Universitat Autònoma de Barcelona

Use of lactic acid bacteria as a preventive strategy against metritis in dairy cows

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MEMÒRIA PRESENTADA PER SANDRA GENÍS PAGÈS

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TUTORITZADA PER ANNA BASSOLS TEIXIDÓ

PER ACCEDIR AL GRAU DE DOCTOR DINS DEL PROGRAMA DE DOCTORAT DE BIOQUÍMICA, BIOLOGIA MOLECULAR I BIOMEDICINA DEL DEPARTAMENT DE BIOQUÍMICA I BIOLOGIA MOLECULAR

FACULTAT DE BIOCIÈNCIES

BELLATERRA, SETEMBRE 2016

Programa de doctorat en bioquímica, biología molecular i biomedicina

Use of lactic acid bacteria as a preventive strategy against metritis in dairy cows

Tesi doctoral, 2016

Departament de Bioquímica I Biologia Molecular
Facultat de Biociències
Universitat Autònoma de Barcelona

Producció de Remugants
Institut de Recerca i Tecnologia Agroalimentàries
Torre Marimon

Memòria presentada per **Sandra Genís Pagès**, per optar al grau de Doctor per la Universitat Autònoma de Barcelona

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Amb el vist-i-plau de la directora de la tesi i de la tutora

Dra. Anna Arís Giralt
Directora

Dra. Anna Bassols Teixidó
Tutora

Acknowledgements

Si algú m'arriba a dir fa uns anys que acabaria treballant amb vaques (dins i fora del laboratori), pensaria que s'havia donat un cop al cap. La vida dóna moltes voltes i mira, una cosa que no hagués provat mai ha acabat agradant-me.

Primer de tot, agrair a l'Anna haver confiat en mi per fer el doctorat i per tota la seva ajuda i suport. A l'Àlex, l'Elena, la Maria i la Marta per tota la seva ajuda estadística, de presentacions, i pels bons moments en general.

A tots els companys de remugants, els que encara hi sou: Bruna, Maria, Georgina, Marçal, Ramon, Laia, Cesc, Sílvia, Anna i Montse; i els que ja heu marxat: Cris, Gemma, Edu i Marine. Moltes gràcies per tots les bones estones, per les vaques voladores, les xerrades, les birres, i fins i tot...l'ajuda als escorxadors!

Thanks to the people from UBC, particularly the Repro team, I learnt a lot from you guys! Thanks for the pizza, the late office nights and the wine, especially the wine!

Gràcies als amics de sempre Anna, Alba, Blanca i Carlos per ser com sou i escoltar-me (i aguantar-me). A les "cuquis", per ampliar horitzons. A la Graci i a l'Abi i a la gent de la uni perquè tot sigui com sempre, encara que geogràficament sigui difícil.

Al pare, la mama i la Gala, gràcies per estar sempre allà, i la Carme per les seves preguntes.

Sense tots vosaltres, aquests anys no haguessin estat el mateix!

Aproximadament el 40% de les vaques lleteres desenvolupen una malaltia uterina durant el post-part que acaba provocant infertilitat. Alguns estudis indiquen que la infecció uterina, causada principalment per *Escherichia coli* durant la primera setmana post-part, està associada amb la metritis, caracteritzada per la inflamació de l'úter on la vaca és incapaç d'eliminar els bacteris patògens. El tractament antibacterià tradicional que s'utilitza per contrarestar la metritis pot no ser efectiu en tots els casos, sobretot quan hi ha una inflamació prolongada.

El primer estudi està enfocat en avaluar l'efecte de 4 possibles probiòtics del grup de bacteris de l'àcid làctic (BAL): *Lactobacillus rhamnosus, Pediococcus acidilactici, Lactobacillus sakei* i *Lactobacillus* reuteri, en un cultiu primari d'endometri amb infecció bacteriana i/o inflamació. Els principals resultats obtinguts van ser que *P. acidilactici* disminuïa la infecció *d'E.* coli, que *L. rhamnosus* reduïa significativament la inflamació cel·lular i que *L. reuteri* era capaç de disminuir la infecció d'*E. coli* quan les cèl·lules epitelials estaven prèviament inflamades. Durant el segon estudi es van provar 4 combinacions diferents de BAL a partir dels resultats obtinguts en el primer estudi. Es va avaluar la capacitat d'aquestes combinacions de disminuir la infecció d'*E. coli* i reduir la inflamació en el mateix cultiu primari. La combinació composta per *L. rhamnous/ P. acidilactici/ L. reuteri* amb una ràtio de 12/12/1 va ser la seleccionada. Llavors es va decidir comprovar que aquesta combinació continués sent eficaç en un model *ex vivo* (explants d'endometri). Els resultats obtinguts confirmaven la capacitat de la combinació de BAL de reduir la inflamació tissular. Per altra banda els assajos amb microscòpia electrònica mostraven un efecte protector de BAL en les cèl·lules epitelials. Hi havia menys necrosis, menys dany mitocondrial i més moc en les cèl·lules tractades amb BAL que en les no tractades. En el tercer estudi es va aplicar la combinació de BAL intravaginalment a vaques i al cap de 3 setmanes es va recol·lectar els seus endometris per obtenir-ne explants i infectar-los amb *E. coli ex vivo.* No es van observar diferències en els marcadors d'inflamació entre les vaques tractades amb BAL o les vaques control, ni en el número de *Lactobacillus* que hi havia a l'endometri de les vaques. Per altra banda, les vaques tractades amb BAL tendien a tenir menys presència d'*E. coli* a la vagina que les vaques control, i a més, expressaven menys *B-defensins i MUC1*, considerats marcadors d'infecció. Finalment, en el quart estudi, es van analitzar els efectes *in vivo* de la

combinació de BAL sobre la incidència de metritis i inflamació de l'endometri en vaques de llet quan s'administrava intravaginalment durant 3 setmanes pre-part i o a l'úter un dia post-part. Els principals resultats obtinguts van ser que el tractament vaginal reduïa un 58% la prevalença de metritis comparada amb el grup control mentre que el tractament endometrial no la variava. No es va observar cap diferència amb els marcadors d'inflamació però els dos tractaments disminuïen l'activitatneutrofílica.

Approximately 40% of dairy cows develop a uterine disease during the post-partum leading to infertility. Several studies indicate that uterine infection, mainly caused by *Escherichia coli* during the first week post-partum, is associated with metritis, characterized by inflammation in the endometrium where the cow is not able to clear pathogenic bacteria. The traditional antimicrobial treatment may lack efficacy, especially in cases of sustained inflammation.

The first study is focused in the evaluation of 4 possible probiotics belonging to the lactic acid bacteria (LAB): *Lactobacillus rhamnosus, Pediococcus acidilactici, Lactobacillus sakei,* and *Lactobacillus reuteri*, in an endometrial primary culture against bacterial infection and inflammation. The main results were that *P. acidilactici* was able to reduce *E. coli* infection, *L. rhamnosus* diminished cellular inflammation, and *L. reuteri* reduced *E. coli* infection when the epithelial cells were inflammated. On the second study, 4 different LAB combinations based on the results of the first study, were tested using the same primary culture. The combination composed by *L. rhamnosus/ P. acidilactici/ L. reuteri* with a ratio of 12/12/1 was selected. Then, this combination was tested in an *ex vivo* model (endometrial explants). The obtained results confirmed the capacity of this LAB combination to reduce tissular inflammation. On the other hand, electron microscopy assays showed a protective effect of LAB in endometrial epithelial cells. There was less necrosis, mitochondrial damage, and more mucus in the surface of LAB-treated cells than not-treated cells. In the third study, LAB combination was applied *in vivo* in the vagina of several cows, and 3 weeks later, the endometrium of those animals were collected. Explants were made from the endometrium and then infected with *E. coli.* No differences were observed in the inflammation markers between LAB-treated and control cows, or in the final quantification of *Lactobacillus* in the endometrium. On the other hand, LAB-treated cows tended to have less presence of *E. coli* in the vagina than control cows and, moreover, they expressed less *B-defensins* and *MUC1*, considerate markers of infection. Finally, on the fourth study, the effects of LAB combination were analyzed *in vivo* quantifying metritis prevalence and endometrial inflammation in dairy cows when the LAB combination was applied intravaginally during 3 weeks pre-partum or intra-uterine, 1 day after calving. The main results were that the vaginal treatment reduced metritis prevalence up to 58% compared with the control cows while no differences were

observed with the endometrial treatment. No differences were found in the inflammation markers whereas both treatments (vaginal and endometrial) were able to modulate neutrophilic activity.

