A combination of lactic acid bacteria regulates *Escherichia coli* infection and inflammation of the bovine endometrium

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ABSTRACT

Uterine function in cattle is compromised by bacterial contamination and inflammation after calving. The objective of this study was to select a combination of lactic acid bacteria (LAB) to decrease endometrium inflammation and *Escherichia coli* infection. Primary endometrial epithelial cells were cultured in vitro to select the most favorable LAB combination modulating basal tissue inflammation and *E. coli* infection. Supernatants were obtained to determine expression of pro-inflammatory cytokines, and *E. coli* infection was evaluated after harvesting the tissue and plate counting. The selected LAB combination was tested in uterus explants to assess its capacity to modulate basal and acute inflammation (associated with *E. coli* infection). The combination of *Lactobacillus rhamnosus*, *Pediococcus acidilactici*, and *Lactobacillus reuteri* at a ratio of 25:25:2, respectively, reduced *E. coli* infection in vitro with (89.77%) or without basal tissue inflammation (95.10%) compared with single LAB strains. Lactic acid bacteria treatment reduced *CXCL8* and *IL1B* expression 4.7- and 2.2-fold, respectively, under acute inflammation. Ex vivo, the tested LAB combination reduced acute inflammation under *E. coli* infection, decreasing IL-8, IL-1 β , and IL-6 up to 2.2-, 2.5-, and 2.2-fold, respectively. In the total inflammation model, the LAB combination decreased IL-8 1.6-fold and IL-6 1.2-fold. Ultrastructural evaluation of the tissue suggested no direct interaction between the LAB and *E. coli*, although pathological effects of *E. coli* in endometrial cells were greatly diminished or even reversed by the LAB combination. This study shows the promising potential of LAB probiotics for therapeutic use against endometrial inflammation and infection.

Key words: *Escherichia coli*, endometrium, inflammation, lactic acid bacteria

INTRODUCTION

Uterine function is often compromised in cattle by bacterial contamination and inflammation after calving (Markusfeld, 1987; Borsberry and Dobson, 1989; Opsomer et al., 2000). Metritis is an acute inflammation that occurs within 21 d and is most common within the first 6 d after parturition. Metritis affects about 20% of lactating dairy cows, with an incidence ranging from 8 to >40% (Galvão, 2011), and is characterized by an enlarged uterus and a watery red-brown fluid discharge, which has a fetid odor (Sheldon et al., 2009). When a persistent clinical disease is found 3 wk after calving, the process is described as purulent vaginal discharge (~20% incidence). However, about 30% of dairy cows have subclinical endometritis (Markusfeld, 1987; Borsberry and Dobson, 1989; Opsomer et al., 2000; Drillich et al., 2001; Sheldon et al., 2006; Lincke et al., 2007), and thus the problem may occur unnoticed. The economic effect of uterine diseases in dairy cows is derived from infertility, increased culling due to failure to conceive, reduced milk production, and the cost of antibiotic treatments. The economic cost of a single case of metritis has been calculated to be about €350 (Drillich et al., 2001; Overton and Fetrow, 2008).

Among the wide range of bacteria that contaminate the bovine uterus, *Escherichia coli* are abundant and are associated with high concentrations of LPS in the uterine lumen and with metritis (Williams et al., 2005). Bacterial infection is generally considered to foster the recruitment of immune cells and lead to acute inflammation in the uterus (Herath et al., 2006). This process coexists with postpartum uterine involution, which causes basal tissue inflammation, leading to an excessive inflammatory status. In some cases, the persistent inflammatory response is also attributable, in part, to reduced immune function before calving (LeBlanc, 2014). In this context, efforts to reduce uterine infec-

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tion and the consequent acute inflammation are of great importance to decrease the final inflammatory status of the uterus and its side effects such as tissue damage or failure to complete involution of the uterus (Walker et al., 2015).

The mechanisms of infection and immunity in the endometrium have mostly been studied in vitro (Herath et al., 2006; Sheldon et al., 2009), whereas the characterization of uterine infection and insights into the pathogenesis of disease often stem from observational studies of spontaneous postpartum uterine infections in vivo (Borsberry and Dobson, 1989; Opsomer et al., 2000; Drillich et al., 2001). The overall inflammatory response to pathogens associated with innate immunity depends on the sum of the actions of multiple cell types rather than just specialized immune cells (Sheldon et al., 2014). The study of tissue explants collected from the endometrium ex vivo allows investigation of potential links between whole-animal clinical signs and cellular function (Borges et al., 2012). Inflammatory mediators, such as cytokines, are important molecules eliciting the cellular response against pathogens, but they also may initiate a cascade of events that may end in important toxic effects on the tissue or induce cell death by necrosis or apoptosis. In this context, morphological assessment of toxic effects at the ultrastructural level by field emission scanning electron microscopy (FESEM) and transmission electron microscopy (TEM) are valuable tools to understand how *E. coli* alone or combined with LAB affects cell health.

