text{LDH total lysis} - \text{LDH uninfected})] \times 100$.

Quantitative real-time PCR. At 2, 4, 6, 12, and 24 h postinfection, total RNA was extracted from BMECs using TRIzol reagent (Invitrogen). Assessment of the quality of the total RNA and subsequent reverse transcription were carried out as described previously (8). The sequences of primers used in this study are listed in Table S1 in the supplemental material. Quantitative reverse transcription-PCR was performed using an ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA). Gene expression data were normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. The relative abundance of mRNA was calculated according to the method of Pfaffl (24).

Immunofluorescence. To evaluate the effect of *L. rhamnosus* GR-1 on the activation of NLRP3, BMECs (6×10^4 cells/well) were plated on glass coverslips in a 24-well flat-bottom culture plate, as described above. At 6 h after *E. coli* challenge, the cells were fixed with 4% paraformaldehyde, treated with 0.2% (vol/vol) Triton X-100, and then blocked with 1% BSA. The cells were then incubated with rabbit polyclonal anti-NLRP3 primary antibody (19771-1-AP; Protein Tech Group, Chicago, IL) at a dilution of 1:50, followed by Cy3-conjugated goat anti-rabbit IgG(H+L) (AP307F; Sigma-Aldrich). DAPI was used for nuclear staining. The cells were observed and photographed using a Nikon Eclipse Ti-U inverted fluorescence microscope equipped with a Nikon DS cooled camera head (Nikon).

Western blotting. At 2, 4, and 6 h postinfection, BMECs were lysed in 1 ml of lysis buffer, composed of 1 ml of radioimmunoprecipitation assay buffer, 5 μ l of protease inhibitor cocktail, and 5 μ l of phenylmethanesulfonyl fluoride (Sigma-Aldrich). The resulting lysates were centrifuged at $13,000 \times g$ for 10 min at 4°C to pellet insoluble material, and the supernatants were used for Western blot analysis. Protein concentrations were determined using a BCA protein assay kit (Pierce Chemical Co., Rockford, IL). The following primary antibodies were used: rabbit polyclonal anti-NLRP3 (19771-1-AP, 1:200 dilution), rabbit polyclonal anti-ASC (10500-1-AP, 1:500 dilution), rabbit polyclonal anti-caspase-1 (22915-1-AP, 1:500 dilution), and mouse anti-GAPDH MAb (60004-1-Ig, 1:2,000 dilution) (Protein Tech Group). Horseradish peroxidase-conjugated Af-

finipure goat anti-mouse IgG(H+L) (SA00001-1) or goat anti-rabbit IgG(H+L) (SA00001-2; Protein Tech Group) were used as secondary antibodies. Densitometric values of Western blot images were obtained from three independent experiments using ImageJ software (National Institutes of Health, Bethesda, MD). Results are presented as the ratio of the NLRP3, ASC, or caspase-1 band intensity to that of GAPDH.

Statistical analysis. Statistical evaluations were performed using SAS statistical software package, version 9.1 (SAS Institute, Inc., Cary, NC). Data were also evaluated using analysis of variance procedures. With regard to small sample sizes, normal distribution and homogeneity of variance were assumed with the UNIVARIATE (Shapiro-Wilk test) and HOVTEST procedures. Differences between means were compared using Tukey's honestly significant difference *post hoc* test. Analysis of differences between groups in the adhesion assay was performed using the two-tailed unpaired Student *t* test. Data were visualized using GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA). A *P* value of <0.05 was considered indicative of statistical significance. All experiments were performed three times.

RESULTS

Cytokeratin-18 expression in cultured BMECs. BMECs were isolated and purified after three passages, and their epithelial origin and purity were determined by evaluating the expression of cytokeratin-18, which is specific for epithelial cells. Both isolated and resuscitated cells exhibited intense positive staining for cytokeratin-18 in the cytoplasmic meshwork of cytokeratin fibrils (see Fig. S1 in the supplemental material).