List of abbreviations

AIEC	Adherent-invasive *Escherichia coli*
AMP	Antimicrobial peptides
BHB	β-hydorxybutyric
CFU	Colony-forming units
DEC	Diarrhoeagenic *Escherichia coli*
DIM	Days in milk
DMEM	Dulbecco's modified eagle medium
DMI	dry matter intake
EAEC	Enteroaggregative *Escherichia coli*
EAF	EPEC adherence factor
EGF	Epithelial growth factor
EHEC	Enterohemorragic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
EnPEC	Endometrial pathogenic *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
ER	Endoplasmatic reticulum
ETEC	Enterotoxic *Escherichia coli*
ExPEC	Extra-intestinal *Escherichia coli*
FESEM	Field emission scanning electron microscopy
FimH	Fimbrae H
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GH	Growth hormone
GHR	Growth hormone receptor
IGF-1	Insuline growth factor 1
IL1R	Interleukin 1 receptor
IL-1β	Interleukine 1 β
IL-6	Interleukine 6
IL-8	Interleukine 8
IR	Insulin resistance
IV	intravenous
LAB	Lactic acid bacteria

LB	Luria Bertrani media
LDH	Lactate dehydrogenase
LPS	lipopolysaccharide
LRR	Leucine-reach repeat
MPEC	Mammary pathogenic *Escherichia coli*
MR	Mannose receptors
MRS	Man, Rogosa, and Shape media
NEB	negative energy balance
NEFA	non-esterified fatty acids
NF-κB	nuclear factor kappa-light-chain -enhancer of activated B cells
NMEC	Neonatal meningitis-associated *Escherichia coli*
NOD	Nuclotide-binding oligomerization-domain protein
O/N	Overnight
PAMP	Pathogen-associated molecular patterns
PAP	P fimbria
PB	Phospate buffer
$PGF_{2\alpha}$	Prostanglandin F2α
plo	pyolysin
PMN	Polymorphonuclear leukocytes
PRR	Pattern-recognition receptors
ROS	Reactive oxygen species
RP	Retained placenta
RPMI	Roswell Park Memorial Institute medium
RT	Rectal temperature
SR	Scavenger receptors
Stx	Shiga toxins
TEM	Transmission electron microscopy
TIR	TOLL/IL1R homology domain
TLR-4	Toll-like receptor 4
TNFα	Tumor necrosis factor α
UPEC	Uropathogenic *Escherichia coli*
WHO	World health organization

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CHAPTER I

General introduction

Block 1: Transition period in dairy cows

The transition period for a dairy cow is from 3 weeks prepartum until 3 weeks postpartum [1]. The term *transition* involves the important physiological, nutritional, and metabolic changes occurring during this period of time. It constitutes a turning point in the productive cycle of a cow from one lactation to the next. How these changes occur and how they are managed, is an issue of great importance because impacts on the lactation performance, clinical and subclinical postpartum diseases, and reproductive efficiency affecting profitability. Health problems during the transition period are clearly a major complicating factor for subsequent reproduction performance, resulting in economic losses. Poor transition also leads to milk income losses.

Calving and the onset of lactation lead to a sudden increase in the energy requirements of the dairy cow. This time is also characterized by a drop in feed intake. During the dry off, cows dry matter intake will be approximately 2% of their body weight. One week before calving, dry matter intake drops to about 1.25% of body weight [2, 3]. This change is believed to be due, in part, to high levels of estrogen prior to calving [4]. Although feed intake decreases, nutrient requirements are high due to calf growth and preparation for milk production. Feed intake after calving is usually less than what they require in spite of the nutrient demands of milk synthesis. This reduction in feed intake is related to physical, behavioral, hormonal and metabolic changes around parturition [5].

This imbalance between the energy that the cow consumes and the energy needed for production demands is termed negative energy balance (NEB). Cows cannot consume sufficient energy-yielding nutrients from voluntary dry matter intake (DMI) after calving to meet energetic requirements for milk production. Consequently, NEB occurs for a period of days to weeks during early lactation [6].

Cows adapt to NEB periods by moving fat reserves through a process known as lipid mobilization. Dairy cows mobilize adipose energy depots and turn skeletal muscle into the major site for use of fat-derived fuels, such as non-esterified fatty acids (NEFA) and ketone bodies, allowing glucose to be redirected for fetal metabolism and lactose synthesis [7].

Metabolic adaptations such as lipid mobilization are accompanied by alterations in inflammatory responses that modify immune function. Lipid mobilization is a physiological adaptation that mammals acquired to survive times of reduced nutrient and energy availability. It is defined as an imbalance between lipogenesis and lipolysis within the adipose tissue [5].

1.1 Non-esterified fatty acids (NEFA) in Immune system

There are several proposed mechanisms by which elevated NEFA can regulate the inflammatory response benefiting or damaging the host. Changes in the concentration and the composition of plasma NEFA directly affect white blood cell function. A first way to modify immune function is by changing the composition of the cellular membrane of blood cells [8]. In animal cells, this membrane is composed of phospholipids. These molecules are formed by different kinds of fatty acids, including saturated, monounsaturated, and polyunsaturated. Fatty acids together with some proteins form a bilayer that surrounds and protect the cells. The fatty acid composition of the cellular membrane is directly affected by the composition of lipids in blood, especially NEFA. Therefore, any change in the content of blood NEFA will be reflected directly in the phospholipid membrane of the white cells [8].

Excessive adipose stores and elevated NEFA concentrations are positive risk factors for several pro-inflammatory periparturient diseases in dairy cows such as mastitis and metritis [9]

In addition to potential hepatic metabolic disease associated with NEB, as ketosis, periparturient dairy cows also undergo a period of reduced immunological capacity during the weeks around calving. The immune dysfunction is not limited to isolated immune parameters; rather is a broad scope and affects multiple functions of various immune cell types. Fat releases NEFA but also glycerol and proinflammatory cytokines such as TNF-α or IL-6. This increment of circulating NEFA can activate the "inflammasome" releasing IL-1β. Then, NEFA can also activate TLR-4, a main receptor for LPS (lipopolysaccharides), which activates NF-κB and leads to the secretion of TNF-α, IL-1β, and IL-8. TNF-α, IL-1β act as intracellular messengers to up-regulate inflammation and increase insulin resistance (IR)[10] .

IR is a physiological condition in which body tissues have lower response to insulin [11] and during the postpartum, dairy cows go through a period or IR as part of the changes they go through to produce milk. Growth hormone (GH) increases near parturition, promoting mobilization of stored nutrients (basically fat from adipose tissue) decreasing insulin concentration to support milk production [12]. This reduction of insulin favors the drain of glucose to the udder. In normal conditions, GH promotes the secretion of insulin-like growth factor (IGF-1) from the liver, but 2 days before calving, GH receptor in the liver is reduced and its expression remains low for 2 weeks. Without IGF-1 to feed back against GH secretion from the hypothalamus, circulating GH levels are high, favoring an increment of NEFA in blood. As NEFA may be used as an alternative source to glucose in peripheral tissues or incorporating directly to milk fat, elevated NEFA contributes to IR (Figure 1)[10].