Because the traditional antimicrobial treatment applied for metritis may lack efficacy and does not typically improve reproductive performance of the treated animals (Gilbert and Schwark, 1992; Otero et al., 2006), the use of probiotic strains of bacteria has been proposed as an alternative to prevent postpartum uterine infections and inflammation (Ametaj et al., 2014; Deng et al., 2015). In our recent work, various individual strains of LAB were shown to protect against *E. coli* infection in vitro and reduce inflammation in epithelial cells (Genís et al., 2016). The hypothesis of the present work is that the combination of LAB strains tested in our previous work (Genís et al., 2016) could reduce inflammation and *E. coli* infection in bovine endometrium. Ex vivo experiments offer a good opportunity to evaluate these effects considering the combination of several cell types and their interactions.

The objective of this study was to establish the best probiotic combination of *Lactobacillus rhamnosus*, *Pediococcus acidilactici*, and *Lactobacillus reuteri* in primary cultures and to assess its capacity to modulate of the uterine inflammation caused by basal tissue inflammation, such as that associated with calving, or by acute factors such as *E. coli* infection.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Pediococcus acidilactici (CECT 5915) and *L. rhamnosus* (CECT 278) were purchased from CECT (Colección Española de Cultivos Tipo, CSIC Valencia, Spain). *Lactobacillus reuteri* (DSM 20016) and *Lactobacillus sakei* (DSM 20100) were acquired from DSMZ (German collection of microorganisms and cell cultures, Leibniz, Germany). A swab sample was taken from the uterus of 4 metritic cows to isolate a pathogenic *E. coli*. Each swab was spread in blood agar, MacConkey agar, and chocolate agar. Final identification of the colonies was made by Vitek (bioMérieux, Craponne, France). The *E. coli* selected was FimH positive (virulence factor) and hemolytic. Bacterial cultures were initiated by inoculating 1 mL of a glycerinate in 9 mL of Luria Bertani medium (10 g/L peptone, 5 g/L yeast extract, and 10 g/L NaCl) for *E. coli* or de Man, Rogosa, and Sharpe medium (Scharlau, Sentmenat, Spain) for *L. rhamnosus*, *P. acidilactici*, and *L. reuteri* at 37°C in static conditions overnight (O/N). Then, bacteria were centrifuged at $6,000 \times g$ for 15 min and resuspended at 1×10^8 cfu/mL with Dulbecco's modified Eagle's medium (DMEM, Gibco, New York, NY) supplemented with 8 µg/mL bovine insulin and 50 µg/mL hydrocortisone. *Escherichia coli* concentration (cfu/mL) was confirmed by serially diluting and plating into MacConkey (Scharlau, Sentmenat, Spain) agar medium. Colonies were counted after O/N growth at 37°C.

Endometrial Primary Culture

Endometrial tissue was obtained at a slaughterhouse from postpubertal nonpregnant cattle with no evidence of genital disease or microbial infection and transported in chilled PBS with 100 µg/mL streptomycin, 100 U/mL penicillin, and 2.5 µg/mL amphotericin B (PEF) to the laboratory. Tissue was then cut into small pieces and incubated with PBS supplemented with $1 \times$ PEF, 0.1 mM EDTA, and 0.1 mM DTT for 10 min at 37°C in 5% CO₂ at 200 rpm. The supernatant was removed, and RPMI 1640 medium (Gibco) with 0.25% collagenase (Gibco) was added and further incubated for 15 min at 37°C in 5% CO₂ at 200 rpm. Supernatant was collected and mixed (1:1) with RPMI 1640 medium enriched with 0.02 mg/mL DNase (Roche, Mannheim, Germany). Cells present in the supernatant were recovered after centrifuging 5 min at $300 \times g$. This process was repeated 3 times. Final cell pellets were resuspended in DMEM with 8 µg/mL bovine insulin, 10 µg/mL gentamycin, 50 µg/mL hydrocortisone, 50 µg/mL streptomycin, 50 IU/mL penicillin, 2.5 µg/

mL amphotericin B, and 10 ng/mL epithelial growth factor (Sigma-Aldrich, St. Louis, MO). Hydrocortisone was included in the medium to foster cell growth (Domènech et al., 2014). Cells were quantified using a hemocytometer and incubated at 160,000 cells/cm² in culture flasks. Epithelial cell phenotype was confirmed by immunofluorescence labeling against anti-cytokeratin (monoclonal anti-cytokeratin pan antibody produced in mouse, 1:500 dilution; Sigma-Aldrich) of at least 90% of isolated cells as previously shown (Genís et al., 2016). For the immunofluorescence, endometrial primary cells were grown on coverslips and fixed with 4% paraformaldehyde in PBS. HeLa cells were used as a positive control, and macrophage differentiated THP-1 cells as a negative control. Coverslips were blocked with 150 μ L of PBS containing goat serum (0.05% vol/vol) and Triton 0.2% for 30 min at room temperature. The primary antibody was incubated in blocking buffer for 2 h at room temperature at a dilution of 1:500 (monoclonal anti-cytokeratin pan antibody produced in mouse; Sigma-Aldrich). After 3 washes with PBS, secondary antibody, an anti-mouse–fluorescein isothiocyanate, was incubated for 1 h at room temperature at a dilution of 1:1,000 (Sigma-Aldrich). Last, cells were washed and dried coverslips mounted on glass slides using Fluoroprep (Biomérieux, Craponne, France) and observed by fluorescence microscopy.

