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ORIGINAL ARTICLE

Lactobacillus rhamnosus GR-1 Limits *Escherichia coli*-Induced Inflammatory Responses *via* Attenuating MyD88-Dependent and MyD88-Independent Pathway Activation in Bovine Endometrial Epithelial Cells

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Abstract—Intrauterine *Escherichia coli* infection after calving reduces fertility and causes major economic losses in the dairy industry. We investigated the protective effect of the probiotic *Lactobacillus rhamnosus* GR-1 on *E. coli*-induced cell damage and inflammation in primary bovine endometrial epithelial cells (BEECs). *L. rhamnosus* GR-1 reduced ultrastructure alterations and the percentage of BEECs apoptosis after *E. coli* challenge. Increased messenger RNA (mRNA) expression of immune response indicators, including pattern recognition receptors (toll-like receptor [TLR]2, TLR4, nucleotide-binding oligomerization domain [NOD]1, and NOD2), inflammasome proteins (NOD-like receptor family member pyrin domain-containing protein 3, apoptosis-associated speck-like protein, and caspase-1), TLR4 downstream adaptor molecules (myeloid differentiation antigen 88 [MyD88], toll-like receptor adaptor molecule 2 [TICAM2]), nuclear transcription factor kB (NF-kB), and the inflammatory cytokines tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-8, IL-10, IL-18, and interferon (IFN)-β, was observed following *E. coli* challenge. However, these increases were attenuated by *L. rhamnosus* GR-1 pretreatment. Our data indicate that *L. rhamnosus* GR-1 ameliorates the *E. coli*-induced disruption of cellular ultrastructure, subsequently reducing the percentage of BEECs apoptosis and limiting inflammatory responses, partly *via* attenuation of MyD88-dependent and MyD88-independent pathway activation. Certain probiotics could potentially prevent postpartum uterine diseases in dairy cows, ultimately reducing the use of antibiotics.

KEY WORDS: *Lactobacillus rhamnosus*; *Escherichia coli*; inflammation; bovine endometrial epithelial cell; apoptosis.

INTRODUCTION

Uterine diseases such as metritis and endometritis are highly prevalent in dairy cows. Reports indicate that almost all dairy cows have bacterial contamination in the uterus within 2 to 3 weeks after calving [1]. The most commonly associated pathogens of postpartum uterine infection are *Escherichia coli*, *Trueperella pyogenes*, and *Fusobacterium necrophorum* [2]. As previous studies reported that after few days of calving, *E. coli* is predominant pathogen which mostly associated with uterine inflammation and increased the susceptibility of endometrium to *T. pyogenes* infection which further impairs the reproductive performance [3, 4].

At present, uterine diseases are commonly treated with antibiotics such as penicillin or third-generation cephalosporins. However, antibiotic resistance and extensive use of antibiotics in food animals are significant growing public health concerns [5]. An efficacious alternative therapy against uterine diseases would have a significant positive impact on the dairy industry by limiting the use of

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antibiotics and consequently reducing economic losses due to these disorders [6].

Probiotics modulate innate and adaptive immunity through both nuclear transcription factor kB (NF-kB) and mitogen-activated protein kinase (MAPK) pathways and then alter subsequent release of cytokines [7, 8]. Nuclear transcription factor kB is vital factor for various cellular processes such as inflammation, immune reaction, cell survival, and apoptosis [9]. Several strains of lactobacilli are used as probiotics to prevent infections within the gastrointestinal and urogenital tracts as well as to ameliorate allergic and inflammatory conditions [10, 11]. *Lactobacillus rhamnosus* GR-1, isolated from the healthy female urethra [12], is used to prevent urinary tract infections, preterm birth, and bacterial vaginosis. These applications highlight the immunomodulatory and antimicrobial activity of *L. rhamnosus* GR-1 [13–15]. Our previous studies demonstrated that pretreatment with *L. rhamnosus* GR-1 ameliorates *E. coli*-induced bovine mammary epithelial cell inflammation and cell damage [16]. Some lactic acid bacteria demonstrate a clear potential to modulate endometrial infection and inflammation in cattle [17–19].

It is known that postpartum involution of the uterus involves remodeling of the extracellular matrix and regeneration of epithelium that overlies the stromal cells to repair the damaged endometrium. Bovine endometrial epithelial cells (BEECs) function as the first line of defense against microbial pathogens and have been used to study the cellular mechanisms underlying bovine physiology and pathology [20]. The endometrium of animals and humans expresses a number of different pattern recognition receptors (PRRs), such as toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) [21, 22]. Innate immune response play an important role with pathogen-associated molecule patterns (PAMPs) in elimination of bacteria from the endometrium which lead to influx of phagocytic cells such as macrophages and neutrophils. The intrauterine accumulation of neutrophils and endometrium damage are main signs of bovine uterine infection [1]. Furthermore, the ultrastructural properties such as size and extension of intracellular organelles are variable in metabolically active and non-active cells [23]. The epithelial and stromal cells of the bovine endometrium express functional TLR4 for detection of lipopolysaccharide (LPS) while TLR1, TLR2, and TLR6 for sensing bacterial lipopeptides [21, 24, 25]. Toll-like receptor 4 is one of the most studied TLRs which activates both myeloid differentiation antigen 88 (MyD88)-dependent and MyD88-independent pathways by LPS. Both LPS and *E. coli* stimulate BEECs to secrete a range of cytokines and chemokines *in vitro* [26]. Lipopolysaccharide could activate the MAPK and NF-kB pathways and increase transcriptional expression of IL-1β, IL-6, IL-8, and IL-18 in bovine endometrial cells [24]. Previous studies reported that the transcriptional expression of inflammatory cytokine is important in development of bovine clinical endometritis in uterine tissue [27]. Moreover, the NLR family member pyrin domain-containing protein 3 (NLRP3), which recognizes microbial components in the cytosol, forms a complex known as the inflammasome along with adaptor protein apoptosis-associated speck-like protein (ASC, encoded by the *Pycard* gene) and the serine protease caspase-1. The inflammasome activates caspase-1 to process pro-forms of IL-1β and IL-18 to the mature biologically active secreted forms [28].

In the present study, the protective effect of the probiotic *Lactobacillus rhamnosus* GR-1 on *E. coli*-induced cell damage and inflammation was assessed in primary BEECs through investigating ultrastructure alterations, percentage of BEECs apoptosis, and messenger RNA (mRNA) expression of immune response indicators.

MATERIALS AND METHODS

Cell Culture and Identification

Primary BEECs were separated from the uterine horns using a previously described procedure [29]. In this study, bovine uterus of the early estrous cycle (days 2–5) was used and the cows had no evidence of genital disease based on visual inspection. Bovine endometrial tissues obtained from three Holstein cows were selected for cell culture after sacrifice. Briefly, the tissues were washed with D-Hank's solution, digested, and then incubated at 37 °C for 1 h with shaking. After filtering, resuspension, and centrifugation, the cells were subsequently suspended in cell culture flasks (Corning Inc., Corning City, NY) using Dulbecco's modified Eagle's medium with Ham's F-12 nutrient mixture (DMEM/F12, pH 7.2; Gibco, Grand Island, NY) containing 10 % fetal bovine serum (Gibco) and 100 U/mL of antibiotic (penicillin and streptomycin) (Invitrogen, Carlsbad, CA). The medium was changed once every 24 h until the cells were spread across the bottom of the culture flask. Fibroblasts were digested with 0.25 % trypsin-EDTA (Gibco, Burlington, Canada). In addition, to verify the origin, BEECs were seeded into 24-well plates on cover slips at a cell density of 1×10^5 per well and analyzed for the expression of the epithelial cell-specific marker cytokeratin, as previously described [25].