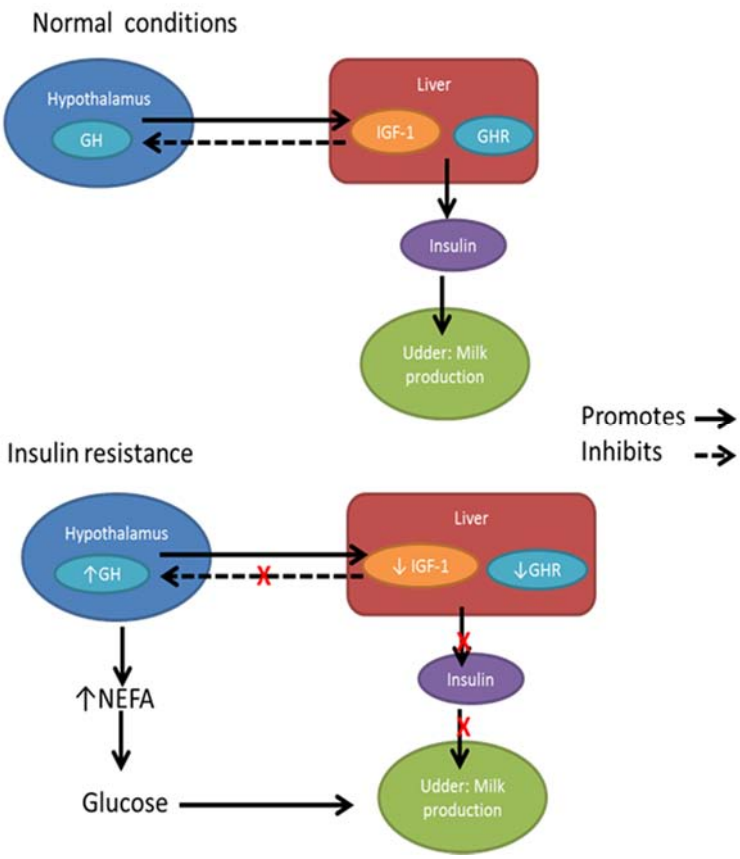


Figure 1: Insulin resistance diagram. In normal conditions, GH binds to GHR in the liver, increasing IGF-1 that results in the synthesis of insulin that will be used for milk production. In the insulin resistance model, GHR expression in the liver is reduced, then there is low IGF-1 to feed back against GH secretion from the hypothalamus, GH levels are high increasing NEFA concentration. NEFA is used as an alternative fuel source to glucose in peripheral tissues or incorporated directly into milk fat.

1.2 Normal postpartum events

The events that must happen after calving, before a cow is likely to conceive again are uterine involution, regeneration of the endometrium, elimination of bacterial contamination of the uterus and return of ovarian cyclical activity [13].

The initial stimulus for these changes is the expulsion of the fetus along with the associated membranes and fluids at calving. Uterine involution is associated with a progressive decrease in vaginal discharge and in uterine and cervical diameters. The cervix decreases from 30 cm immediately after parturition to approximately 2 cm by day 7 postpartum [14]. The decrease in the uterine diameter follows a similar pattern to the cervical diameter though the cervix usually involutes more slowly than the uterus [15]. Epithelial regeneration is complete by about 25 days after calving, but the deeper layers of the tissues are not fully restored until 6-8 weeks after parturition. The normal uterus is able to efficiently clear bacterial infection, by 3-4 weeks postpartum, the number of bacteria and the variety of species has diminished substantially in healthy cows. The genital tract should have little evidence of the previous pregnancy by 6 weeks after calving and be capable of establishing the next pregnancy.

1.3 Peripartum diseases

Disease incidence is the highest in the 30 days immediately after calving, and 30 to 50% of high-producing cows may be affected by some disease around parturition [16] increasing with age due to the increased occurrence of production related diseases [17]. All peripartum dairy cattle experience a period of insulin resistance, reduced feed intake, negative energy balance, lipolysis, and weight loss in early lactation. Furthermore, levels of calcium in blood are low after calving; the immune function is reduced from 1-2 weeks before until 2-3 after parturition, plus there is bacterial contamination of the uterus for 2-3 weeks after calving [16]. All these factors contribute to the apparition of the following diseases:

1.3.1 Hypocalcemia:

Hypocalcemia is a common metabolic disorder that occurs at the onset of lactation. The dysregulation of calcium at the onset of lactation is largely a result of a dairy cow's

inability to manage maternal calcium homeostasis while secreting calcium into the milk [18]. Blood calcium concentration is around 2.5 mM but when lactation starts the colostrum has a calcium concentration of 62-75 mM and milk of 25-30 mM [19]. This represents a ≈30-10 fold difference and this demand for calcium from the mammary gland leads to depletion of circulating and maternal calcium stores, often provoking periparturient hypocalcemia.

Hypocalcemia can be a clinical (total blood calcium below 6 mg/dL) or subclinical (below 8 mg/dL) disease [20]. It can have its clinical manifestation between 24 hours pre-natal until 72 hours after delivery [21]. Intravenous administration of Ca (usually 8 to 10 g Ca in the form of Ca borogluconate) are used to keep the cow alive long enough for intestinal and bone Ca transport mechanisms to adapt [22]. In its clinical form, hypocalcemia is affecting 3.45% of North American cows. It reaches 6.17% in Europe and 3.5% in Australia [23]. Taking subclinical cases into account, the incidence of subclinical hypocalcemia is 50% in older cows [20].

Subclinical hypocalcemia can be present for the first 40 days after a normal delivery in cows with high milk production [24]. In these cases, the alterations of blood calcium levels are lower, but also dramatically affect the health and welfare of animals. There are no clinical signs of the hypocalcemia itself, but food intake decreases and muscle contraction can be reduced, affecting ruminal, intestinal and abomasal mobility [20].

Hypocalcemia is associated with an increased incidence of displaced abomasum [25], uterine prolapsed and retention of fetal membranes [26]. It has also been linked with higher incidence of metritis and mastitis due to the reduction in the immune cell functions [27].

1.3.2 Ketosis:

Ketosis is a common disease of adult cattle. It typically occurs in dairy cows in early lactation and is t is characterized by an intense adipose mobilization and a high glucose demand. Adipose mobilization is accompanied by high blood serum concentrations of non-esterified fatty acids (NEFAs). During periods of intense gluconeogenesis, a large portion of serum NEFAs is directed to ketone body synthesis in the liver. Thus, the

clinicopathologic characterization of ketosis includes high serum concentrations of NEFAs and ketone bodies and low concentrations of glucose. Prevalence of ketosis during the first 6 weeks postpartum is between 7-14%, where the peak prevalence is during the first 2 weeks. The clinical diagnosis of ketosis is based on presence of risk factors (early lactation), clinical signs, and ketone bodies in urine or milk. BHB (β-hydroxybutyric) concentrations in milk may be measured by a dipstick method that is available in some countries, or by an electronic device. Treatment of ketosis is aimed at reestablishing normoglycemia and reducing serum ketone body concentrations. Bolus IV administration of 500 mL of 50% dextrose solution is a common therapy [28]. Cows with ketosis have 2.0 times more risk of metritis, cystic ovarian disease and left displaced abomasum [29].

Several authors found that ketosis was a risk factor for endometritis particularly in multiparous cows [30, 31].

1.3.3 Abomasum displacement:

The abomasum (or true stomach) normally lies on the floor of the abdomen, but can become filled with gas and rise to the top of the abdomen, when it is said to be 'displaced'. The abomasum is more likely to be displaced to the left (LDA) than the right (RDA). The majority of cases occur soon after calving. During pregnancy the uterus displaces the abomasum, so that after calving the abomasum has to move back to its normal position, increasing the risk of displacement. The symptoms are loss of appetite, reduction in milk yield, reduction in rumination time, and mild diarrhea. Treatment requires replacing the abomasum in its normal position. Preferably, the veterinarian also prevents recurrence by tacking the abomasum to the body wall.

Surgery can be performed, however is not always necessary. Often the abomasum can be returned to its usual place by casting and rolling the animal onto its back, permitting the abomasum to "float" back into its normal position.