Ex Vivo Bovine Endometrial Explants

Segments of endometrial tissue were obtained at a slaughterhouse from 20 postpubertal nonpregnant cattle with no evidence of genital disease or microbial infection and transported in chilled PBS with 100 μ g/mL streptomycin, 100 U/mL penicillin, and 2.5 μ g/mL amphotericin B to the laboratory. The tissue was cut into 0.8-cm² diameter segments using skin biopsy punches (Kai Medical, Seki, Japan). The explants were plated in 24-well plates (1 explant/well) and incubated with RPMI 1640 medium (Gibco) supplemented with

10% FBS, 50 μ g/mL streptomycin, 50 IU/mL penicillin, and 2.5 μ g/mL amphotericin B in 5% CO₂ at 37°C. These experiments were repeated on 4 different days (each day involving 5 independent animals to reach a total of 20 cows) and using in each experiment 6 replicates/animal for each ex vivo treatment. Sample size was established taking into account the animal variability observed in previous gene expression and ex vivo experiments (data not shown)

In Vitro Experiments: LAB Treatment, Inflammation, and E. coli Infection

Primary epithelial cells were plated at 80,000 cells/well in 24-well plates with DMEM supplemented with 8 μ g/mL bovine insulin and 50 μ g/mL hydrocortisone and kept O/N at 37°C and 5% CO₂. Cells were incubated with 6 replicates O/N with 4 LAB combinations (Table 1) and positive controls (*P. acidilactici* at a ratio of 25 bacteria/epithelial cell, in the model without basal inflammation, and with *L. reuteri* at ratio of 2 bacteria/endometrial cell, in the presence of basal inflammation). The basal inflammation was induced with 10 μ g/ μ L of IL-1 β as described elsewhere (Genís et al., 2016). Cells were then challenged with 4 $\times$ 10⁶ cfu/well of *E. coli* for 6 h. Infection without LAB was used as an infection control and incubation with medium instead of *E. coli* as a negative control. If cells were submitted to previous basal inflammation, the negative control was cells with IL-1 β and medium. Cells were washed twice with chilled PBS, and associated bacteria were released with 500 μ L of 0.9% NaCl/well. Associated *E. coli* was quantified by serially diluting and plating the cell extract onto MacConkey agar media. Colonies were counted after O/N growth at 37°C. Cells used for gene expression analyses were washed twice with PBS and harvested with 500 μ L of TriZol (5Prima, Gaithersburg, MD) for RNA extraction.

Ex Vivo Experiments: LAB Treatment, Inflammation, and E. coli Infection

Explants were incubated in 6 replicates O/N with the selected LAB combination and then challenged with 1.5 $\times$ 10⁶ cfu/well of *E. coli* O/N. Infection without LAB was used as an infection control and incubation with media instead of *E. coli* as a negative control. When *E. coli* infection was evaluated in endometrial explants under inflammation, IL-1 β was added at 10 ng/well 1 h before LAB administration. In this case explants inflamed and infected were used as an infection control, and explants inflamed and treated with LAB were used as a negative control. Supernatant was collected for further IL-8, IL-1 β , and IL-6 ELISA quantification.

Table 1. Combinations of lactic acid bacteria (LAB; *Lactobacillus rhamnosus*, *Pediococcus acidilactici*, and *Lactobacillus reuteri*) assessed in vitro for their ability to reduce inflammation and infection with *Escherichia coli* in a uterine explant model for dairy cows¹

Combination	Ratio		
	<i>L. rhamnosus</i>	<i>P. acidilactici</i>	<i>L. reuteri</i>
A	100	25	0
B	25	25	0
C	100	25	2
D	25	25	2

¹Ratio of each LAB corresponds to the number of LAB vs. endometrial epithelial cells.

Quantitative RT-PCR Analyses

One microgram of RNA was reverse transcribed to cDNA using the PrimeScript RT reagent kit (Takara, Shiga, Japan) following manufacturer's instructions. Quantitative PCR (qPCR) was performed for bovine *CXCL8* and *IL1B* using specific primers (Table 2). A total reaction volume of 20 μ L was used, containing 50 ng of cDNA, 10 μ L of SYBR Green Fluorescent (Bio-Rad, Hercules, CA), and the optimized primer concentration for each gene (Table 2). The qPCR amplifications were cycled as follows: an initial denaturing step of 10 min at 95°C, followed by 40 cycles of 10 s at 95°C, 15 s at the optimized annealing temperature for each gene, 30 to 60 s at 72°C, and a final extension of 10 min at 72°C. Relative gene expression was calculated using the Pfaffl method (Pfaffl, 2001) with *GAPDH* as a reference gene control, and all the qPCR were performed in triplicate. *GAPDH* was chosen because it is stably expressed in a variety of cells, including uterine tissues (Wathes et al., 2009; Galvão et al., 2011). Moreover, we checked that no differences in the expression of *GAPDH* were found among treatments.

Enzyme-Linked Immunosorbent Assay

Concentrations of IL-1 β , IL-6, and IL-8 were measured in supernatants from explants by sandwich ELISA according to the manufacturers' instructions: Bovine IL-1 β ELISA Kit (ESS0027; ThermoFisher Scientific, Cramlington, UK); Bovine IL-6 ELISA Kit (ESS0029; ThermoFisher Scientific); Human CXCL8/IL-8 DuoSet (DY208, Vitro S.A., Spain). The Human CXCL8/IL-8 DuoSet has previously been validated for measuring bovine IL-8 (Rinaldi et al., 2008; Borges et al., 2012). Bovine IL-1 β ELISA Kit and Bovine IL-6 ELISA Kit are specific for the measurement of natural and recombinant IL-1 β and IL-6, respectively.