Bacterial Strains and Growth Conditions

Lactobacillus rhamnosus GR-1 (ATCC 55826; American Type Culture Collection, Manassas, VA) was cultured in De Man, Rogosa, and Sharpe (MRS) broth (Oxoid, Hampshire, United Kingdom) for 24 h at 37 °C under microaerophilic conditions. For all experiments, bacteria were diluted 1:100 in MRS broth and cultured for 8 h to mid-log phase (optical density at 600 nm [OD_{600}] of 0.5).

Escherichia coli O111:K58(B4) (CVCC1450; China Institute of Veterinary Drug Centre, Beijing, China) used for induction of endometritis was grown in Luria-Bertani (LB) broth (Oxoid, Basingstoke, England) at 37 °C with constant shaking. Bacteria were diluted 1:100 in LB broth and cultured for 3 h to mid-log phase (OD_{600} of 0.5).

SEM

Bovine endometrial epithelial cells were seeded into 6-well plates at a density of 4×10^5 per well. On day 2, confluent monolayers of BEECs were treated under the following conditions: (i) DMEM alone, (ii) *E. coli* (4×10^5 colony forming units [CFU]/well) infection alone, (iii) *L. rhamnosus* GR-1 (4×10^7 CFU/well) incubation alone for 3 h, or (iv) pre-incubation with *L. rhamnosus* GR-1 (4×10^7 CFU/well) for 3 h prior to addition of *E. coli* (4×10^5 CFU/well). At 6 h after *E. coli* challenge, BEECs were collected and prepared for scanning electron microscopy (SEM) as described previously [30]. Briefly, BEECs were fixed overnight in 3 % glutaraldehyde and dehydrated using an alcohol series followed by 100 % acetone. Samples were then critically point dried and sputter coated with gold before visualization under an environmental scanning electron microscope (Quanta 200 FEG; FEI, Eindhoven, The Netherlands).

TEM

Bovine endometrial epithelial cells were grown and challenged as described above and then harvested and fixed in 3 % glutaraldehyde for 24 h at room temperature. The fixed cells were post-fixed in 1 % osmium tetroxide, dehydrated using a graded ethanol series (30, 50, 70, 80, 90, and 100 %), embedded in Epon (Energy Beam Sciences, Agawam, MA), and sliced into ultrathin (50–60 nm) sections using a Leica EM UC6 ultramicrotome (Leica Microsystems, Wetzlar, Germany). The sections were stained with 3 % uranyl acetate and lead citrate, and observed under a transmission electron microscope (H7500; Hitachi, Tokyo, Japan).

Flow Cytometry

An annexin V–fluorescein isothiocyanate (FITC) apoptosis assay kit (KeyGEN BioTECH, Nanjing, China) was used to measure apoptosis [31]. Briefly, BEECs in the four treatment groups were collected at 3, 6, and 9 h after *E. coli* challenge and then washed twice with pre-chilled PBS and stained with FITC-conjugated annexin V and propidium iodide. Specially, positive cells were incubated with camptothecin (KGA8151, KeyGEN BioTECH, Nanjing, China). Samples were assessed within an hour using a FACScalibur flow cytometer (BD Biosciences) equipped with FlowJo software.

qRT-PCR

Cells were grown and challenged as described above in a 6-well plates. Bovine endometrial epithelial cells were collected at 10 h after *E. coli* challenge, and then total RNA was extracted using TRIzol reagent kit (Invitrogen) as according to previous study [32]. The concentration and purity (OD_{260}/OD_{280} absorption ratio >1.9) of the total RNA were determined using a NanoDrop ND-2000C spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). One microgram of total RNA was then reverse-transcribed into cDNA using the GoScript reverse transcription system (Promega, Madison, WI). Quantitative real-time PCR was performed with SYBR Premix Dimer-Eraser (TakaRa Biotechnology, Inc., Shiga, Japan) using an ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA). Primer sequences are listed in Table 1. All reactions were conducted in three replicates. Glyceraldehyde-3-phophate dehydrogenase (GAPDH) was used as an internal control to normalization of our data as it is a housekeeping gene [17], and fold change in gene expression was calculated using the $2^{-\Delta\Delta C_T}$ method. The relative expression of target gene mRNA in each group was calculated using the following equations:

$$\Delta C_T = C_{T \text{ target gene}} - C_{T \text{ GAPDH}} \text{ and } \Delta\Delta C_T$$

$$= \Delta C_{T \text{ treated group}} - \Delta C_{T \text{ control group}}.$$

Statistical Analysis

Before statistical analyses, data were transformed by log when necessary to achieve a normal distribution. All

Table 1. Sequences of Oligonucleotide Primers Used for Real-Time PCR, Length of the Respective PCR Products, and Gene Accession Numbers

Gene product	Primer		Product size (bp)	GenBank accession
	Direction	Sequence (5′ to 3′)		
TLR2	F	CATTCCCTGGCAAGTGGATTATC	201	AY634629
	R	GGAATGGCCTTCTTGTCAATGG		
TLR4	F	TATGAACCACTCCACTCGCTC	207	DQ839566
	R	CATCATTTGCTCAGCTCCCAC		
NOD1	F	TGGTCACTCACATCCGAAAC	218	NM_001256563
	R	AGGCCTGAGATCCACATAAG		
NOD2	F	GCAGAAACACAGATGAGAGAGAC	220	NM_001002889.1
	R	GGAAACCATTCAAGGAAGCCG		
MyD88	F	AAGTACAAGCCAATGAAGAAAGAG	102	NM_001014382.2
	R	GAGGCGAGTCCAGAACCAG		
TOLLIP	F	GGCGTGGACTCTTTCTACCT	112	NM_001039961.1
	R	CGACCTTGCCCTGCTTCA		
NF-kB	F	ATACGTCGGCCGTGTCTAT	187	XM_005226864.2
	R	GGAACTGTGATCCGTGTAG		
TICAM2	F	GGAACTGTGATCCGTGTAG	116	NM_001046456.1
	R	GAGTTGTATTTGTGCTGCCTGT		
IRF3	F	GCATCCCTTGGAAGCACG	180	NM_001029845.3
	R	CCTCCGCTAAACGCAACAC		
IFN-β	F	CCTGTGCCTGATTTCATCATGA	97	NM_174350.1
	R	GCAAGCTGTAGCTCCTGGAAAG		
NLRP3	F	GTTGTCCGTTTCCTCTTT	151	NM_001102219.1
	R	TGGGCTCAATCTGTAGTG		
ASC	F	CAGCGGACGAGCTCAAAAAG	149	NM_174730.2
	R	ACCGTACGCCTCCAGATAGT		
Caspase-1	F	GGGATGGTATGGGTAAACGA	107	XM_002692921.2
	R	TCCTTCTCTATGTGGGCTTTC		
IL-1β	F	CCTCGGTTCCATGGGAGATG	119	NM_174093.1
	R	AGGCACTGTTCCTCAGCTTC		
IL-6	F	GCTGAATCTTCCAAAAATGGAGG	215	NM_173923.2
	R	GCTTCAGGATCTGGATCAGTG		
IL-18	F	TCAGATAATGCACCCCAGACC	78	NM_174091.2
	R	GATGGTTACGGCCAGACCTC		
TNF-α	F	TCCAGAAGTTGCTTGTGCCT	144	NM_173966.3
	R	CAGAGGGCTGTTGATGGAGG		
IL-8	F	ACACATTCCACACCTTTCCAC	149	AF232704
	R	ACCTTCTGCACCCACTTTTC		
IL-10	F	CCTTGTCGGAAATGATCCAGTTTT	67	NM_174088.1
	R	TCAGGCCCGTGGTTCTCA		
GAPDH	F	GTCTTCACTACCATGGAGAAGG	201	NM_001034034
	R	TCATGGATGACCTTGGCCAG		

TLR toll-like receptor, *NOD* nucleotide-binding oligomerization domain, *MyD88* myeloid differentiation primary response 88, *TOLLIP* toll-interacting protein, *NF-kB* nuclear transcription factor kB, *TICAM2* toll-like receptor adapter molecule 2, *IRF* interferon regulatory factor, *NLRP* NOD-like receptor family member pyrin domain-containing protein, *ASC* apoptosis-associated speck-like protein containing a CARD, *TNF-α* tumor necrosis factor, *F* forward primer, *R* reverse primer

quantitative data are presented as the mean ± SEM. Data were subjected to one-way analysis of variance, followed by Tukey's post hoc test using the IBM SPSS Statistics 21 (SPSS Inc., Chicago, IL) software package. A *P* value of <0.05 was considered indicative of statistical significance. All experiments were performed three times.