1.3.4 Peripartum uterine diseases:

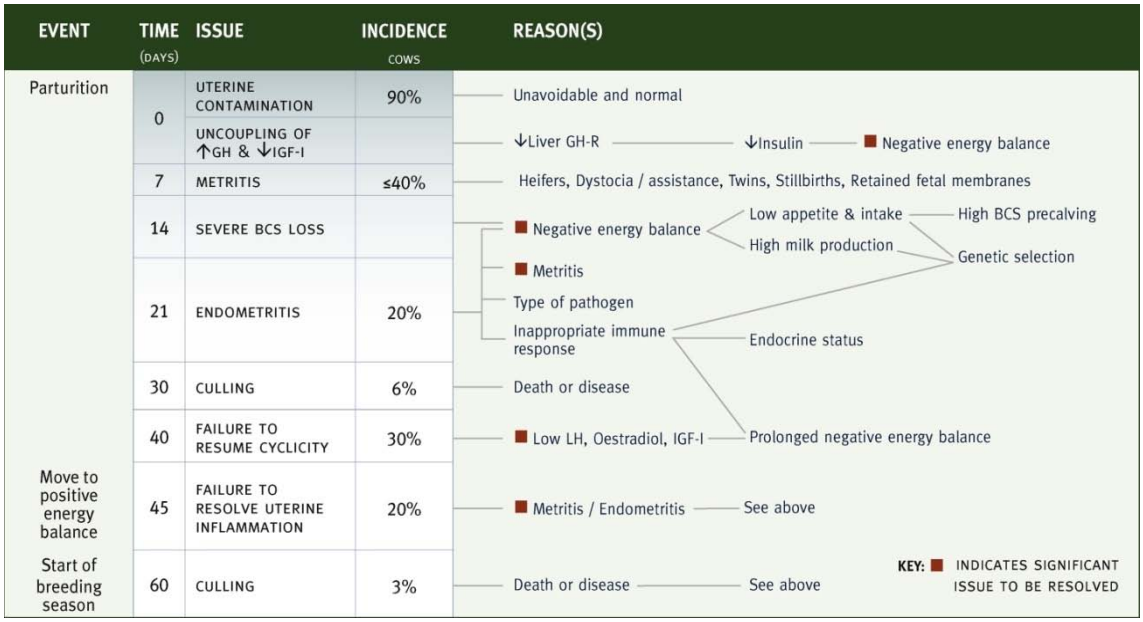
The resolution of post-partum uterine infection and inflammation has been identified as one of the most important events that has to happen for the establishment of successful

pregnancy in dairy cattle [32]. Uterine contamination at parturition or in the following days is unavoidable and normal with 80-100% of animals having bacteria in the uterine lumen in the first 2 weeks post-partum [33]. Pathogenic bacteria frequently persist, causing uterine disease. Although many of these bacteria are eliminated during the first 5 weeks post-partum, around 40% of this animals develop clinical disease in the uterus [34]. On average, 35% of cattle develop acute clinical uterine disease within a week of parturition (metritis), 20% have a persistent clinical disease 3 weeks after calving (endometritis) and approximately 30% have a chronic subclinical inflammation of the uterus (subclinical endometritis)[34-36].

The uterus of post-partum cows is usually contaminated (that is different from infected) with a range of bacteria, but this is not consistently associated with clinical disease. Infection implies the adhesion of pathogenic organisms to the mucosa, colonization or penetration of the epithelium, and/or the release of bacterial toxins that lead to establishment of uterine diseases. Depending on the immune response of the cow and the species and number (load or challenge) of the bacteria, a uterine disease will be developed.

The most typical uterine diseases are the following: retained placenta, metritis, and endometritis.

Figure 2: Major problems in diary cows in the postpartum period. Schematic presentation of time, issue, incidence and reasons of uterine diseases [32].



1.3.4.1 Retained placenta:

Retained fetal membranes or, retained placenta (RP), is the failure to pass the placenta within 24h postpartum [37]. Some authors define RP at 12h instead of 24, as 95% of the cows expel the placenta during the 12 first hours so no distinction is required. If the placenta is retained, it will take an average of 7 days to be finally expelled. The incidence in healthy dairy cows is 5-15%. This incidence is increased by abortion, dystocia, twin birth, stillbirth, hypocalcemia, heat stress, increasing age, premature birth or induction of parturition, placentitis, and nutritional disturbances [38]. At the same time, cows with RP have an increased risk for metritis, displaced abomasum, and mastitis [39-41].

Retention of fetal membranes is mediated by impaired migration of neutrophils to the placental interface in the periparturient period. The decreased neutrophil function extends into the postpartum period and probably mediates the recognized complications of RP.

Diagnosis is usually straightforward as degenerating, discolored, ultimately fetid membranes are seen hanging from the vulva <24h after calving. No signs of systemic illness are usually found. Manual removal of RP is not recommended and is potentially harmful [42, 43]. Untreated cows expel the membranes in 2-11 days. It is not clear if all the cases need systemic antibiotic treatment but oxytocin, estradiol, $PGF_{2\alpha}$, and oral calcium preparations are frequently used.

1.3.4.2 Metritis:

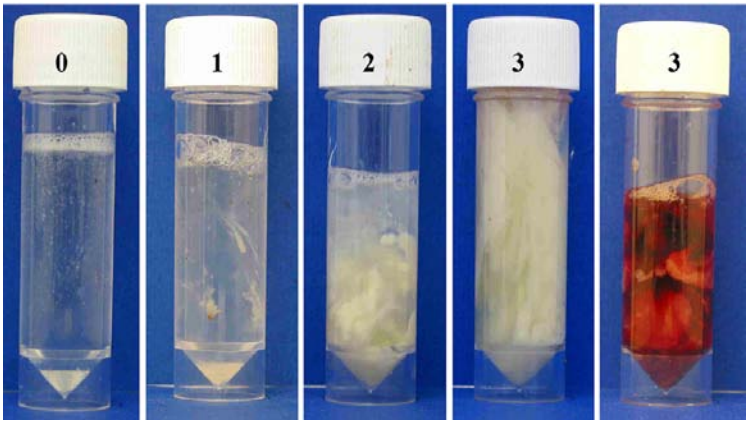
Clinical metritis is inflammation of uterus, usually due to bacterial infection, occurring within 21 days (most commonly within 10 days) after parturition. It is characterized by systemic signs of sickness, including fever, red-brown watery foul-smelling uterine discharge, dullness, inappetance, elevated heart rate, and low production [33]. Metritis affects up to 40% of dairy cows and the total estimated cost of a single case of metritis is about 317€ taking in account direct and indirect costs [44]. Direct costs are treatment costs and reduction in milk production. In the other hand, indirect costs involve increment in number of inseminations, increase at calving interval and, culling increase.

Some studies calculated that the annual cost of uterine disease in the European Union is €1.4 billion and in the United States is $650 million [45].

Microbial contamination of the uterus occurs shortly after parturition by a number of opportunistic bacteria, including *Escherichia coli, Trueparella pyogenes,* and the anaerobes *Prevotella* sp,. *Fusobacterium necrophorum,* and *Fusobacterium nucleatum*. *E. coli* is particularly prevalent in the first week postpartum and is associated with metritis, with increased risk of infection with *T. pyogenes* in weeks 2 and 3, and with endometritis [10]. Cows with severe metritis ate 2-6 kg dry matter less than healthy cows in the 2-3 weeks preceding the clinical signs of metritis [46]. The largest risk factor for metritis is RP, but other conditions that may impair feed intake and immune function also increase the risk of metritis. It is generally considered that bacterial contamination of the uterus combined with the tissue repair due to calving, initiates inflammation of the endometrium and in occasions, deeper layers of the uterus [47, 48]. This sustained inflammation may be attributable to the reduce immune function before calving [49].

It is very important to diagnose and treat metritis early in the post-calving period. Every cow should receive a postpartum examination early in lactation (first 10 days) as part of routine heard health program. Clinical metritis can be diagnosed by presence of a >50% pus in a purulent vaginal discharge and fever > 39.5.

Figure 3: Vaginal discharge scale. Grade 0 (clear or translucent mucus), 1 (mucus containing flecks of white or off-white pus), 2 (exudate containing < 50% pus) or 3 (exudate containing ≥ 50% purulent material, usually white or yellow but occasionally sanguineous)[33].



Because of metritis infectious nature, antibiotics are considered beneficial for its treatment. These antibiotics are given by intrauterine route, systemically, or both. Dairy

cows with metritis have been treated with systemic penicillin or ampicillin administrations in conjunction with intrauterine instillations of oxytetracycline or ampicillin and cloxacillin [50].

The innate immune response to bacteria is key to rapidly clearing infection [51]. Recruitment of hematopoietic immune cells and the inflammatory response, including secretion of cytokines and chemokines, all combine to clear the bacterial infection and restore hemostatic function of the endometrium. When the physical barriers (cervix and mucosal mechanisms) are breached during parturition, bacterial pathogens can rapidly invade the uterus to establish infection. This can result in clinical disease if cellular and humoral defense mechanisms are overwhelmed [52].

1.3.4.3 Endometritis:

Endometritis is inflammation of the uterus without systemic illness. It is characterized by muco-purulent uterine discharge associated with chronic bacterial infection of the uterus, occurring later than 3 weeks postpartum. Histologically, endometritis is characterized by disruption of the endometrial epithelium, infiltration of inflammatory cells and accumulations of lymphocytes, vascular congestion, and stromal edema [53]. Diagnosis of subclinical (cytologic) endometritis requires use of endometrial cytology, ultrasonography, or endometrial biopsy, because other signs are often absent.