Field Emission Scanning Electron Microscopy

Microdrops of *L. rhamnosus*, *P. acidilactici*, and *L. reuteri* cultures were directly deposited for 2 min onto silicon wafers (Ted Pella Inc., Redding, CA), excess of sample blotted with Whatman filter, air-dried, and

immediately observed without coating in nearly native state with a FESEM Zeiss Merlin (Oberkochen, Germany) equipped with a high resolution in lens secondary electron detector and operating at 1 kV.

Triplicate samples of epithelial cell cultures with or without LAB treatment and with *E. coli* infection, except for the negative control, were fixed with 3% (vol/vol) glutaraldehyde (Merck, Darmstadt, Germany) in phosphate buffer (PB) 0.1 M at pH 7.2 (Sigma-Aldrich, Steinheim, Germany) for 2 h at room temperature, rinsed 4 times with PB, postfixed with 1% (wt/vol) osmium tetroxide (TAAB Lab, Reading, UK) in PB for 2 h at 4°C, rinsed 4 times in PB, dehydrated in ethanol (Panreac, Barcelona, Spain), and dried with CO₂ in a critical point drying system (Bal-Tec AG, Bakzers, Liechtenstein). Samples were mounted on stubs and observed without coating with a Zeiss Merlin microscope under the same conditions as LAB samples.

A qualitative approach was used for the analysis of 10 randomly distributed areas for each treatment. The parameters observed were general structure of the epithelium (size and shape of cells, ultrastructure, and amounts of ultrastructure of microvilli), presence of *E. coli* and LAB on the cell surface, and cell debris.

Transmission Electron Microscopy

For the assessment of potential changes in the ultrastructure, samples of epithelial cultures treated with PBS, *E. coli*, and *E. coli* with LAB were fixed O/N in 2% (wt/vol) paraformaldehyde (TAAB Lab) and 2.5% (vol/vol) glutaraldehyde (Merck) in 0.1 M PB (Sigma-Aldrich, Steinheim, Germany), postfixed for 2 h with 1% (wt/vol) osmium tetroxide (TAAB Lab) containing 0.8% (wt/vol) potassium hexacyanoferrate (Sigma-Aldrich) in PB, sequentially dehydrated in acetone, embedded in Eponate 12 resin (Ted Pella Inc.), and polymerized at 60°C for 48 h. Ultrathin sections (70 nm) of selected areas obtained with Leica ultracut UCT microtome (Leica Microsystems, Mannheim, Germany) were placed on 200-mesh carbon-coated copper grids and contrasted with conventional uranyl acetate and lead citrate solutions. Ultrathin sections were then observed in a TEM Jeol JEM-1400 (Jeol Ltd., Tokyo, Japan) operating at 80 kV and equipped with a CCD

Table 2. Sequence, annealing temperature (At), concentration (μ M), and amplicon size (bp) of forward and reverse primers used for quantitative PCR and accession number or reference

Gene	Forward primer	Reverse primer	At, °C	μ M	Accession no. or reference
<i>GAPDH</i>	GCATCGTGGAGGGACTTATGA	GGGCCATCCACAGTCTTCTG	52	0.125	NM_001034034
<i>CXCL8</i>	TTGAGAGTGGACCACTGC	TGCACCCAGTTTCCCTTGG	55	0.5	Arnold et al., 2004
<i>IL1B</i>	TGGGAGATGGAAACATCCAG	TTTATTGACTGCACGGGTGC	50	0.3125	M37211

Gatan (Gatan Inc., Pleasanton, CA) ES1000W Erlangshen camera.

A semiquantitative analysis was performed on 10 ultrathin sections randomly chosen from 3 different areas per treatment. The parameters studied were presence of mucus on the cell surface, mitochondrial damage, and cell death (necrosis or apoptosis). For each treatment, a value following a scale from healthy, without alteration (–) to high alteration (+++) was assigned for each parameter. Briefly, mucus was observed as a electrodense layer coating surface cells; edematous mitochondria was considered when the organelles showed higher size, round shape, nonhomogeneous matrix electrodensity, and/or disorganized or disappeared cristae; necrotic cells showed nonhomogeneous and disorganized cytosol and conserved nucleus (see representative images in Figure 8).

Statistical Analyses

Before statistical analyses, data were transformed by log or square root when necessary to achieve a normal distribution. Results herein are expressed as means of nontransformed data $\pm$ SEM obtained with normalized data (unless otherwise stated). Data were analyzed using a mixed-effects model (SAS Inst. Inc., Cary, NC), considering treatment and infection as fixed effects and animal (the donor for the cell cultures) as a random effect. Data from TEM were analyzed using a Fisher exact test (SAS Inst. Inc.), assigning a value from 0 (–) to 3 (+++) for each parameter. Differences were considered significant when $P < 0.05$.

RESULTS

Selection of LAB Combination In Vitro

The best combination of LAB strains tested in our previous work (Genís et al., 2016) was selected in vitro taking into account its ability to reduce inflammation and *E. coli* infection.