RESULTS

Effect of *L. rhamnosus GR-1* on Disruption of the Ultrastructure of BEECs Infected with *E. coli*

Under SEM, untreated control BEECs were arranged neatly and closely, and microvilli appeared to be in good

condition (Fig. 1a), whereas the microvilli disappeared and the separation between the nucleus and cytoplasm was disrupted in cells infected with *E. coli* (Fig. 1b). The surface of BEECs incubated with *L. rhamnosus* GR-1 alone remained intact (Fig. 1c). Pretreatment with *L. rhamnosus* GR-1 reduced the degree of disruption of the cell surface at 6 h after *E. coli* challenge (Fig. 1d).

In transmission electron microscopy (TEM), untreated control cells exhibited homogeneous electron density, intact mitochondrial structures, and an intact nuclear membrane (Fig. 2). In contrast, *E. coli* infection resulted in cytoplasm cavitation and hazy mitochondrial structures. Pretreatment with *L. rhamnosus* GR-1 reduced the degree of disruption of cell structures at 6 h after *E. coli* challenge.

Effect of *L. rhamnosus* GR-1 on Apoptosis of BEECs

Apoptosis of BEECs was investigated using flow cytometry. As shown in Fig. 3, pretreatment with

L. rhamnosus GR-1 resulted in a decrease in the percentage of BEECs apoptosis during *E. coli* infection at 3 and 9 h.

Effect of *L. rhamnosus* GR-1 on TLRs and NODs mRNA Expression

Real-time PCR analysis at 10 h demonstrated that pretreatment with *L. rhamnosus* GR-1 reduced TLR2, TLR4, NOD1, and NOD2 mRNA expression induced by *E. coli* infection (Fig. 4).

Effect of *L. rhamnosus* GR-1 on MyD88-Dependent and MyD88-Independent Pathways

The expression of MyD88, toll-interacting protein (TOLLIP), NF-kB, toll-like receptor adaptor molecule 2 (TICAM2), interferon regulatory factor 3 (IRF3), and IFN-β was determined by quantitative reverse transcription PCR (qRT-PCR). Pretreatment with *L. rhamnosus* GR-1 suppressed MyD88, NF-kB, TICAM2, and IFN-β
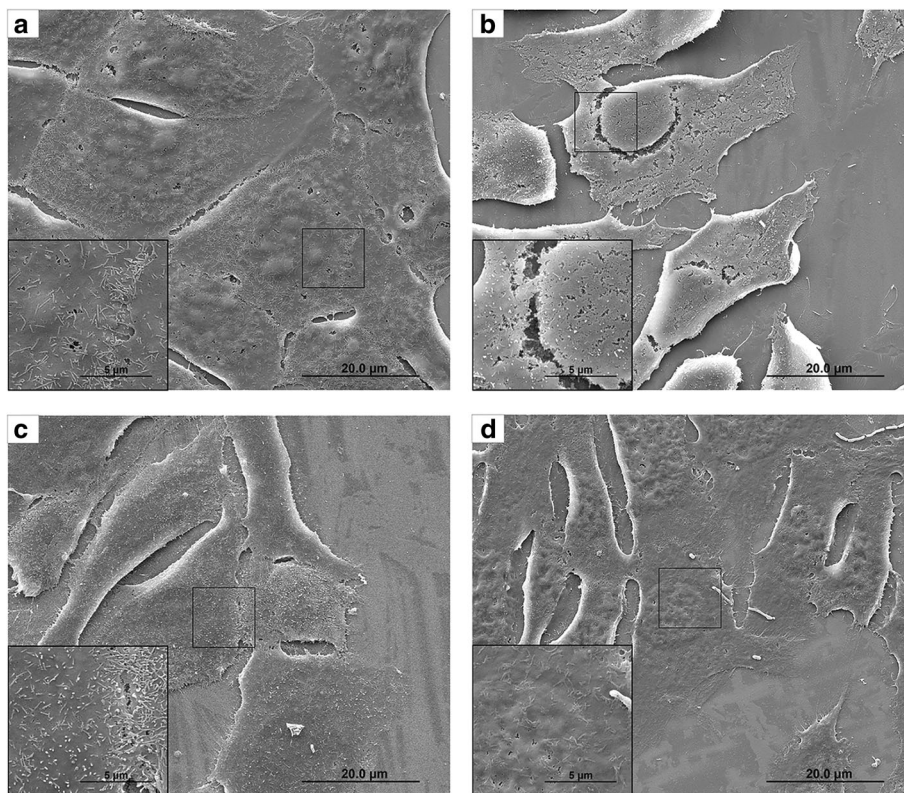