The prevalence of endometritis in 43 studies ranged from 2.2% to 37.3% [37], the main problem is that many studies do not define explicitly endometritis. Endometritis increase mean days open by 15, decrease the relative risk of pregnancy by 150 DIM by 31%, and reduce the rate at which cows become pregnant by 16% [54]. As uterine inflammation occurs in all cows during uterine involution, the factors responsible for failure to resolve the endometrial inflammation at the start of the breeding period seem to be critical. Detection and treatment of suffering animals must be detected as soon as possible but on the average dairy farm, disease detection is done by a veterinarian and only during routine herd health checks. That means that in many cases, early warning signs of disease go unnoticed until the disease is in its full clinical stage, making it more difficult to treat. As a result chronic endometritis may still be present at the moment that cows should become pregnant [29]. The costs of endometritis include reproductive inefficiency,

culling, treatment cost, milk discard, labor, and increased risk of residues in food products.

A wide variety of therapies have been reported, including systemically or locally administrated antibiotics, or systemically injected $PGF_{2\alpha}$. The principle of the endometritis' therapy is to reduce the load of pathogenic bacteria and enhance uterine defense and repair mechanisms, and thereby halt and reverse inflammatory changes impairing fertility [53].

Block 2. Bacteria causing metritis

Uterine infection was most commonly associated with the presence of *Escherichia coli*, *Trueperella pyogenes, Fusobacterium necrophorum,* and *Prevotella or Bacteroides* species.

E. coli infections are mostly found during the first days / week after calving, this germ has been thought to pave the way for subsequent infections with other bacteria or viruses. Nowadays, there is a lot of research going on to find out whether there are typical *E. coli* strains bearing specific virulence factors involved. On the other hand, *T. pyogenes* often in combination with some of the above mentioned anaerobes, causes the most severe endometrial lesions [41].

2.1 *Escherichia coli*

E. coli is a Gram-negative, facultative anaerobic, rod-shaped bacterium which has high diversity of subspecies that goes from commensal bacteria to highly adapted pathogens.

Pathogen species cause a huge variety of diseases such as extra-intestinal infections, gastroenteritis and uropathogenic diseases. There are 6 different types of strains, also known as virotypes that typically cause diarrhea (DEC), differentiated by their pathogenic capacity:

- Enteropathogenic *E. coli* (EPEC): induces a profuse diarrhea. Pathogenesis of EPEC involves a plasmid-encoded protein referred as EPEC adherence factor (EAF) that enables localized adherence of bacteria to intestinal cells

and non fimbrial adhesin (intimin), which is an outer membrane protein that mediates the final steps of adherence.

- Enterotoxigenic *E. coli* (ETEC): it also induces diarrhea. The main feature is the production of fimbria and enterotoxins.

- Enteroinvasive *E. coli* (EIEC): penetrates and multiply with epithelial cells of the colon causing widespread cell destruction.

- Enterohemorragic *E. coli* (EHEC): It causes bloody diarrhea. It is characterized by the production of Shiga toxins (Stx). It is considerated as moderately invasive.

- Enteroagreggative *E. coli* (EAEC): its distinguishing feature is their ability to attach to tissue culture cells in an aggregative manner.

- Adherent-invasive *E. coli* (AIEC): They invade the host intestinal epithelial cells and multiply, causing destruction and acute inflammatory response.

On the other hand, there are several varieties that can be found outside the gut, they are known as Extra-intestinal *E. coli* (ExPEC). *E. coli* strains isolated from infections outside the intestinal tract are:

- Uropathogenic *E. coli* (UPEC): The bacteria colonize from the faces or perineal region and ascend the urinary tract to the bladder. The adhesin most associated with UPEC is the P fimbria (PAP).
- Neonatal meningitis-associated *E. coli* (NMEC): *E. coli* strains invade the blood stream. The K-1 antigen is considered the major determinant of virulence among these strains of *E. col*

Two specific animal pathogenic subgroups have been proposed:

- Mammary pathogenic *E. coli* (MPEC) [55]
- Endometrial pathogenic *E. coli (*EnPEC) [56]

2.1.1 Endometrial pathogenic *E. coli* (EnPEC):

It is assumed that the infection with Gram-negative *E. coli* is the first step in the process to develop metritis in dairy cows. There is a wide genetic diversity of *E. coli* in the environment and feces. So the most common assumption was that these genetically diverse fecal *E. coli* randomly contaminate the endometrium to cause metritis. However, it has been demonstrated that strains of DEC and ExPEC, are able to infect tissues different than the endometrium. Sheldon et al. demonstrated in 2010 a different type of *E. coli* strain, named Endometrial pathogenic *E. coli* (EnPEC).

EnPEC is more adherent and invasive for endometrial epithelial and stromal cells, compared with other strains of *E. coli*, isolated from the uterus of clinically unaffected animals. The endometrial epithelial and stromal cells produced more prostaglandin E_2 and IL-8 in response to lipopolysaccharide (LPS) purified from EnPEC compared with non-pathogenic *E. coli*. LPS seems to be the major virulence factor of EnPEC, stimulating TLR4-dependent inflammatory responses by endometrial cells [57]. The EnPEC causes metritis when infused into the uterus of mice with accumulation of neutrophils and macrophages in the endometrium. Its infusion, is only associated with bacterial invasion of the endometrium and myometrium [56, 58]. EnPEC tend to be phylogenetically distant from the majority of ExPEC, and more closely related to human intestinal pathogens [59]. However, the EnPEC have acquired, via horizontal transfer, DNA encoding iron acquisition systems and a virulence plasmid, similar to that found in several ExPECs. On the other hand, EnPEC have few virulence factors typical of enteric *E. coli*, although they possess the gene encoding the *FimH* adhesion factor, and it fosters adhesion of EnPEC to endometrial cells [58, 60].

2.2 *Trueperella pyogenes:*

T. pyogenes also previously known as *Corynebacterium pyogenes, Actinomyces pyogenes,* and *Arcanobacerium pyogenes*, is a non-motile facultative anaerobic Gram-positive bacterium. Is commonly found in the urogenital, gastrointestinal, and upper respiratory tracts of cattle, goats, horses, musk deer, pigs, and sheep, causing abscesses, mastitis, metritis, and pneumonia. It usually invades after physical or microbial induced trauma.

It is associated with the severity of postpartum endometritis in cattle, and causes uterine disease when infused into the uterus of cattle [61-63]. Some authors postulated that the most severe endometrial lesions are caused by *T. pyogenes* [34, 47]. All the strains isolated from the uterus express *plo* [64], that encodes a cholesterol-dependent cytotoxin called pyolysin, which is both a host-protective antigen and a virulence factor. Cholesterol-dependent cytotoxin molecules are attracted to cholesterol-rich domains in cell membranes, where they aggregate to form a pore, leading to osmotic death of the cell [65]. It has also been proved the capacity of pyolysin to kill endometrial epithelial and stromal cells *in vitro*.

2.3 *Fusebacterium necrophorum:*

F. necrophorum is a road-shaped species of Gram-negative bacteria. Is an obligate anaerobe and is a common inhabitant of the alimentary tract of humans and animals. Along with *T. pyogenes,* and *Prevotella* sp. act synergistically to enhance the likelihood and severity of uterine disease [66]. It is recognized to be highly relevant in the establishment of uterine infections in cattle [67].

2.4 *Prevotella* species:

Prevotella is a genus of Gram negative bacteria, and are members of the oral and vaginal flora. It has been isolated from cows with metritis. *Prevotella* has been linked with a more severe clinical disease and increased endometrial inflammation [68].

2.5 *Bacteroides* species:

Bacteriodes is a genus of Gram negative, obligate anaerobic bacteria. They are part of the mammal gastrointestinal flora but some species have been described as opportunistic human pathogens. It has been isolated from the uterus of metritic cows being found that metritic cows increase *Bacteroides* population in spite of *Proteobacteria* [69].

Block 3: Innate immune response

3.1 Immune system:

The physiologic function of the immune system is to defend against infectious microbes. However, even noninfectious foreign substances can elicit immune responses. Even

more, mechanisms that normally protect individuals from infection and eliminate foreign substances are themselves capable of causing tissue injury and disease in some situations. So, in summary, immunity is a reaction to foreign substances, including microbes, as well as macromolecules such as proteins and polysaccharides, regardless of the physiologic or pathologic consequence of such reaction.