Escherichia coli Infection. The effect of the LAB combinations on *E. coli* infection is presented in Figure 1A. The *E. coli* count was reduced by 95.1% ($P < 0.0001$) when using LAB combination D (Table 1; *L. rhamnosus* ratio 25, *P. acidilactici* ratio 25, and *L. reuteri* ratio 2); whereas, it was increased ($P < 0.0001$) with LAB combinations A and B (56.0 and 75.6%, respectively) relative to culture with *E. coli* inoculum alone. No differences were found with LAB combination C. Furthermore, LAB combination D was able to surpass *P. acidilactici*, used as positive control, which reduced *E. coli* infection by 89.7%.

Figure 1. *Escherichia coli* infection of primary endometrium cultures. Viable cell counts (cfu/mL) of *E. coli* for each combination of lactic acid bacteria (explained in Table 1) in an endometrium in the absence (A) or in the presence of a basal inflammation (B). Bars represent means $\pm$ SE. Bars with an asterisk differ ($P < 0.01$) from *E. coli*. *P. acidilactici* = *Pediococcus acidilactici*; *L. reuteri* = *Lactobacillus reuteri*.

The probiotic potential and modulatory effects of the LAB combinations against endometrial *E. coli* infection were similar in the presence (Figure 1A) or absence of a basal inflammation (Figure 1B). Under basal inflammation, *E. coli* infection was reduced with LAB combination D (89.78%; $P < 0.01$), and no differences were found with other LAB combinations. Lactic acid bacteria combination D was also able to surpass the decrease of infection induced by *L. reuteri* alone (73.5%), which was used as a positive control to reduce infection under basal inflammation.

Innate Immunity Under Acute Inflammation. The expression of *CXCL8* and *IL1B* genes was used as markers of innate immunity under acute inflammation caused by *E. coli* treatment of either LAB-treated or nontreated endometrial cells. The abundance of *CXCL8* mRNA was affected ($P < 0.01$) by LAB treatment when cells were not infected (Figure 2A, open bars) and also ($P < 0.0001$) in the presence of infection (Figure 2A, gray bars). Specifically, when cells were treated with the LAB combinations C and D, 1.7- and 2.1-fold increments in *CXCL8* expression in epithelial cells were observed, respectively, compared to nontreated cells

(negative control). No differences were observed when LAB combinations A and B were applied to epithelial primary cultures of endometrium.

The expression of the *CXCL8* gene increased 21.8-fold with respect to the negative control (Figure 2A, gray bars), but it was down-regulated when *E. coli* was applied with LAB combinations A, B, C, or D (11.8-, 2.0-, 4.7-, and 4.7-fold reduction, respectively; Figure 2A, gray bars).

The gene expression of *IL1B* was affected by LAB treatment ($P < 0.0001$) when cells were uninfected (Figure 2B, open bars) and also ($P < 0.0001$) in the presence of infection (Figure 2B, gray bars). Cells treated with LAB combinations A and C expressed more *IL1B* than the negative control (13.9- and 21.7-fold, respectively). No differences were found with the other LAB combinations (Figure 2B, open bars).

The mRNA abundance of *IL1B* in the endometrium infected with *E. coli* increased 137.5-fold with respect to the negative control. However, this increment was reduced when cells were treated with either LAB combination (5.5-, 3.1-, 10.6-, and 2.2-fold, for A, B, C, and D, respectively; Figure 2B, open bars).

Innate Immunity Under Basal and Acute Inflammation. The gene expression of *CXCL8* in the basal tissue inflammation model of endometrial cells (cells pretreated with 10 $\mu\text{g}/\mu\text{L}$ of $\text{IL-1}\beta$) was affected by the different LAB combinations both in the presence ($P < 0.0001$; Figure 3A, gray bars) and in the absence of an acute inflammation caused by *E. coli* infection ($P < 0.0001$; Figure 3A, open bars). Pretreatment of the endometrium with $\text{IL-1}\beta$ increased the expression of *CXCL8* gene by 6.2-fold, but it only increased 1.4-fold when the basal inflammation was induced in the presence of LAB combination B (Figure 3A, open bars).

Infection with *E. coli* increased 2.35-fold the expression of *CXCL8* by epithelium under a basal inflammation. When cells were treated with LAB combinations A or C, a decreased expression of *CXCL8* was observed (2.6- and 2.2-fold, respectively), whereas, the LAB combination D only tended ($P = 0.08$) to decrease *CXCL8* expression, and no differences were found with the LAB combination B (Figure 3B).

The mRNA abundance of *IL1B* in the endometrium in the presence of basal inflammation was affected by LAB combination when acute inflammation was induced (Figure 3B, gray bars; $P < 0.0001$). The inflamed endometrium expressed 49-fold more *IL1B* than the negative control. However, no differences were observed when the LAB treatments were applied (Figure 3B, open bars). *Escherichia coli* infection (acute inflammation) raised the expression of *IL1B* 1.93-fold compared to uninfected cells under basal inflammation. When the

LAB combinations were applied, a reduction (relative to negative control) was observed in all cases: 2.1-fold for LAB combination A, 1.7-fold for LAB combination B, 2.3-fold for LAB combination C, and 1.9-fold for LAB combination D (Figure 3B, gray bars).