Fig. 1. Effect of *L. rhamnosus* GR-1 on disruption of the cell structure of BEECs infected with *E. coli*. Structural features of BEECs were observed by scanning electron microscopy at 6 h after *E. coli* challenge. BEECs were cultured with medium alone (**a**), challenged with *E. coli* only (**b**), pretreated with *L. rhamnosus* GR-1 only for 3 h (**c**), or pretreated with *L. rhamnosus* GR-1 for 3 h prior to exposure to *E. coli* (**d**). *Scale bars* at 20 and 5 μm. The data are representative of three independent experiments.
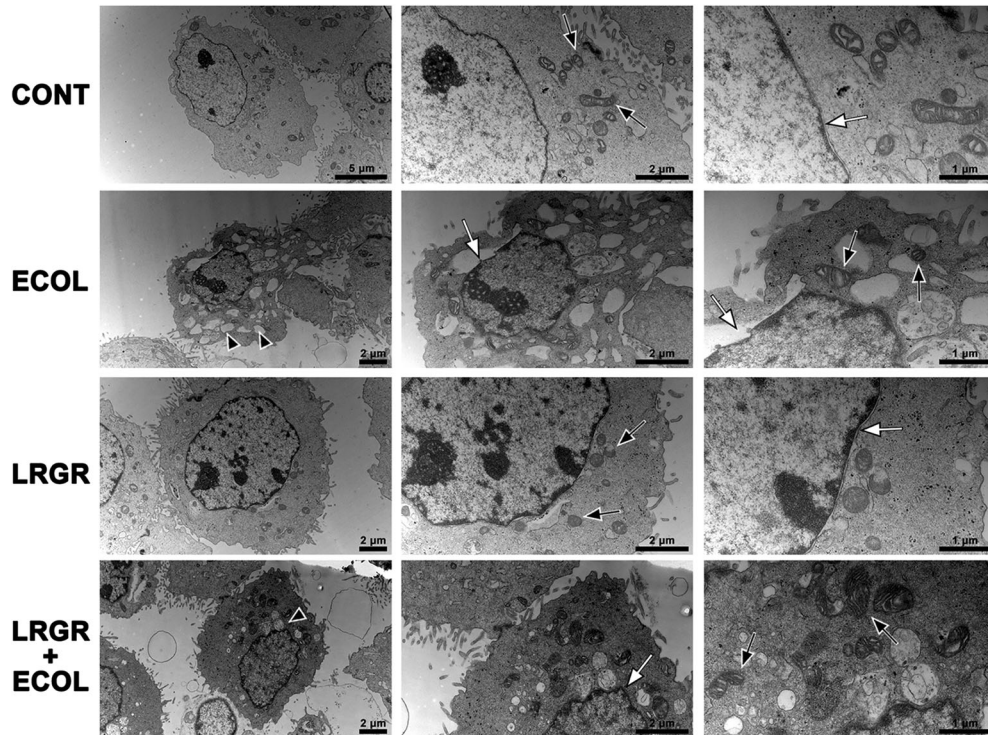
Fig. 2. Effect of *L. rhamnosus* GR-1 on disruption of the subcellular structure of BEECs infected with *E. coli*. The subcellular structure of BEECs was observed by transmission electron microscopy at 6 h after *E. coli* challenge. *Black arrows* indicate mitochondria, *white arrows* indicate the nuclear membrane, and *black arrowheads* indicate cytoplasm cavitations. *CONT* DMEM alone, *ECOL E. coli* (4×10^5 CFU/well) infection alone, *LRGR* pretreated with *L. rhamnosus* GR-1 (4×10^7 CFU/well) only for 3 h, (−3 h) *LRGR + ECOL* pretreated with *L. rhamnosus* GR-1 (4×10^7 CFU/well) for 3 h prior to exposure to *E. coli* (4×10^5 CFU/well). The data are representative of three independent experiments.

expression during *E. coli* infection at 10 h. However, pre-incubation with *L. rhamnosus* GR-1 had no effect on the increases in TOLLIP and IRF3 expression at 10 h in cells challenged with *E. coli* (Fig. 5).
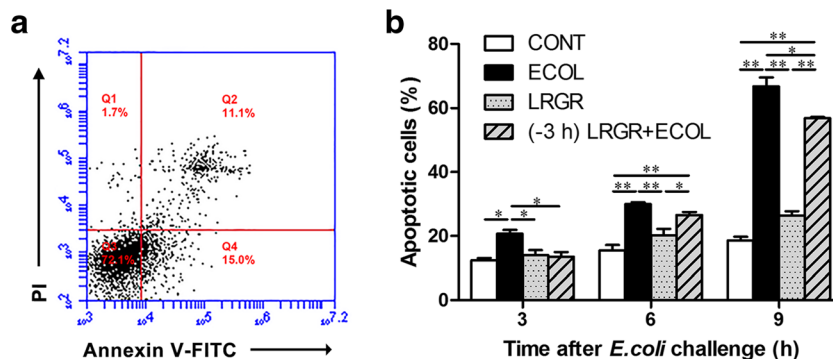

Fig. 3. Effect of *L. rhamnosus* GR-1 on apoptosis of BEECs infected with *E. coli*. BEECs were collected from the indicated cultures at 3, 6, and 9 h after *E. coli* challenge. Cell distribution was assessed based on annexin V binding and propidium iodide (*PI*) uptake. FITC and PI fluorescence were measured by flow cytometry using FL-1 and FL-2 filters, respectively. **a** Representative two-dimensional scatter plots of annexin V *versus* PI. **b** Percentage of apoptotic cells. *CONT* DMEM alone, *ECOL E. coli* (4×10^5 CFU/well) infection alone, *LRGR* pretreated with *L. rhamnosus* GR-1 (4×10^7 CFU/well) only for 3 h, (−3 h) *LRGR + ECOL* pretreated with *L. rhamnosus* GR-1 (4×10^7 CFU/well) for 3 h prior to exposure to *E. coli* (4×10^5 CFU/well). Data are presented as the means ± SEM of three independent experiments. *$P < 0.05$; **$P < 0.01$.
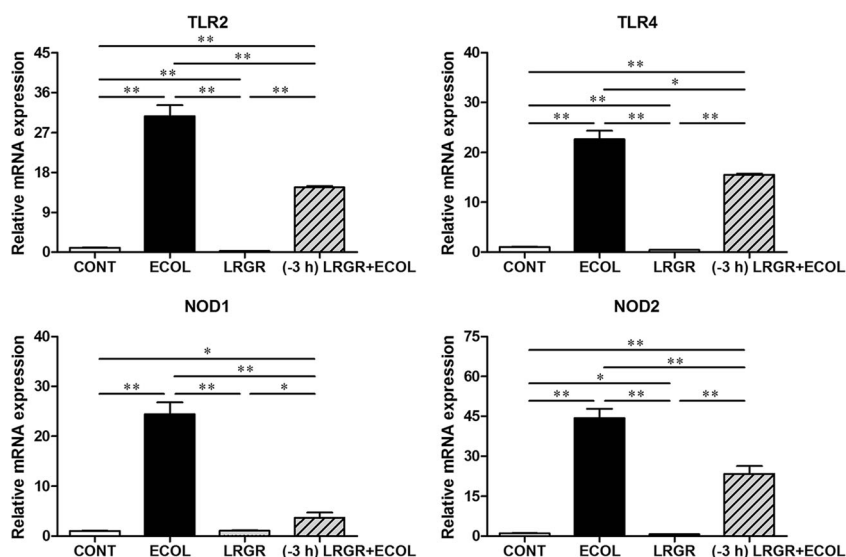
Fig. 4. Changes in TLRs and NODs mRNA expression during *E. coli* infection in BEECs pretreated with *L. rhamnosus* GR-1. The relative expression of mRNAs encoding TLR2 (**a**), TLR4 (**b**), NOD1 (**c**), and NOD2 (**d**) in BEECs collected from the indicated cultures at 10 h after *E. coli* challenge was analyzed using qRT-PCR. *CONT* DMEM alone, *ECOL E. coli* (4×10^5 CFU/well) infection alone, *LRGR* pretreated with *L. rhamnosus* GR-1 (4×10^7 CFU/well) only for 3 h, *(−3 h) LRGR + ECOL* pretreated with *L. rhamnosus* GR-1 (4×10^7 CFU/well) for 3 h prior to exposure to *E. coli* (4×10^5 CFU/well). Data are presented as the means ± SEM of three independent experiments. *$P < 0.05$; **$P < 0.01$.

Effects of *L. rhamnosus* GR-1 on NLRP3 Inflammasome Activation

Expression of the NLRP3 inflammasome was assessed by qRT-PCR. Pretreatment with *L. rhamnosus* GR-1 inhibited *E. coli*-induced increases in the expression of NLRP3, ASC, and caspase-1 at 10 h (Fig. 6).

Pretreatment with *L. rhamnosus* GR-1 Ameliorated *E. coli*-Induced Cytokine Production

Pre-incubation with *L. rhamnosus* GR-1 attenuated *E. coli*-induced increases in IL-1β, IL-18, IL-8, IL-6, IL-10, and TNF-α mRNA expression at 10 h after *E. coli* challenge (Fig. 7).