Pretreatment with live and UV-irradiated *L. rhamnosus* GR-1 reduced the adhesion of *E. coli* to BMEC monolayers. At 6 h after *E. coli* challenge, pretreatment with live and UV-irradiated *L. rhamnosus* GR-1 led to a decrease in the *E. coli* adhesion rate to 50% ($P = 0.001$) and 63% ($P = 0.013$), respectively. The number of adherent *E. coli* was about $3.38 \times 10^4 \pm 8.11 \times 10^2$ CFU, but this inhibitory effect was not observed in cell cultures pretreated with heat-killed *L. rhamnosus* GR-1, *L. rhamnosus* GR-1 supernatant, or DMEM acidified with lactic acid (Fig. 1). Internalization of *E. coli* by BMECs was not observed. Compared to *E. coli* infection only, the number of *E. coli* was unaltered in the supernatant fraction subjected to different pretreatment conditions (see Fig. S2 in the supplemental material).

Pretreatment with *L. rhamnosus* GR-1 ameliorated *E. coli*-induced disruption of BMEC ultrastructure. Under scanning electron microscopy, untreated control BMECs appeared plump, with numerous microvilli, whereas microvilli were missing from cells infected with *E. coli*, and the cells exhibited atrophy and various degrees of collapse (see Fig. S3A and B in the supplemental material). At 6 h after *E. coli* challenge, *E. coli* cells could be seen attached to the surface of BMECs. The cell membrane of BMECs incubated with *L. rhamnosus* GR-1 alone remained intact, and the cells exhibited numerous microvilli and a plump surface (see Fig. S3C in the supplemental material). Pretreatment with *L. rhamnosus* GR-1 reduced the degree of disruption of the cell membrane for up to 6 h after *E. coli* challenge (see Fig. S3D in the supplemental material).

Under conventional TEM (Fig. 2), untreated control BMECs exhibited a normal appearance (intact cytoplasm and organelles), including numerous microvilli and tight junctions (often between adjacent cells), homogeneous electron density, intact mitochondria structures, and intact endoplasmic reticulum structures in the cell cytoplasm. However, *E. coli* infection resulted in rupture of microvilli, loosening of the cytoplasmic matrix structure and nu-

FIG 1 Pretreatment with live and UV-irradiated *L. rhamnosus* GR-1 reduced the adhesion of *E. coli* to BMEC monolayers. BMECs were collected from the indicated BMEC cultures at 6 h after *E. coli* challenge. An adhesion assay using *E. coli* alone served as a reference. The adhesion rate was defined as the adhered *E. coli* population on the BMECs pretreated with different conditions relative to the adhered *E. coli* population in the reference experiment. Data are presented as the means $\pm$ the standard deviations (SD) of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

clear cavitation, hazy mitochondrial structures, and degeneration into myelin-like figures. BMECs incubated with *L. rhamnosus* GR-1 alone maintained a normal appearance. Pretreatment with *L. rhamnosus* GR-1 reduced the degree of disruption of BMEC subcellular structures for up to 6 h after *E. coli* challenge.

Effect of *L. rhamnosus* GR-1 on the morphology of monolayer BMECs. Light microscopy was used to determine whether *L. rhamnosus* GR-1 has any direct effect on BMECs in monolayers infected with *E. coli* (see Fig. S4 in the supplemental material). In DMEM alone, the BMEC monolayer had a cobblestone appearance, and the density of the proliferating monolayer was conserved. Following *E. coli* challenge, the monolayer became disrupted and the cells exhibited age-associated vacuolation. Cells incubated with *L. rhamnosus* GR-1 alone maintained normal morphology. Moreover, pretreatment with *L. rhamnosus* GR-1 reduced the extent of cell damage induced by *E. coli* infection for up to 6 h after *E. coli* challenge.

Pretreatment with *L. rhamnosus* GR-1 ameliorated *E. coli*-induced BMEC death. Cell death was quantified by monitoring the release of LDH 6 h after *E. coli* challenge (see Fig. S5 in the supplemental material). The percentage of dead cells increased in cultures subjected to *E. coli* infection alone ($P = 0.001$) but not in cultures of cells pretreated with *L. rhamnosus* GR-1.