Immunity is divided in two parts determined by the speed and specificity of the reaction. These are named the innate and the adaptive responses, although in practice there is much interaction between them.

The innate response provides immediate defense against infection while the adaptive immunity is the response from the immune system that is stimulated by exposure to infectious agents and increase in magnitude and defensive capabilities with each successive exposure to a particular microbe. In this study we will focus in the innate immunity response [70].

3.2 Innate response:

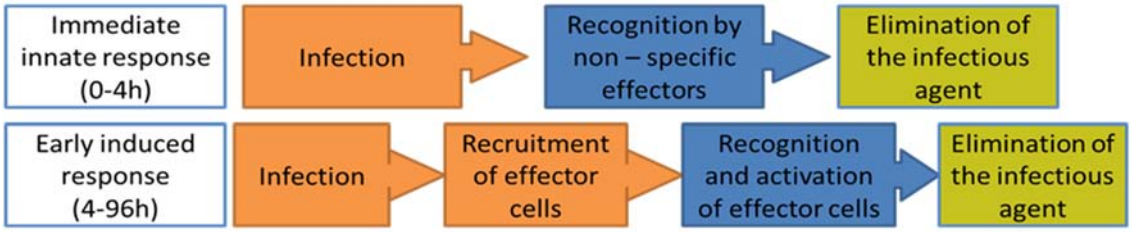
The term innate immunity, also known as non-specific immune system, is used to include physical, chemical, and microbiological barriers, but more usually encompasses the elements of the immune system (neutrophils, monocytes, macrophages, complement, cytokines, and acute phase proteins) which provide immediate host defense [71].

It is a rapid response as it is the first line of defense, because of that it is not selective, not targeting specific species of invaders.

Microorganisms not always cause diseases. Most of them are detected and eliminated in minutes or hours by defense mechanisms that do not require a long period of induction to be activated. Those are the mechanisms belonging to the innate response.

The innate immunity mechanisms act immediately and are followed by the early induced response, that are activated by the infection, but do not provide long term immunity protection (Figure 4) [72].

Figure 4: Innate immune response diagram



3.2.1 Immediate innate response:

The elements of the immediate innate response include anatomical barriers and secretory molecules. Among the mechanical anatomical barriers are the skin and internal epithelial layers, the movement of the intestines and the oscillation of broncho-pulmonary cilia.

The epithelial surfaces form a physical barrier that is very impermeable to most infectious agents, acting as the first line of defense against invading organisms. The contact with the microorganisms may be produced through external or internal epithelial surfaces: gastrointestinal, respiratory and urogenital tracts [73]. The epithelial cells are efficiently sealed by tight junctions that separate and protect from the external media. Mucosal membranes are surrounded by mucus, which is a viscous fluid with glycoproteins named mucins. Bacteria and other microorganism are trapped inside the mucus and the infection may be prevented or retarded [74].

There are several types of direct antimicrobial proteins and peptids secreted by epithelial cells or other immune mediators.

3.2.2 Early induced response:

When a microorganism goes through the epithelial barrier and starts to replicate inside the host tissue, it is usually recognized by tissue-resident macrophages or neutrophils. Both phagocytes have a key role in the innate immunity as they are able to recognize, ingest and destroy most of the pathogens without the help of the adaptive immune response [70].

3.2.3 Inflammation:

Inflammation is an evolutionarily conserved response underlying many physiological and pathological processes. In response to stimuli associated with infection and tissue injury, components of innate and adaptive immunity initiate coordinated responses and trigger inflammation [75]. In normal conditions, inflammation will help the body to adapt and overcome adverse stimuli, reaching finally homeostasis.

Two types of inflammation have been lately described: acute and subacute inflammation.

3.2.3.1 Acute inflammation:

Classic signs of inflammation are redness, swelling, heat and pain. In response to acute inflammatory stimuli, the body increases the expression and release of inflammatory mediators including cytokines, chemokines, adhesion molecules, eicosanoids, and complement proteins. These molecules form regulatory networks that promote increased blood flow to the infected tissue, immune cell infiltration and activation, and systemic responses, including increased body temperature, increased heart rate, and decreased appetite. If Gram-negative bacteria infects the tissue, LPS released from the bacterial outer membrane is the main pathogen component initiating inflammatory responses, triggering the production of pro-inflammatory cytokines, that will start the migration of leukocytes to the site of infection [76]. To be more specific, this response is triggered by receptors of the innate immune system, such as Toll-like receptors (TLRs) and NOD (nucleotide-binding oligomerization-domain protein). This recognition is mediated by tissue-resident macrophages and mast cell which will release cytokines and chemokines attracting primarily neutrophils. Once the neutrophils reach the tissue, they will be activated by direct contact with the pathogens or through the actions of cytokines, and they will release the toxic content of their granules. If the immune response has been successful, the infectious agent has been eliminated [75].

3.2.3.2 Subacute inflammation:

Subacute or chronic inflammation causes mild increases in inflammatory mediators that contribute to chronic and progressive changes in tissue function [76]. Pathologic inflammation appears to differ from physiologic inflammation in severity and duration. Several evidences indicate that the same inflammatory effectors are involved in disease

as in physiologic inflammation, only changing in the magnitude, regulation, duration and effectiveness of the response [10].

3.2.4 Bacterial recognition:

There are two types of microbial inducers: pathogen-associated molecular patterns (PAMPs) and virulence factors.

The innate immune system recognizes microorganisms via a limited number of pattern-recognition receptors (PRRs). PRRS are able to recognize microbial components, the PAMPs that are essential for the survival of the microorganism and, in consequence, difficult for the microbe to alter.

Different PRRs react with specific PAMPs, showing different expression pattern, activating specific signaling pathways, which lead to distinct antipathogenic responses.

Some of the most important PRRs that are involved in the pathogen recognition are Toll-like receptors (TLR), Mannose receptors (MR), Scavenger receptors (SR), and NOD proteins.

TLRs principally bind PAMPs found in prokaryotes [77], being TLR4 the receptor recognizing lipopolysaccharide (LPS) belonging to *E. coli*.

3.2.5 TLR:

Toll like receptors (TLR) are type I integral membrane glycoproteins characterized by the extracellular domains containing varying numbers of leucin-rich-repeat (LRR) motifs and a cytoplasmatic signaling domain homologous to that of the interleukin 1 receptor (IL-1R), termed the Toll/IL-1R homology (TIR) domain [78]. Based on their primary sequences, TLRs can be divided into several subfamilies, each of which recognizes related PAMPs:

- Lipids recognition: TLR1, TLR2, and TLR6

- Nucleic acids recognition: TLR7, TLR8, and TLR9

- LPS recognition: TLR4

Various immune cells express TLRs, as macrophages, dendritic cells, B cells, some specific types of T cells, and non-immune cells as fibroblasts and epithelial cells. Its expression is modulated in response to pathogens, cytokines, and environmental stresses. They may be expressed extra- or intracellularly. In the case of this thesis, we will focus in the role of TLR4 because it is the receptor that recognizes PAMPs belonging to *E. coli* causing metritis.

Receptor TLR4 recognizes LPS, present in the cell wall of Gram negative bacteria, triggering a signaling cascade and production of proinflammatory cytokines and chemokines leading to the induction of genes, mainly Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), involved in antimicrobial host defense [79].