Figure 2. Innate immune response in primary endometrial cultures under an acute inflammation. The y-axis represents the arithmetic mean fold change relative to expression of a reference gene (*GAPDH*). (A) Relative expression of *CXCL8* when cells were treated with lactic acid bacteria (LAB) combinations in the absence (open bars) or in the presence of an *Escherichia coli* infection (gray bars). (B) Gene expression of *IL1B* in uninfected (open bars) and infected (gray bars) cells. Bars represent means $\pm$ SEM (A) or SE (B) for the different treatments. †Bars differ ($P < 0.01$) from the negative control; *bars differ ($P < 0.01$) from *E. coli* control. Negative control and *E. coli* control differed at $P < 0.0001$.

LAB Combination for Ex Vivo Experiments

The LAB combination selected to conduct the ex vivo experiments was the combination D (*L. rhamnosus* ratio 25, *P. acidilactici* ratio 25, and *L. reuteri* ratio 2).

Ex Vivo Acute Inflammation Model. The secretion of IL-8, IL-1 β , and IL-6 was evaluated after an acute inflammation produced by *E. coli* infection of either LAB-treated or nontreated explants.

Interleukin-8 secretion was affected ($P < 0.0001$) by *E. coli* infection, and an interaction ($P < 0.0001$) between infection and treatment was also observed (Figure 4A). When the probiotic combination was applied to explants, IL-8 secretion increased 1.6-fold compared to the negative control. When infection was induced, the secretion of IL-8 increased 3.7-fold compared to the negative control. However, in the presence of the probiotic combination the secretion of IL-8 decreased by 2.20-fold. No differences were found in the secretion of IL-8 between the explants treated with probiotic in the presence or absence of an *E. coli* infection.

The secretion of IL-1 β was affected by LAB treatment ($P < 0.0001$), infection ($P < 0.0001$), and the interaction between treatment and infection ($P < 0.0001$, Figure 4B). The probiotic combination increased the secretion of IL-1 β by 2.1-fold compared to the negative control. When the explants were infected with *E. coli*, the secretion of IL-1 β increased 6.3-fold, but the increase was lesser when the infection of *E. coli* was induced in cells treated with LAB (2.5-fold). Hence, the probiotic combination was able to reduce by 1.2-fold the final secretion of IL-1 β in the presence of an *E. coli* infection.

The secretion of IL-6 was affected by infection ($P < 0.0001$) and the interaction between infection and treatment ($P < 0.0001$, Figure 4C). The probiotic combination increased the secretion of IL-6 1.8-fold compared to the negative control. When the explants were infected by *E. coli*, secretion of IL-6 increased 4.5-fold, whereas when explants were pretreated with the probiotics, IL-6 secretion decreased 2.2-fold.

Ex Vivo Total Inflammation Model. The secretion of IL-8, IL-1 β , and IL-6 was evaluated in LAB-treated and untreated endometrial explants after inducing inflammation (basal + acute inflammation).

The secretion of IL-8 was affected by treatment ($P < 0.0001$), infection ($P < 0.0001$), and the interaction between treatment and infection ($P < 0.0001$, Figure 5A). *Escherichia coli* infection increased the secretion of IL-8 1.8-fold compared to the negative control. When the LAB treatment was previously applied, secretion of IL-8 decreased 1.6-fold. No differences were observed between the negative control and explants pretreated with LAB.

Figure 3. Innate immune response of primary endometrial cultures under a total (acute and basal) inflammation. The y-axis represents the arithmetic mean fold change relative to an expression of a reference gene (*GAPDH*). (A) Expression of *CXCL8* gene when cells were treated with lactic acid bacteria (LAB) combinations in the absence (open bar) or in the presence of an *Escherichia coli* infection (gray bars). (B) Gene expression of *IL1B* in infected (gray bars) and uninfected (open bars) cells. Bars represent means $\pm$ SEM for the different treatments. †Bars differ ($P < 0.05$) from IL-1 β ; *bars differ ($P < 0.05$) from *E. coli* + IL-1 β . Negative control and *E. coli* control differed at $P < 0.0001$.

Secretion of IL-1 β was affected by infection ($P < 0.0001$) and the interaction between treatment and infection ($P < 0.0005$, Figure 5B). *Escherichia coli* infection increased the secretion of IL-1 β 1.1-fold compared to the negative control. When the noninfected explants were treated with LAB, an increment of 1.04-fold was observed compared to the negative control and no differences were perceived in comparison with the infected

explants. The combination of the LAB and *E. coli* increased the secretion of IL-1 β 1.1-fold compared with the infected explants.

Figure 4. Innate immune response in endometrial explants treated ex vivo under an acute inflammation. Secretion (pg/mL) of IL-8 (A), IL-1 β (B), and IL-6 (C). Dots represent means for each treatment and cow, whereas lines represent the means for each treatment. Columns with different letters (a–d) differ ($P < 0.0001$). LAB = lactic acid bacteria; *E. coli* = *Escherichia coli*.

Secretion of IL-6 was affected by infection ($P < 0.0001$) and the interaction between treatment and infection ($P < 0.0002$, Figure 5C). The LAB treatment increased the secretion of IL-6 1.1-fold compared to the negative control. When the explants were infected with *E. coli*, the secretion of IL-6 increased 1.6-fold. Although *E. coli* induced the explants treated with probiotics to secrete more IL-6 than those not infected (1.2-fold), the probiotic combination resulted in 1.2-fold reduction of the secretion of IL-6.