FIG 2 Effect of *L. rhamnosus* GR-1 on *E. coli*-induced disruption of BMEC subcellular structure. The subcellular structure of BMECs collected from the indicated BMEC cultures at 6 h after *E. coli* challenge was observed using TEM. Black arrows indicate microvilli, white arrows indicate mitochondria, black arrowheads indicate tight-junction structures, white arrowheads indicate endoplasmic reticulum structures, and double white arrows indicate myelin-like figures. The data are representative of three independent experiments.

FIG 3 Changes in TLR and NLR mRNA expression during *E. coli* infection in BMECs preincubated with *L. rhamnosus* GR-1. The relative expression of mRNAs for the *TLR2* (A), *TLR4* (B), *NOD1* (C), and *NOD2* (D) genes in BMECs collected from the indicated BMEC cultures at 2, 4, 6, 12, and 24 h after *E. coli* challenge was analyzed using quantitative real-time PCR. Data are presented as the means $\pm$ the standard errors of the mean (SEM) of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Effect of *L. rhamnosus* GR-1 on TLR- and NOD-mediated inflammatory signaling. TLR2 mRNA expression was upregulated in cells pre-incubated with *L. rhamnosus* GR-1 compared to untreated control BMECs at 4 and 6 h after *E. coli* challenge ($P = 0.01$ and $P < 0.001$, respectively; Fig. 3A). At 12 and 24 h after *E. coli* challenge, TLR2 mRNA expression was increased in cells subjected to different treatments.

TLR4 mRNA expression in cells only infected with *E. coli* was elevated compared with untreated control BMECs at 4, 6, 12, and 24 h after *E. coli* challenge but not in cells incubated with *L. rhamnosus* GR-1 only ($P = 0.023$, $P = 0.001$, $P < 0.001$, and $P < 0.001$, respectively; Fig. 3B).

NOD1 mRNA expression was elevated in cells only infected with *E. coli* compared with untreated control BMECs at 4, 6, 12, and 24 h after *E. coli* challenge ($P = 0.039$, $P < 0.001$, $P < 0.001$, and $P = 0.002$, respectively; Fig. 3C). An increase in *NOD1* mRNA expression was also observed in cells preincubated with *L. rhamnosus* GR-1 (but not in cells incubated with *L. rhamnosus* GR-1 only) compared with untreated control BMECs at 6, 12, and 24 h after *E. coli* challenge ($P = 0.012$, $P = 0.016$, and $P = 0.033$, respectively).

Compared with untreated control BMECs, there was an increase in *NOD2* mRNA expression at 12 and 24 h after *E. coli* challenge in cells only infected with *E. coli* but not in cells preincubated with *L. rhamnosus* GR-1 or cells incubated with *L. rhamnosus* GR-1 only ($P = 0.004$ and $P = 0.001$, respectively; Fig. 3D).

Effect of *L. rhamnosus* GR-1 on NLRP3 inflammasome activation. Immunofluorescence staining showed an increase in

NLRP3 activation in cells only infected with *E. coli* compared with untreated control BMECs at 6 h after *E. coli* challenge, and this increase was attenuated by pretreatment with *L. rhamnosus* GR-1. BMECs incubated with *L. rhamnosus* GR-1 only did not exhibit an increase in NLRP3 activation compared with untreated control BMECs (Fig. 4).

Western blot analysis revealed that there was also an increase in NLRP3 protein expression at 2, 4, and 6 h after *E. coli* challenge in cells only infected with *E. coli* compared with untreated control BMECs ($P < 0.001$, $P = 0.001$, and $P < 0.001$, respectively; Fig. 5A). Compared with cells only infected with *E. coli*, NLRP3 protein expression was lower at the above-mentioned time points in cells incubated with *L. rhamnosus* GR-1 only ($P = 0.008$, $P = 0.043$, and $P < 0.001$, respectively) and in cells preincubated with *L. rhamnosus* GR-1 ($P = 0.001$, $P < 0.001$, and $P = 0.012$, respectively). Compared to untreated control BMECs, a transient increase in ASC protein expression was observed at 2 h after *E. coli* challenge ($P < 0.001$; Fig. 5B). However, a decrease in ASC protein expression was observed at 4 h after *E. coli* challenge in cells only infected with *E. coli* but not in cells incubated with *L. rhamnosus* GR-1 only ($P = 0.026$). At 6 h after *E. coli* challenge, compared to untreated control BMECs, ASC protein expression was decreased both in cells only infected with *E. coli* and in cells preincubated with *L. rhamnosus* GR-1, but not in cells incubated with *L. rhamnosus* GR-1 only ($P < 0.001$).