DISCUSSION

Bacterial infection of the female genital tract is common in cattle, particularly after parturition, and can cause debilitating disease, infertility, and even mortality [2]. It has been demonstrated that *E. coli* is typically the first pathogen associated with uterine disease after parturition [25, 33]. *Escherichia coli* infection induced production of proinflammatory cytokines, including IL-1 and TNF-α lead to apoptosis in a variety of cell types, such as endometrial

epithelial cells and bovine mammary tissue [34, 35]. *Lactobacillus rhamnosus* GR-1 is a lactic acid bacterium that is part of the normal microbiota in urethra. The important mode of action of probiotic including *L. rhamnosus* GR-1 and other commensal lactobacilli has immunomodulation and anti-apoptotic activity [8, 36]. In a recent study, cows treated intravaginally with a mixture of three lactic acid bacteria had lower incidence of metritis and total uterine infections [19]. It is in agreement that TLRs and NLRs on endometrial tissues are critical components of the innate immune system for detecting PAMPs [21, 37].

In this study, we investigated the protective effect of probiotic *L. rhamnosus* GR-1 in attenuating *E. coli*-induced cell ultrastructure alterations and the percentage of BEECs apoptosis, as well as attenuation of inflammatory responses in BEECs. Our results indicated that *L. rhamnosus* GR-1 had no negative influence on the ultrastructure of BEECs and that pretreatment with the probiotic reduced the severity of injury caused by *E. coli*. Our previous studies have shown that *L. rhamnosus* pretreatment suppressed enterotoxigenic *Escherichia coli*-induced apoptosis of intestinal epithelial cells [38]. In the present study, pretreatment with *L. rhamnosus* GR-1 also suppressed the apoptosis of BEECs caused by *E. coli* at 3 and 9 h after challenge, which is in line with the ultrastructure results. This finding is consistent with previous studies
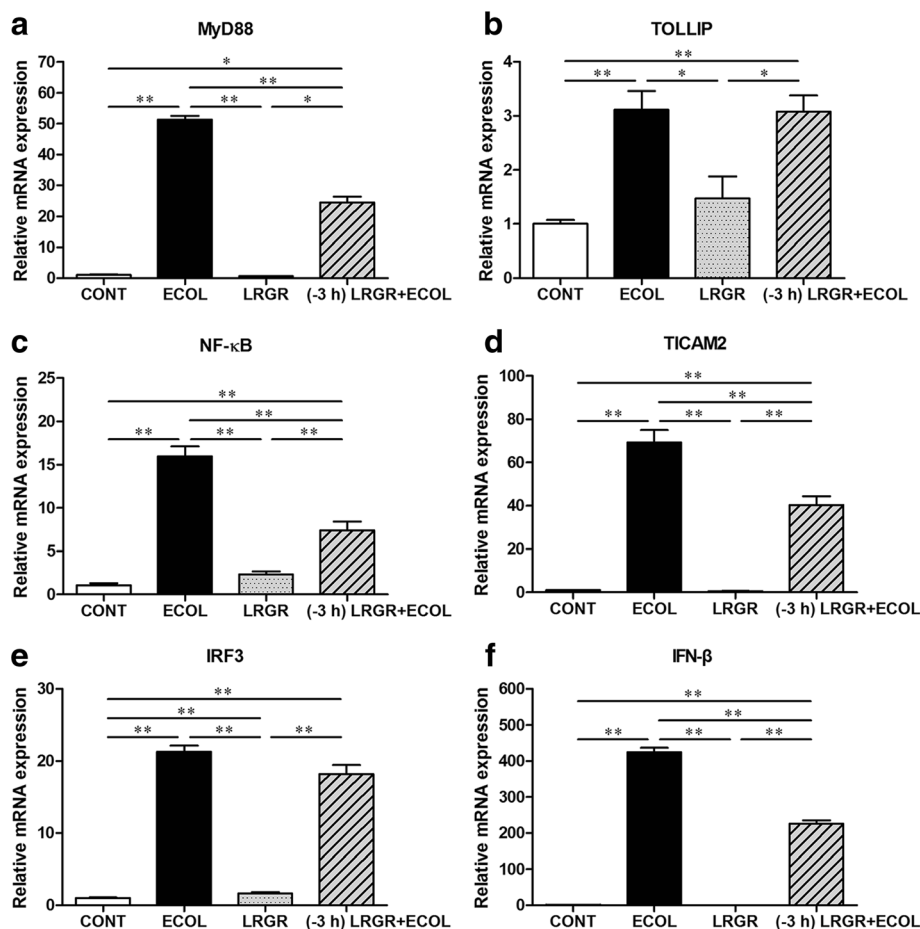
Fig. 5. Effect of *L. rhamnosus* GR-1 on both MyD88-dependent and MyD88-independent pathways in BEECs following *E. coli* challenge. The relative expression of mRNAs encoding MyD88 (**a**), TOLLIP (**b**), NF-kB (**c**), TICAM2 (**d**), IRF3 (**e**), and IFN-β (**f**) in BEECs collected from the indicated cultures at 10 h after *E. coli* challenge was analyzed by qRT-PCR. *CONT* DMEM alone, *ECOL E. coli* (4×10^5 CFU/well) infection alone, *LRGR* pretreated with *L. rhamnosus* GR-1 (4×10^7 CFU/well) only for 3 h, (−3 h) LRGR + ECOL pretreated with *L. rhamnosus* GR-1 (4×10^7 CFU/well) for 3 h prior to exposure to *E. coli* (4×10^5 CFU/well). Data are presented as the means ± SEM of three independent experiments. *$P < 0.05$; **$P < 0.01$.

in which *Lactobacillus rhamnosus* GG prevents cytokine-induced apoptosis in two different intestinal epithelial cell models [36]. Bacterial toxins or the proinflammatory mediators can cause cell death by apoptosis during inflammation [34]. In the current study, *L. rhamnosus* GR-1 may decrease the proinflammatory cytokines such as IL-1β and TNF-α through inhibiting the activation of NF-kB which suppressed the apoptosis of BEECs.

As a PRRs, TLR4 expression can be upregulated by LPS [39]. Even though the interaction between LPS and TLR4 is the best-studied paradigm for inflammation in the endometrium, cellular responses to PAMPs that bind TLR1, TLR2, TLR5, and TLR6 have also been reported in a range of species, including the intact bovine