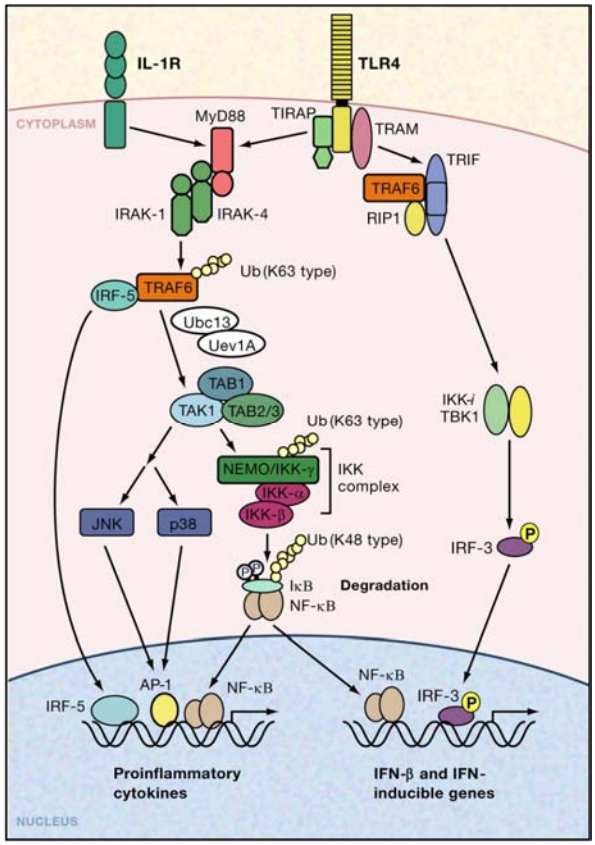


Figure 5: Signalling patways of the Toll-like receptors. TLRs and IL-1R share common signaling pathways in general. Stimulation with their ligands (IL-1 and LPS, respectively) recruits TIR-domain-containing adeptors including MyD88 (Myeloid differentiation primary response gene 88) and TIRAp to the receptor, and the subsequent formation of comples of IRAKs, TRAF6, and IRF-5 is induced. TRAF6 acts as an E3 ubiquitinligase and catalyzes the K63-linked polyubiquitin chain on TRAF6 itself and NEMO with E2 ubiquitin ligase complex of UBC13 and UEV1A. This ubiquitination activates the TAK1 comples, resulting in the phosphorylation of NEMO and activation of the IKK complex. Phosphorylated IκB undergoes K48-linked ubiquitination and degradation by the proteasome, Freed NF-κb translocates into the nucleus and initiatesexpression of pro-inflammatory cytokine gens. Simultaneously, TAK1 activates the MAP kinase cascades, leading to the activation of AP-1, which is also criticial for the inductin of cytokine genes. TLR4 triggers the MYD88-in dependent, TRIFF-dependent signaling pathway via TRAM to induce type I IFNs. TRIF activates NF-κB and IRF-3, resulting in the induction of proinflammatory cytokine genes and type I IFNs. TRAF6 and RIP1 induce NF-κB activation and TBK1/IKK-*i* phosporylate IRF-3, which induces the translocation of IRF-3 [79].

3.2.6 Cytokines:

Cytokines are a broad and loose category of small proteins (~5-20 kDa) that are important in cell signaling. They are released by different type of cells and have specific effect in the interactions and communications between cells. Cytokines may act in the cells that secrete them (autocrine action), on nearby cells (paracrine action), or in some instances on distant cells (endocrine action). It is common for different cell types to secrete the same cytokine to act in several different cell types (pleiotropy). They are also redundant in their activity, meaning several functions can be stimulated by different cytokines. They are often produced in a cascade, as one cytokine stimulates its target cell to make additional cytokines. They can act synergically or antagonistically.

There are pro-inflammatory and anti-inflammatory cytokines. Pro-inflammatory cytokines are produced mainly by activated macrophages and are involved in the up-regulation of the inflammatory reactions but also by other cell types. Some of the most relevant pro-inflammatory cytokines in the endometrium are IL-1β, TNF-α, or IL-6. In the other hand, anti-inflammatory cytokines are immunoregulatory molecules that control pro-inflammatory cytokine response. IL-10 and IL-4 are anti-inflammatory cytokines.

Chemokines, belonging to the cytokine family, can induce direct chemotaxis in nearby responsive cells. The major role of chemokines is to act as chemoattractant to guide the migration of cells. Their release is often stimulated by pro-inflammatory cells such as IL-1β. Chemokines function mainly as chemoattractants for leukocytes, recruiting monocytes, neutrophils and other effector cells from the blood to sites of infection or tissue damage. One example of chemokine is IL-8 [80].

Table 1: Principal cytokines involved in metritis

Name	Source	Target(s)
IL-1	macrophages, endothelia, epithelia	endothelia (↑ coagulation, ↑ inflammation), hepatocyties (↑ acute phase proteins), hypothalamus (↑ fever)
TNF	macrophages, T lymphocytes	endothelia (↑ coagulation, ↑ inflammation), hepatocyties (↑ acute phase proteins), neutrophils (↑ activation), hypothalamus (↑ fever)

IL-6	macrophages, endothelia, T lymphocytes	hepatocytes (↑ acute phase proteins), B lymphocytes (↑ proliferation)
IL-8	monocytes, neutrophils, macrophages and NK cells	chemoattractant for neutrophils, basophils and T cells; activates neutrophils to degranulate

3.2.7 Antimicrobial peptides:

Antimicrobial peptides (AMPs) are oligopeptides with a varying number (from five to over a hundred) of amino acids. They have a broad spectrum of targets ranging from viruses to parasites. In animals, AMPs are mostly found in tissues that are exposed to airbone pathogens, but can also be found in other tissues as endometrium; and are part of the first line of the innate immune response [81] against virus, bacteria, and fungi [82]. Several types of eukaryotic cells are involved in AMP production such as lymphs, epithelial cells in gastrointestinal and genitourinary systems, phagocytes, and lymphocytes of the immune system [83].

Defensins are a family of mammalian defensins, a small cysteine-rich cationic proteins found in vertebrates and invertebrates. β-defensins, the most widely distributed, are antimicrobial peptides implicated in the resistance of epithelial surfaces to microbial colonization. They have the ability to strengthen the immune system but can also enhance the adaptative immune system by chemotaxis of monocytes, T-lymphocytes, dendritic cells and mast cells to the infection site [84, 85]. Defensins are abundant in neutrophils' granules.

3.2.8 Neutrophils:

Neutrophils, also called polymorphonuclear leukocytes (PMN), are the most abundant type of granulocytes and the most abundant type of white blood cells (40% to 75%). They form an essential part of the innate immune system, as one of its principal effector cells. Its function is to attack microbes that have breached the epithelial barriers and entered into tissues or the circulation. The cytoplasm contains granules of two types: the majority, called specific granules, are filled with enzymes such as lysozyme, collagenase, and elastase. The rest are lysosimes containing enzymes and other microbial substances [70].

Blood-derived PMNs are the main effector cells removing bacteria from the uterus after calving [34]. However, neutrophil killing ability is lower in cows with endometritis [86] and it is also impaired by the state of negative energy balance around parturition [87].