Compared to untreated control BMECs, caspase-1 protein expression was elevated at 4 and 6 h after *E. coli* challenge both in cells only infected with *E. coli* ($P < 0.001$) and in cells preincubated with *L. rhamnosus* GR-1 ($P < 0.001$), but not in cells incu-

FIG 4 Pretreatment with *L. rhamnosus* GR-1 attenuated NLRP3 activation. Immunofluorescence staining for NLRP3 (red) in BMECs collected from the indicated BMEC cultures at 6 h after *E. coli* challenge. DAPI was used for nuclear staining (blue). Representative confocal immunofluorescence images show expression of NLRP3. Scale bars, 50 μ m. Data are representative of three independent experiments.

bated with *L. rhamnosus* GR-1 only (Fig. 5C). At 6 h after *E. coli* challenge, caspase-1 protein expression was lower in cells preincubated with *L. rhamnosus* GR-1 than in cells only infected with *E. coli* ($P = 0.002$).

Effect of *L. rhamnosus* GR-1 on cytokine mRNA expression in BMECs. Compared with untreated control BMECs, increased IL-1 β mRNA expression was observed at 2, 4, and 6 h after *E. coli* challenge in cells only infected with *E. coli* but not in cells incubated with *L. rhamnosus* GR-1 only or cells preincubated with *L. rhamnosus* GR-1 ($P < 0.001$, $P = 0.009$, and $P = 0.001$, respectively; Fig. 6A). This increase did not extend beyond 12 h post-*E. coli* challenge. The profile of changes in IL-8 mRNA expression was similar to that of IL-1 β mRNA expression (Fig. 6C).

IL-18 mRNA expression was elevated in cells only infected with *E. coli* compared to untreated control BMECs at 4, 6, 12, and 24 h after *E. coli* challenge ($P = 0.025$, $P < 0.001$, $P = 0.003$, and $P = 0.001$, respectively; Fig. 6B). The *E. coli*-induced increase in IL-18 mRNA expression was attenuated at 6, 12, and 24 h after *E. coli* challenge by preincubation with *L. rhamnosus* GR-1 ($P < 0.001$, $P = 0.008$, and $P = 0.011$, respectively). Compared to untreated control BMECs, increased IL-18 mRNA expression was also observed at 12 h after *E. coli* challenge in cells incubated with *L.*

rhamnosus GR-1 only but not in cells preincubated with *L. rhamnosus* GR-1 ($P = 0.016$).

Compared to untreated control BMECs, there was an increase in tumor necrosis factor alpha (TNF- α) mRNA expression at various time points after challenge in cells only infected with *E. coli* ($P < 0.001$; Fig. 6D). TNF- α mRNA expression was lower in cells preincubated with *L. rhamnosus* GR-1 ($P = 0.001$, $P < 0.001$, $P = 0.007$, $P = 0.002$, and $P = 0.003$, respectively) than in cells only infected with *E. coli*. Compared to untreated control BMECs, IL-6 mRNA expression was upregulated 4 h after *E. coli* challenge in cells only infected with *E. coli* but not in cells incubated with *L. rhamnosus* GR-1 only or cells preincubated with *L. rhamnosus* GR-1 (Fig. 6E). Compared to untreated control BMECs, IL-10 mRNA expression was elevated at 6, 12, and 24 h after *E. coli* challenge in cells subjected to different treatments (Fig. 6F).