endometrium [40, 41]. Our results show that pre-incubation with *L. rhamnosus* GR-1 inhibits the *E. coli*-induced increase in TLR2 and TLR4 mRNA expression levels at 10 h. Other studies have reported that probiotic lactobacilli suppress *Candida albicans*-induced TLR2, IL-8, and TNF-α expression, inhibit the NF-kB signaling pathway [42], and downregulate IL-6 and IL8 expression in cells infected with *E. coli* [8]. In BEECs, TLR4 and MyD88-dependent signaling pathways are essential for the response to LPS [24]. We therefore examined the expression of the MyD88-dependent signaling adaptor molecule as well as that of inflammatory cytokines, as we expected to find that the expression of MyD88, TOLLIP, NF-kB, and various inflammatory cytokines (IL-1β, TNF-α, IL-6,
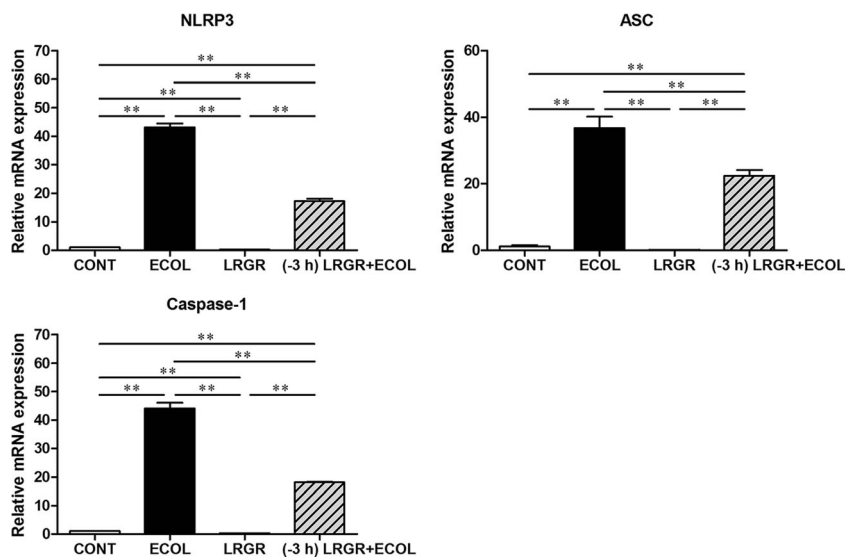
Fig. 6. Effect of *L. rhamnosus* GR-1 on inflammasome mRNA expression in BEECs following *E. coli* challenge. The relative expression of mRNAs encoding NLRP3 (**a**), ASC (**b**), and caspase-1 (**c**) in BEECs collected from the indicated cultures at 10 h after *E. coli* challenge was analyzed using qRT-PCR. *CONT* DMEM alone, *ECOL* E. coli (4×10^5 CFU/well) infection alone, *LRGR* pretreated with *L. rhamnosus* GR-1 (4×10^7 CFU/well) only for 3 h, *(−3 h) LRGR + ECOL* pretreated with *L. rhamnosus* GR-1 (4×10^7 CFU/well) for 3 h prior to exposure to *E. coli* (4×10^5 CFU/well). Data are presented as the means ± SEM of three independent experiments. *$P < 0.05$; **$P < 0.01$.

IL-8, and IL-10) is upregulated when BEECs are challenged with *E. coli*. We found that with the exception of TOLLIP, pre-incubation of BEECs with *L. rhamnosus* GR-1 inhibited the upregulation of the abovementioned factors at 10 h.

We also examined another signaling pathway involved in the activation of TLR4, the MyD88-independent pathway, which requires signal transduction intermediaries such as TICAM1 and TICAM2, and activates IRF3, ultimately inducing the production of IFN-β [43]. In this study, *E. coli* challenge increased expression of the adaptor molecule TICAM2, transcription factor IRF3, and IFN-β mRNA, whereas pre-incubation with *L. rhamnosus* GR-1 inhibited these increases in expression, except for IRF3. Consistent with our results, both the MyD88-dependent and MyD88-independent pathways involving TLR4 are reportedly activated by LPS in BEECs [25]. Collectively, our results indicate that both MyD88-dependent and MyD88-independent signaling pathways may be involved in regulating the expression of inflammatory cytokines in BEECs pretreated with *L. rhamnosus* GR-1 upon exposure to *E. coli*.

In contrast to TLRs, which are cell-surface pathogen receptors, the NLRs proteins NOD1 and NOD2 are cytoplasmic and are known primarily for their critical functions in inflammatory responses and host defense against microbial pathogens [44]. These NLRs can also initiate signal transduction mechanisms through stimulation of NF-kB and IRF. Recent reports have demonstrated a cooperative role for TLR4 and NOD1 as revealed by the mobilization of mature hematopoietic stem cells to the spleen upon *E. coli* K12 infection [45]. Our data suggest that the levels of NOD1 and NOD2 mRNA expression are the same as the levels of TLR2 and TLR4 mRNA expression. Based on these results, we hypothesize that *L. rhamnosus* GR-1 ameliorates *E. coli*-induced inflammatory responses in BEECs *via* cooperation between TLRs and NLRs.

Furthermore, diverse pathogenic and endogenic stimuli can activate the NLRP3 inflammasome *via* the cytoplasmic innate receptor NLRP3, ASC, and the effector caspase-1 assembly [46], resulting in the release of IL-1β and IL-18 [47]. To assess activation of the NLRP3 inflammasome in this study, the expression of all three of the abovementioned components was evaluated. We found that along with IL-1β and IL-18, the levels of NLRP3, ASC, and caspase-1 mRNA expression were all upregulated by *E. coli* challenge and downregulated at 10 h in BEECs pretreated with *L. rhamnosus* GR-1 before *E. coli* challenge. This finding is consistent with our previous results indicating that *L. rhamnosus* GR-1 pretreatment attenuates *E. coli*-induced increases in NLRP3 inflammasome expression in bovine mammary epithelial cells [16].
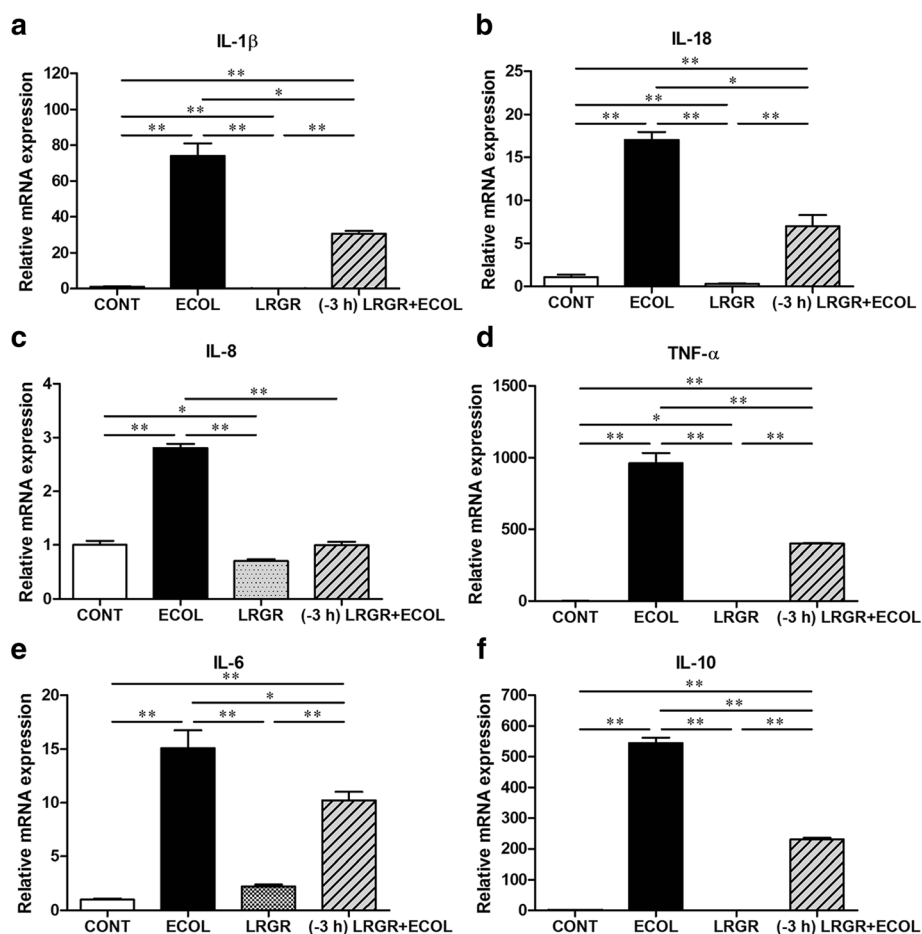
Fig. 7. Effect of *L. rhamnosus* GR-1 pretreatment on cytokine mRNA expression in BEECs following *E. coli* challenge. The relative expression of mRNAs encoding IL-1β (**a**), IL-18 (**b**), IL-8 (**c**), TNF-α (**d**), IL-6 (**e**), and IL-10 (**f**) in BEECs collected from the indicated cultures at 10 h after *E. coli* challenge was analyzed by qRT-PCR. *CONT* DMEM alone, *ECOL* E. coli (4×10^5 CFU/well) infection alone, *LRGR* pretreated with *L. rhamnosus* GR-1 (4×10^7 CFU/well) only for 3 h, *(−3 h) LRGR + ECOL* pretreated with *L. rhamnosus* GR-1 (4×10^7 CFU/well) for 3 h prior to exposure to *E. coli* (4×10^5 CFU/well). Data are presented as the means ± SEM of three independent experiments. *$P < 0.05$; **$P < 0.01$.