Figure 5. Innate immune response in endometrial explants treated ex vivo under a total inflammation (basal plus acute). Secretion (pg/mL) of IL-8 (A), IL-1 β (B), and IL-6 (C). Dots represent means for each treatment and cow, whereas lines are the means for each treatment. Columns with different letters (a–d) differ ($P < 0.0001$). LAB = lactic acid bacteria; *E. coli* = *Escherichia coli*.

Field Emission Scanning Electron Microscopy

The study of LAB by FESEM showed bacteria with similar size and shape in each culture and also after incubation with primary endometrial epithelia (Figure 6A, 6B). Moreover, *E. coli* bacteria were also observed in contact with epithelial cells either incubated alone or with the LAB combination D (Figure 6C, 6D). *Escherichia coli* alone or *E. coli* and LAB appeared in low numbers in the surface of the epithelial cells and in no case formed biofilms or interactions between each other (Figure 6C, 6D). The abundance of *E. coli* was lower in samples treated with LAB than in those infected with *E. coli* alone ($P < 0.05$).

A qualitative assessment of epithelial cells was performed by FESEM, and a healthy epithelium was observed in all treatments (epithelial cells with normal size and shape and normal appearance of microvilli), but in cultures infected with *E. coli*, abundant areas with cell debris and bacilli were observed on the surface of epithelial cells (Figure 7). These areas of damaged epithelial cells were less abundant in the control and in the LAB pre-incubated cells than in cells infected with *E. coli*.

Transmission Electron Microscopy

Quantitative data by TEM clearly illustrated the highest incidence of necrosis among all samples infected with *E. coli*, indicating an obvious harmful effect of this pathogen in our model ($P = 0.07$, Figure 8). In contrast, cell death decreased with the use of LAB probiotics. A similar pattern between treatments was found for mitochondrial damage (edematous mitochondria; $P < 0.01$). No morphological signs of apoptosis were found in any of the treatments.

DISCUSSION

Understanding the mechanisms of infection and immunity normally involves the use of in vitro studies. However, the generation of cell cultures usually disrupts the tissue architecture and the spatial arrangement of cells, which are often necessary for normal function (Vogel and Sheetz, 2006; Healy et al., 2014). The use of tissue explants provides the opportunity to study the overall inflammatory response to pathogens depending on the sum of actions of multiple cell types rather than just epithelial or specialized immune cells. In this study, endometrial tissue from nonpregnant cows collected in the slaughterhouse was used to make explants. It is important to take into account that it is an experimental approach and the tissue does not come from actual postpartum cows. The LAB combinations tested herein

were selected from the results obtained during previous experiments (Genís et al., 2016). Briefly, 3 doses of a combination of 4 LAB were tested (*L. rhamnosus*, *P. acidilactici*, *L. reuteri*, and *L. sakei*) for their capacity to modulate basal inflammation in an endometrial primary culture infected with *E. coli*. The main results obtained in our previous work were that *P. acidilactici* ratio 25 (25 bacterial cells to 1 epithelial cell) was able to inhibit *E. coli* infection and down-regulate total inflammation; *L. rhamnosus* ratios 100 and 25 were able to reduce total inflammation but increased the extent of *E. coli* infection with the greatest dose; and *L. reuteri* ratio 2 was able to reduce *E. coli* infection in the presence of a basal inflammation (Genís et al., 2016).

Overall, all the biochemical and ultrastructural results herein demonstrated that the best LAB combination to fight *E. coli* infection in the endometrium was the LAB combination D composed of *L. rhamnosus*, *P. acidilactici*, and *L. reuteri* in a ratio of 25:25:2 (Figures 1 and 2). *Lactobacillus reuteri* played a key role in the LAB combination as indicated by the comparison of LAB combination D and B, which differed only in the presence (D) or absence (B) of this bacterium. The presence of *L. reuteri* (D) reduced 21.6- and 83.8-fold the extent of *E. coli* infection compared with the levels achieved with the LAB combination B with or without a basal inflammation, respectively. Despite the importance of *L. reuteri*, the mixture with *L. rhamnosus* and *P. acidilactici* was necessary to improve the performance obtained with *L. reuteri* and *P. acidilactici* alone, which had previously been selected as the best LAB strains inhibiting infection with or without inflammation, respectively (Genís et al., 2016). These results confirmed our hypothesis that an adequate combination of probiotics would efficiently counteract an endometrial infection by *E. coli*. The LAB combination D not only most efficiently reduced the *E. coli* infection (Figure 1), but it also led to the greatest reduction of acute and basal inflammation (Figures 2 and 3). Probably, in the case of the combination D applied to epithelial cultures, the reduction of count (cfu) of *E. coli* (Figure 1) was the main cause behind the decreased expression of proinflammatory cytokines during the infection (Figure 2), because lower bacterial counts cause less acute inflammation.

The healthy uterus is able to efficiently clear bacterial infection, and once bacteria have gained access to the uterus, the most important component of uterine defense is nonspecific phagocytosis by neutrophils (LeBlanc, 2008). Parturition in dairy cattle is associated with impairment of polymorphonuclear leukocyte phagocytosis and with a decrease in the ability to fight bacterial infections (Kehrli et al., 1989). A decrease in neutrophils' activity during the periparturient pe-

Figure 6. Representative images of lactic acid bacteria (LAB) and *Escherichia coli*. (A) General view and detail of LAB cultures. (B) Detail of LAB incubated with epithelial cells. General view and details of *E. coli* incubated (C) alone or (D) with LAB combination (*Lactobacillus rhamnosus*, *Pediococcus acidilactici*, *Lactobacillus reuteri*). Bar size: 1 µm.