DISCUSSION

The teat surfaces, cisterns, and canals of uninfected cows can harbor a diverse microflora that includes probiotic bacteria (25, 26). However, the application of probiotics as an alternative treatment for mastitis has several limitations. First, probiotics exert their immunomodulatory effects via the gut, promoting mucosal acti-

FIG 5 Western blot detection of NLRP3, ASC, and caspase-1. Representative panels showing expression of NLRP3 (A, left panels), ASC (B, left panels), and caspase-1 (C, left panels) by BMECs collected from the indicated BMEC cultures at 2, 4, and 6 h after *E. coli* challenge. The intensities of NLRP3, ASC, and caspase-1 bands were determined using Quantity One software. Expression of GAPDH was measured as an internal control. Results are presented as the ratio of NLRP3, ASC, or caspase-1 band intensity to that of GAPDH (right panels). Data are presented as the means $\pm$ the SEM of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

vation in response to pathogen infection in the intestinal ecosystem. Oral ingestion of probiotics has many other beneficial effects on intestinal health, but how the mechanism underlying these effects extends beyond the local environment of gut to the mammary glands remains largely unknown. Second, our previous studies demonstrated that pretreatment with high doses of *L. rhamnosus* (10^{12} CFU/day) may counteract the beneficial immunomodulatory effects (7, 8). Accordingly, the dose effect of dietary direct-fed microbials in ameliorating mastitis in dairy cows needs to be studied in more detail. Third, the means of probiotic supplementation (e.g., oral capsules, mixed into the feed or intramammary inoculation) in cattle needs to be thoroughly researched.

Bacterial adhesion to host epithelial cells is a key step in the initiation of infection. In the present study, preincubation of BMECs with live and UV-irradiated *L. rhamnosus* GR-1 had no direct killing effect on *E. coli* cells but did reduce the level of adhesion of *E. coli* to 50 and 63% of that observed in BMECs infected with *E. coli*. Surface layer proteins and exopolysaccharides of probiotic bacteria may play an important role in facilitating prior colonization and the exclusion of pathogenic bacteria by repressing the formation of pathogenic biofilms via inhibiting adhesion (27, 28). The reduction in *E. coli* adhesion mediated by *L. rhamnosus* GR-1 may be due to steric hindrance arising from competition for attachment sites between *L. rhamnosus* GR-1 and *E. coli*.

The innate immune response of BMECs is initiated following detection of pathogens via specialized PRRs. Activation of TLR2 signaling triggered by *L. rhamnosus* is most likely mediated by peptidoglycans, lipoproteins, and lipoteichoic acids from Gram-positive bacteria. Certain *Lactobacillus* strains may exert anti-inflammatory effects via TLR2 activation (29, 30). In addition, activation of TLR2 signaling was shown to induce neutrophil recruitment and restrict invasion of *E. coli* P4 into mammary gland epithelial cells in a murine mastitis model (31). Based on these results, we hypothesize that *L. rhamnosus* GR-1-mediated activation of TLR2-dependent signaling may induce recruitment and activation of inflammatory cells, representing an important primary line of host defense in *E. coli*-induced mastitis.

In the present study, *E. coli* challenge increased TLR4 mRNA expression, whereas preincubation with *L. rhamnosus* GR-1 attenuated this increase. Consistent with our results, the probiotics *L. amylovorus* and *L. jensenii* were shown to suppress the proinflammatory responses triggered by *E. coli* via downregulation of TLR4-dependent NF- κ B and MAPK activation, as well as via modulation of the negative regulators Tollip, Bcl-3, A20, MKP-1, and inhibitory IL-1R-associated kinase M (IRAK-M) (32, 33). In an *in vitro* mouse enterocyte model, LPS-mediated TLR4 signaling could be inhibited by activation of TLR9 with bacterial DNA via IRAK-M (34). NLRs are the cytoplasmic counterparts of TLRs and cooperate in the innate immune response to pathogens (35). Our

FIG 6 Effect of *L. rhamnosus* GR-1 pretreatment on cytokine mRNA expression in BMECs following *E. coli* challenge. The relative expression of mRNAs for the *IL1 β* (A), *IL18* (B), *IL8* (C), *TNF α* (D), *IL6* (E), and *IL10* (F) genes in BMECs collected from the indicated BMEC cultures at 2, 4, 6, 12, and 24 h after *E. coli* challenge was analyzed by quantitative real-time PCR. Data are presented as the means $\pm$ the SEM of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

data indicate that *L. rhamnosus* GR-1 limits excessive and harmful inflammatory responses by attenuating the *E. coli*-induced increases in NOD1 and NOD2 expression.