The use of *L. rhamnosus* GR-1 to prevent *E. coli* infection in BEECs leads to promising prospectives on new strategies to effectively treat uterine disease in dairy cows. Besides, the BEECs are disrupted or sloughed during parturition and the postpartum period to expose the stroma to PAMPs [24]. The stromal cells are adjacent to the vasculature, so their elaboration of cytokines and chemokines may have more impact on uterine diseases. So, we considered that future studies on stromal cells and larger clinical studies will be essential for ultimate realization the potential of *L. rhamnosus* GR-1 in prevention of uterine disease.

In conclusion, our findings suggest that pretreatment with *L. rhamnosus* GR-1 ameliorates *E. coli*-induced disruption of cellular ultrastructure in BEECs, subsequently reducing the percentage of BEECs apoptosis and limiting inflammatory responses, partly *via* attenuation of MyD88-dependent and MyD88-independent pathway activation. Our data suggest that pretreatment with *L. rhamnosus* GR-1 could potentially prevent postpartum uterine diseases in dairy cows, ultimately reducing the use of antibiotics. However, determining the potential of *L. rhamnosus* GR-1 in this regard will require further research.

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REFERENCES

1. Sheldon, I.M., J. Cronin, L. Goetze, G. Donofrio, and H.J. Schuberth. 2009. Defining postpartum uterine disease and the mechanisms of infection and immunity in the female reproductive tract in cattle. *Biology of Reproduction* 81: 1025–1032.

2. Sheldon, I.M., E.J. Williams, A.N. Miller, D.M. Nash, and S. Herath. 2008. Uterine diseases in cattle after parturition. *Veterinary Journal* 176: 115–121.

3. Machado, V.S., M.L. Bicalho, R.V. Pereira, L.S. Caixeta, J.H. Bittar, G. Oikonomou, R.O. Gilbert, and R.C. Bicalho. 2012. The effect of intrauterine administration of mannose or bacteriophage on uterine health and fertility of dairy cows with special focus on *Escherichia coli* and *Arcanobacterium pyogenes*. *Journal of Dairy Science* 95: 3100–3109.

4. Williams, E.J., D.P. Fischer, D.E. Noakes, G.C. England, A. Rycroft, H. Dobson, and I.M. Sheldon. 2007. The relationship between uterine pathogen growth density and ovarian function in the postpartum dairy cow. *Theriogenology* 68: 549–559.

5. Dolejska, M., Z. Jurcickova, I. Literak, L. Pokludova, J. Bures, A. Hera, L. Kohoutova, J. Smola, and A. Cizek. 2011. Inc*N* plasmids carrying *bla* CTX-M-1 in *Escherichia coli* isolates on a dairy farm. *Veterinary Microbiology* 149: 513–516.

6. Machado, V.S., M.L. Bicalho, E.B. Meira Junior, R. Rossi, B.L. Ribeiro, S. Lima, T. Santos, et al. 2014. Subcutaneous immunization with inactivated bacterial components and purified protein of *Escherichia coli*, *Fusobacterium necrophorum* and *Trueperella pyogenes* prevents puerperal metritis in Holstein dairy cows. *PLoS One* 9, e91734.

7. Kim, Y.G., T. Ohta, T. Takahashi, A. Kushiro, K. Nomoto, T. Yokokura, N. Okada, and H. Danbara. 2006. Probiotic *Lactobacillus casei* activates innate immunity via NF-kappaB and p38 MAP kinase signaling pathways. *Microbes and Infection* 8: 994–1005.

8. Karlsson, M., N. Scherbak, G. Reid, and J. Jass. 2012. *Lactobacillus rhamnosus* GR-1 enhances NF-kappaB activation in *Escherichia coli*-stimulated urinary bladder cells through TLR4. *BMC Microbiology* 12: 15.

9. Baldwin, A.S. 2012. Regulation of cell death and autophagy by IKK and NF-kappaB: critical mechanisms in immune function and cancer. *Immunological Reviews* 246: 327–345.

10. Reid, G., A.W. Bruce, N. Fraser, C. Heinemann, J. Owen, and B. Henning. 2001. Oral probiotics can resolve urogenital infections. *FEMS Immunology and Medical Microbiology* 30: 49–52.

11. Collado, M.C., E. Isolauri, S. Salminen, and Y. Sanz. 2009. The impact of probiotic on gut health. *Current Drug Metabolism* 10: 68–78.

12. Chan, R.C., A.W. Bruce, and G. Reid. 1984. Adherence of cervical, vaginal and distal urethral normal microbial flora to human uroepithelial cells and the inhibition of adherence of gram-negative uropathogens by competitive exclusion. *Journal of Urology* 131: 596–601.

13. Kim, S.O., H.I. Sheikh, S.D. Ha, A. Martins, and G. Reid. 2006. G-CSF-mediated inhibition of JNK is a key mechanism for *Lactobacillus rhamnosus*-induced suppression of TNF production in macrophages. *Cellular Microbiology* 8: 1958–1971.

14. Reid, G., and J. Burton. 2002. Use of *Lactobacillus* to prevent infection by pathogenic bacteria. *Microbes and Infection* 4: 319–324.

15. Yang, S., W. Li, J.R. Challis, G. Reid, S.O. Kim, and A.D. Bocking. 2014. Probiotic *Lactobacillus rhamnosus* GR-1 supernatant prevents lipopolysaccharide-induced preterm birth and reduces inflammation in pregnant CD-1 mice. *American Journal of Obstetrics and Gynecology* 211: 44.e41–44.e12.

16. Wu, Q., M.C. Liu, J. Yang, J.F. Wang, and Y.H. Zhu. 2016. *Lactobacillus rhamnosus* GR-1 ameliorates *Escherichia coli*-induced inflammation and cell damage via attenuation of ASC-independent NLRP3 inflammasome activation. *Applied and Environmental Microbiology* 82: 1173–1182.

17. Genís, S., À. Bach, F. Fàbregas, and A. Arís. 2016. Potential of lactic acid bacteria at regulating *Escherichia coli* infection and inflammation of bovine endometrium. *Theriogenology* 85: 625–637.

18. Ametaj, B.N., S. Iqbal, F. Selami, J.F. Odhiambo, Y. Wang, M.G. Ganzle, S.M. Dunn, and Q. Zebeli. 2014. Intravaginal administration of lactic acid bacteria modulated the incidence of purulent vaginal discharges, plasma haptoglobin concentrations, and milk production in dairy cows. *Research in Veterinary Science* 96: 365–370.

19. Deng, Q., J.F. Odhiambo, U. Farooq, T. Lam, S.M. Dunn, and B.N. Ametaj. 2015. Intravaginal lactic acid bacteria modulated local and systemic immune responses and lowered the incidence of uterine infections in periparturient dairy cows. *PLoS One* 10, e0124167.

20. MacKintosh, S.B., H.J. Schuberth, L.L. Healy, and I.M. Sheldon. 2013. Polarised bovine endometrial epithelial cells vectorially secrete prostaglandins and chemotactic factors under physiological and pathological conditions. *Reproduction* 145: 57–72.