Figure 7. Representative images of epithelial cells of negative control (C-), *Escherichia coli* and lactic acid bacteria (LAB) with *E. coli*. (A) General view of epithelial cell surface showing similar aspect in the 3 treatments. (B) Detail of cell surface of the 3 treatments showing microvilli longer and more abundant in epithelia treated with *E. coli* alone and *E. coli* with LAB than with control cells. (C) Detail of *E. coli* bacilli (rod-shape bacterium) with cells covered with cell debris (misshapen remnants of degraded organelles and other cell components) or detail of *E. coli* with LAB associated with cells with a normal appearance. Bar size for general view = 5 μm ; for the rest = 1 μm .

riod has been suggested to influence the occurrence of uterine infections in both cows (Cai et al., 1994; Hammon et al., 2006) and buffalos (Patra et al., 2013). The phagocytic capacity of neutrophils remains high throughout the peripartum period, but their killing capacity is impaired (Hussain, 1992).

Several studies demonstrate the capacity of *L. rhamnosus* to activate neutrophils (Seow et al., 2010; Kandasamy et al., 2011). Fish receiving a feed supplemented with *P. acidilactici* also presented elevated proportions of circulating neutrophils (Standen et al., 2013), and *L. reuteri* recruited neutrophils against pneumovirus in mice (Garcia-Crespo et al., 2013).

Because IL-8 is the main chemokine recruiting neutrophils, the increase in IL-8 concentration observed when the LAB probiotic combinations were applied to the explants (Figures 4 and 5) could be responsible for the

recruitment of neutrophils. This action, in combination with the previously demonstrated ability of LAB to reduce *E. coli* cfu, would protect the tissue against *E. coli* infection and consequent acute inflammation (with or without basal inflammation). Hence, the inflammatory response was less pronounced in LAB-treated and infected explants than in the explants infected but not treated with LAB (Figures 4 and 5). The LAB probiotics likely prepared the tissue pre-infection to efficiently respond against pathogens and avoid a high inflammatory response that could be deleterious for the uterus.

The FESEM images (Figures 6 and 7) demonstrated that no direct interaction occurred between the different LAB strains or with *E. coli* or between bacteria and endometrial cells, and that no biofilms formed. The presence of LAB may have reduced through competition mechanisms the direct interaction of *E. coli* with

the epithelial cells. The absence of biofilm formation or direct contact between LAB and *E. coli* suggests that the mode of action of LAB could be related to the presence of extracellular bioactive peptides and bacteriocins (important antimicrobial metabolites produced by LAB) (Hor and Liong, 2014). In this context, pediocin PA, a bacteriocin produced by *P. acidilactici*, has been described to inactivate *Listeria* and other food pathogens (Woraprayote et al., 2013). Also, a recent study showed that increased secretion of MUC3 mucin leads to reduced adhesion of enteropathogenic *E. coli* and enterohemorrhagic *E. coli* strains (Mack et al., 2003; Pan et al., 2013). The bacterial exopolysaccharide re-

uteran, synthesized by *L. reuteri*, has also been shown to reduce enterotoxigenic *E. coli* colonization of piglet jejunal epithelial cells (Zhang et al., 2015). Further studies will be carried out to demonstrate the involvement of those factors in the LAB protection against *E. coli* infection of bovine endometrium.

Cytokines and necrosis reflect the progression of inflammation (Perdomo et al., 1994; Ajonuma et al., 2010). In general, the loss of epithelial cells increases the risk of infections, inflammation, and other deleterious effects in the tissues (Perdomo et al., 1994). LAB treatment reduced mitochondrial damage in *E. coli*-infected cells compared to *E. coli*-infected cells without LAB

(Figure 8). Our ultrastructural findings are relevant because mitochondrial damage and cell death compromise the tridimensional organization of epithelial cells and hampers their barrier function, which is crucial for endometrium homeostasis (Bryant and Mostov, 2008). These results complemented our biochemical data because intracellular signaling pathways of inflammation actively participate in tissue homeostasis (Eritja et al., 2012) and may induce morphological changes (Saito et al., 2014).

CONCLUSIONS

A LAB combination consisting of *L. rhamnosus*, *P. acidilactici*, and *L. reuteri* in a ratio of 25:25:2 showed the best potential to modulate *E. coli* infection and endometrial inflammation in vitro compared with the individual LAB strains separately. Furthermore, the optimal LAB combination was able to reduce the secretion of inflammation markers (IL-8, IL-1 β , and IL-6) in the ex vivo acute inflammation model and in the ex vivo total inflammation model (IL-8 and IL-6). We also conclude that the physiological and morphological effects (namely proinflammatory cytokine production and necrosis) of the exposure of uterine cells to *E. coli* may be reduced in the presence of a combination of LAB. This ability to modulate and reduce or even neutralize the main pathological effects of *E. coli* infection demonstrates a promising potential of LAB probiotics as a therapeutic and preventive alternative against metritis in the periparturient cow.

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