Activation of TLRs or NLRs leads to NLRP3 inflammasome activation, resulting in upregulation in the transcription of caspase-1 and proinflammatory cytokine genes (19, 36). Consistent with immunofluorescence results, Western blot analyses revealed that the expression of both NLRP3 and caspase-1 protein is increased during *E. coli* infection, whereas this increase is attenuated by preincubation with *L. rhamnosus* GR-1. We also found that *L. rhamnosus* GR-1 pretreatment attenuates *E. coli*-induced increases in IL-1 β and IL-18 mRNA expression. In an asbestos inhalation model, *Nlrp3*^{-/-} mice showed diminished recruitment of inflammatory cells to the lungs, paralleled by lower cytokine

production, indicating that NLRP3 inflammasomes play a role in pulmonary inflammatory diseases (37). Furthermore, *Nlrp3*^{-/-} mice are more susceptible to experimental colitis and exhibit reduced expression of IL-1 β and the anti-inflammatory cytokine IL-10 (38). In addition to anti-inflammatory mediators (e.g., IL-10) released by *L. rhamnosus* GR-1, our data also suggest that *L. rhamnosus* GR-1-mediated limitation of the production of IL-8, IL-6, and TNF- α is perhaps associated with synergistic responses of TLR-, NLR-, and NLRP3-mediated signaling. Lactate reduces inflammation and organ injury through negative regulation of TLR4-mediated activation of the NLRP3 inflammasome and production of IL-1 β via arrestin β 2 and G-protein-coupled receptor 81 (20). We therefore hypothesize that parallel to competitive inhibition of pathogen adhesion, *L. rhamnosus* GR-1 attenuates *E.*

coli-induced NLRP3 inflammasome activation in association with lactate production.

Unexpectedly, contradictory to increases in the expression of NLRP3 and caspase-1 protein expression, expression of the adaptor protein ASC is decreased in BMECs infected with *E. coli*, even in cells pretreated with *L. rhamnosus* GR-1. It is possible that in addition to NLRP3, other NLRs activate caspase-1 in an ASC-independent manner (36). Lethal toxin-induced caspase-1 activation in the NLRP1b inflammasome is associated with caspase-1 ubiquitination, which occurs independently of ASC expression and caspase-1 autoproteolysis (39). Alternatively, apart from ASC, a mitochondrial antiviral signaling protein located in the outer membrane may serve as a new adaptor to promote NLRP3 mitochondrial localization and activation (40). Taken together, our findings suggest that *L. rhamnosus* GR-1 ameliorates *E. coli*-induced inflammation and cell damage via attenuation of ASC-independent NLRP3 inflammasome activation. Further studies are needed to elucidate whether a noncanonical ASC-independent pathway may be involved in NLRP3 inflammasome activation or whether other CARD-containing NLRs, such as NLRC4, play key roles in the beneficial effect of the probiotic strain *L. rhamnosus* GR-1 in preventing *E. coli* infection.

The use of *L. rhamnosus* GR-1 to prevent *E. coli* O111 infection in BMECs leads to promising perspectives on new strategies to improve the efficiency of mastitis treatment. This BMEC model provides an *in vitro* framework for evaluating response to *Lactobacillus*-based intervention in bovine mastitis. However, the primary results of this study require further confirmation with other *Lactobacillus* strains to determine whether the effects are specific to *L. rhamnosus* GR-1 or general for many lactobacilli. Undoubtedly, future *in vivo* studies will be essential for ultimate realization of the potential of urethra-derived *L. rhamnosus* GR-1 in preventing mastitis. Characterization of the potential residual pathogenic activity should also be considered when *L. rhamnosus* GR-1 is proposed as an antimastitis probiotic.

In conclusion, our findings suggest that *L. rhamnosus* GR-1 pretreatment ameliorates *E. coli*-induced inflammation and cell damage, partly through impeding the adhesion of *E. coli* to BMECs, subsequently ameliorating *E. coli*-induced disruption of BMEC morphology and ultrastructure and limiting detrimental inflammatory responses via promotion of TLR2 and NOD1 synergism and attenuation of ASC-independent NLRP3 inflammasome activation.

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