21. Turner, M.L., J.G. Cronin, G.D. Healey, and I.M. Sheldon. 2014. Epithelial and stromal cells of bovine endometrium have roles in innate immunity and initiate inflammatory responses to bacterial lipopeptides *in vitro* via Toll-like receptors TLR2, TLR1, and TLR6. *Endocrinology* 155: 1453–1465.

22. King, A.E., A.W. Horne, S. Hombach-Klonisch, J.I. Mason, and H.O. Critchley. 2009. Differential expression and regulation of nuclear oligomerization domain proteins NOD1 and NOD2 in human endometrium: a potential role in innate immune protection and menstruation. *Molecular Human Reproduction* 15: 311–319.

23. Roshangar, L., S. Abdollahifard, A. Majdi, A. Zarrintan, A. Ghasemzade, L. Farzadi, S.S. Rad, and J.S. Rad. 2013. Study of ultrastructure and apoptosis in the endometrium of women with or without endometriosis. *Iranian Journal of Reproductive Medicine* 11: 399–404.

24. Cronin, J.G., M.L. Turner, L. Goetze, C.E. Bryant, and I.M. Sheldon. 2012. Toll-like receptor 4 and MYD88-dependent signaling mechanisms of the innate immune system are essential for the response to lipopolysaccharide by epithelial and stromal cells of the bovine endometrium. *Biology of Reproduction* 86: 51.

25. Fu, Y., B. Liu, X. Feng, Z. Liu, D. Liang, F. Li, D. Li, et al. 2013. Lipopolysaccharide increases Toll-like receptor 4 and downstream Toll-like receptor signaling molecules expression in bovine endometrial epithelial cells. *Veterinary Immunology and Immunopathology* 151: 20–27.

26. Sheldon, I.M., A.N. Rycroft, B. Dogan, M. Craven, J.J. Bromfield, A. Chandler, M.H. Roberts, S.B. Price, R.O. Gilbert, and K.W. Simpson. 2010. Specific strains of *Escherichia coli* are pathogenic for the

endometrium of cattle and cause pelvic inflammatory disease in cattle and mice. *PLoS One* 5, e9192.

27. Galvão, K.N., N.R. Santos, J.S. Galvão, and R.O. Gilbert. 2011. Association between endometritis and endometrial cytokine expression in postpartum Holstein cows. *Theriogenology* 76: 290–299.

28. Turner, M.L., G.D. Healey, and I.M. Sheldon. 2012. Immunity and inflammation in the uterus. *Reproduction in Domestic Animals* 47(Suppl 4): 402–409.

29. Fortier, M.A., L.A. Guilbault, and F. Grasso. 1988. Specific properties of epithelial and stromal cells from the endometrium of cows. *Journal of Reproduction and Fertility* 83: 239–248.

30. Horne, A.W., E.N. Lalani, R.A. Margara, T.A. Ryder, M.A. Mobberley, and J.O. White. 2005. The expression pattern of MUC1 glycoforms and other biomarkers of endometrial receptivity in fertile and infertile women. *Molecular Reproduction and Development* 72: 216–229.

31. Liu, D., Y. Yang, Q. Liu, and J. Wang. 2011. Inhibition of autophagy by 3-MA potentiates cisplatin-induced apoptosis in esophageal squamous cell carcinoma cells. *Medical Oncology* 28: 105–111.

32. Ma, J.L., Y.H. Zhu, L. Zhang, Z.Y. Zhuge, P.Q. Liu, X.D. Yan, H.S. Gao, and J.F. Wang. 2011. Serum concentration and mRNA expression in milk somatic cells of toll-like receptor 2, toll-like receptor 4, and cytokines in dairy cows following intramammary inoculation with *Escherichia coli. Journal of Dairy Science* 94: 5903–5912.

33. Földi, J., M. Kulcsár, A. Pecsi, B. Huyghe, C. de Sa, J.A. Lohuis, P. Cox, and G. Huszenicza. 2006. Bacterial complications of postpartum uterine involution in cattle. *Animal Reproduction Science* 96: 265–281.

34. Long, E., A.V. Capuco, D.L. Wood, T. Sonstegard, G. Tomita, M.J. Paape, and X. Zhao. 2001. *Escherichia coli* induces apoptosis and proliferation of mammary cells. *Cell Death and Differentiation* 8: 808–816.

35. Okazaki, M., T. Matsuyama, T. Kohno, H. Shindo, T. Koji, Y. Morimoto, and T. Ishimaru. 2005. Induction of epithelial cell apoptosis in the uterus by a mouse uterine ischemia-reperfusion model: possible involvement of tumor necrosis factor-alpha. *Biology of Reproduction* 72: 1282–1288.

36. Yan, F., and D.B. Polk. 2002. Probiotic bacterium prevents cytokine-induced apoptosis in intestinal epithelial cells. *Journal of Biological Chemistry* 277: 50959–50965.

37. Lappas, M. 2013. NOD1 and NOD2 regulate proinflammatory and prolabor mediators in human fetal membranes and myometrium via nuclear factor-kappa B. *Biology of Reproduction* 89: 14.

38. Zhang, W., Y.H. Zhu, J.C. Yang, G.Y. Yang, D. Zhou, and J.F. Wang. 2015. A selected *Lactobacillus rhamnosus* strain promotes EGFR-independent Akt activation in an enterotoxigenic *Escherichia coli* K88-infected IPEC-J2 cell model. *PLoS One* 10, e0125717.

39. Herath, S., D.P. Fischer, D. Werling, E.J. Williams, S.T. Lilly, H. Dobson, C.E. Bryant, and I.M. Sheldon. 2006. Expression and function of Toll-like receptor 4 in the endometrial cells of the uterus. *Endocrinology* 147: 562–570.

40. Young, S.L., T.D. Lyddon, R.L. Jorgenson, and M.L. Misfeldt. 2004. Expression of Toll-like receptors in human endometrial epithelial cells and cell lines. *American Journal of Reproductive Immunology* 52: 67–73.

41. Borges, A.M., G.D. Healey, and I.M. Sheldon. 2012. Explants of intact endometrium to model bovine innate immunity and inflammation *ex vivo. American Journal of Reproductive Immunology* 67: 526–539.

42. Wagner, R.D., and S.J. Johnson. 2012. Probiotic *lactobacillus* and estrogen effects on vaginal epithelial gene expression responses to *Candida albicans. Journal of Biomedical Science* 19: 58.

43. Fitzgerald, K.A., D.C. Rowe, B.J. Barnes, D.R. Caffrey, A. Visintin, E. Latz, B. Monks, P.M. Pitha, and D.T. Golenbock. 2003. LPS-TLR4 signaling to IRF-3/7 and NF-kappaB involves the toll adapters TRAM and TRIF. *Journal of Experimental Medicine* 198: 1043–1055.

44. Van Gorp, H., A. Kuchmiy, F. Van Hauwermeiren, and M. Lamkanfi. 2014. NOD-like receptors interfacing the immune and reproductive systems. *FEBS Journal* 281: 4568–4582.

45. Burberry, A., M.Y. Zeng, L. Ding, I. Wicks, N. Inohara, S.J. Morrison, and G. Núñez. 2014. Infection mobilizes hematopoietic stem cells through cooperative NOD-like receptor and Toll-like receptor signaling. *Cell Host & Microbe* 15: 779–791.

46. Latz, E. 2010. The inflammasomes: mechanisms of activation and function. *Current Opinion in Immunology* 22: 28–33.

47. Man, S.M., and T.D. Kanneganti. 2015. Regulation of inflammasome activation. *Immunological Reviews* 265: 6–21.