3.3 Uterine immunity in cattle:

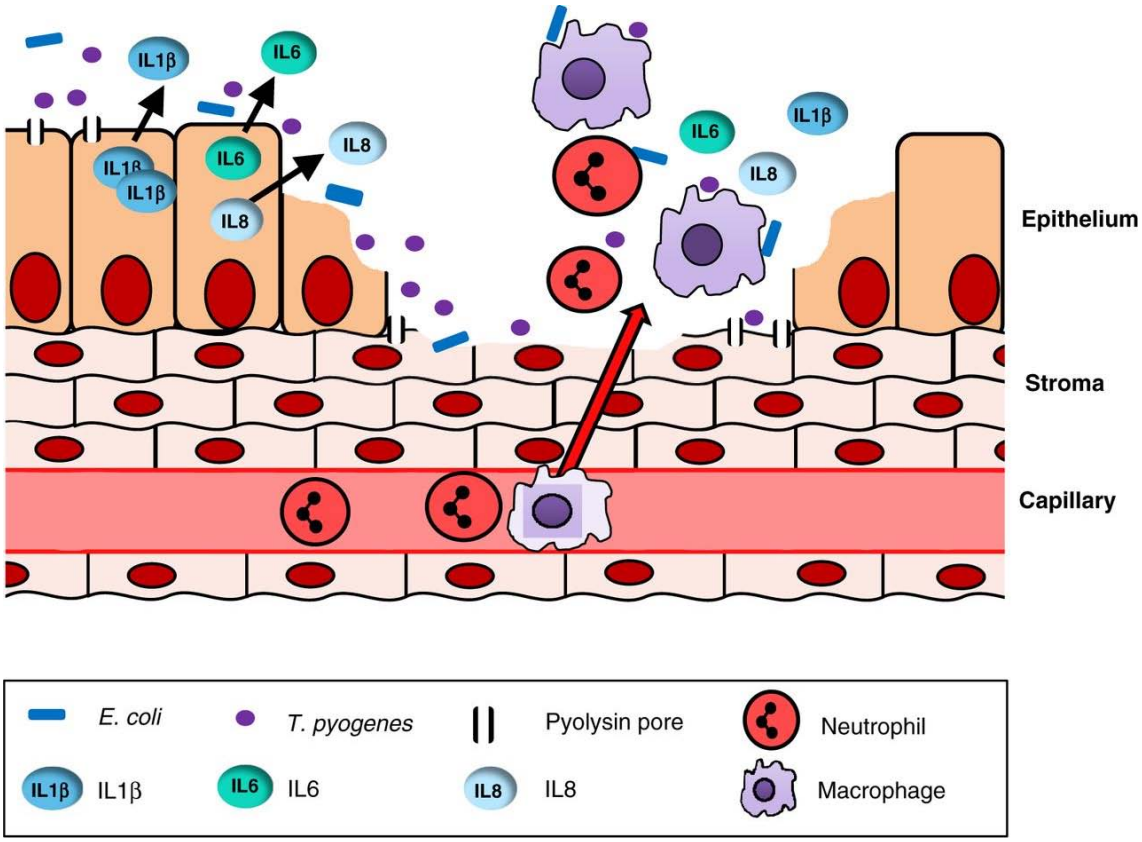
Mammalian pregnancy involves regulation of uterine immunity to facilitate implantation and survival of the fetus. The classic view is that immunity is suppressed during gestation, and that this immunosuppressive mechanisms associated with pregnancy persist in the endometrium after parturition, probably predisposing to uterine disease [34]. Progesterone maintains uterine quiescence throughout gestation, but before parturition the influence of progesterone diminishes rapidly. It has been described in different species an increment of inflammatory mediators, such as IL-8, IL-1β, IL-6, and TNF-α, and an influx of leukocytes, once the influence of progesterone is reduced [49]. Most dairy cows experience several weeks of substantial reduction in immune function around calving, typically reaching a nadir approximately 1 week postpartum [88]. Another feature of the post-partum bovine uterus is the accumulation of CD14+ monocytes and macrophages, that once activated, will enhance the local inflammatory response by attracting neutrophils and monocytes, which will clear apoptotic cells, and help remodeling the caruncle (a small, fleshy protuberance of mucocutaneus tissue), and helping to the successful release of the fetal membrane. As stated before, the uterus must recover fast after parturition in order to be ready for the establishment of the next pregnancy. This recovery involves tissue repair, regeneration of the epithelium lining the endometrium, and elimination of bacteria [89].

3.4 Endometrial response to uterine infection

Host responses to bacterial infection of the uterus from cows are characterized by inflammation of the endometrium with the recruitment of macrophages and neutrophils, along with the accumulation of pus in the lumen.

Figure 5: Pathogenic bacteria cause inflammation in the endometrium. After parturition, *Escherichia coli*, *Trueperella pyogenes* and other bacteria invade the uterus, and the pathogenic bacteria adhere to and invade the endometrium, particularly where there is tissue damage. *T. pyogenes* also produces pyolysin, which creates transmembrane pores in endometrial cells, causing cell death and further tissue damage. Endometrial epithelial and stromal cells sense

pathogen-associated and damage-associated molecular patterns via innate immune receptors, such as TLRs, which leads to the release of cytokines and chemokines, such as IL1B, IL6 and IL8 (black arrows). These inflammatory mediators attract and activate haematopoietic cells, particularly neutrophils and macrophages, to the site of infection (red arrow), in order to clear the invading microbes and resolve the tissue damage [89].



Once the bacteria reach the endometrium they release LPS. The LPS is recognized by TLR-4 expressed by epithelial cells. Activation of TLRs initiates signaling cascades, activating NF-κB, resulting in the synthesis and production of pro-inflammatory cytokines and chemokines such IL-8, IL-1β, and IL-6 that mobilize and activate immune cells [79, 90]. In the specific case of cows, it is associated with the entrance of neutrophils into the uterus [91]. In summary, pathologic inflammation only seems to differ from physiological response in severity, duration and time postpartum in which is most pronounced.

Block 4: Probiotics

The term "probiotics" has been amended by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) to "Live microorganisms, which, when administrated in adequate amounts, confer health benefit on the host" [92]. However, this definition is not accepted by European Food Safety

Authority (EFSA) or the U.S. Food and Drug Administration (FDA) since they insist that the health claim incorporated in the definition is not measurable due to the fact that commercial markets have outpace the ability of science to substantiate the evidence [93]. Other definitions advanced through the years have been restrictive by specification of mechanisms, site of action, delivery format, method, or host. Probiotics have been shown to exert a wide range of effects. An important aspect to mention when claiming the health benefits of probiotics is that a beneficial effect can only be demonstrated by *in vivo* studies [94]. *In vitro* studies can be used to characterize a possible mechanism of probiotic action, determine the safety of probiotic microorganisms or convey other knowledge of probiotic strains, in summary, it provides the first step evaluating the probiotics [93].

Probiotics are used in human and animal health; being called "live biotherapeutics" when used as human drugs. In the case of animals, probiotics are mainly used as feed additives to regulate intestinal homeostasis. In the case of this thesis, we will focus in probiotics used to regulate infection and inflammation and to be applied in the uteri-vaginal tract.

The most important properties of strains that may be considered probiotics are [95, 96]:

- Adhesion properties [97]
- Antimicrobial activity against pathogenic microorganisms [97]
- Reduce pathogen adhesion to surfaces [93]
- Enhancing viability of probiotics [93]
- Production of bacteriocins and bacteriocin-like substances [98]

The most common types of microorganisms used as probiotics are lactic acid bacteria (LAB) and bifidobacteria, although other bacteria and some yeasts are also used [93, 98].

4.1 Lactic Acid Bacteria

Lactic acid bacteria (LAB) are Gram-positive, non-spore forming cocci, coccobacilli or rods. Generally, they are not respiratory and lack catalase. They ferment glucose to lactic acid, or to lactic acid, CO_2, and ethanol. They are aerotolerant anaerobes.

The term LAB is conventionally reserved for genera in the order *Lactobacillaes,* which includes *Lactobacillus, Leuconostoc, Pediococcus, Lactococcus,* and *Streptococcus*. They

can convert hexose sugars to lactic acid thus producing an acid environment which inhibits the growth of several species of harmful bacteria [99].

Lactobacilli are normally present in the vagina, gastrointestinal tract and gut of humans [99] and they are also present in the vagina of dairy cows [100]. Vaginal lactobacilli inhibit the growth of genitourinary pathogenic microorganisms through mechanisms of competitive exclusion of pathogens, stimulation of the host immune system, and production of specific antibacterial compounds such as lactic acid, hydrogen peroxide, and antimicrobial peptides [101]

4.1.1 *Lactobacillus rhamnosus:*

It is a short Gram-positive facultative anaerobic rod that often appears in chains. Some strains of *L. rhamnosus* are used as probiotics. It is known that *L. rhamnosus* is able to survive the acid and bile of the stomach and intestine, colonize the digestive tract, and balance intestinal microflora [102, 103]. It has also been proved that *L. rhamnosus* is able to protect the human urogenital tract by excreting biosurfactants to inhibit the adhesion of pathogens and it is extremely adherent to uroepithelial and vaginal cells [104].

4.1.2 *Pediococcus acidilactici:*

It is a Gram-positive facultative anaerobic cocci that is often found in pairs or tetrads. *P. acidilactici* is a homofermentative bacterium that can grow in a wide range of pH, temperature, and osmotic pressure, therefore being able to colonize the digestive tract. *P. acidilactici* has a wide range of potential benefits which are being studied. It has been used as a probiotic supplement to treat constipation, diarrhea, and enhancing immune response among birds and small animals [105-107]. It is also know that can prevent *E. coli* colonization in the small intestine of pigs [108]. It is known that *Pediococci* produce antimicrobial compounds such as organic acids, hydrogen peroxide, and antimicrobial peptides including pediocin AcH/PA-1 [100].

4.1.3 *Lactobacillus sakei:*

L. sakei is a Gram-positive facultative heterofementative rod that has usually been used in the food industry, being proposed to enhance microbial safety of non-fermented meat

products [109]. It has been identified that *L. sakei* produces bacteriocins, specifically it produces sakacin T and sakacin X [110].

4.1.4 *Lactobacillus reuteri:*

L. reuteri Gram-positive facultative anaerobic rod and some of its strains are used as probiotics. They naturally inhibit the gut of mammals and birds. *L. reuteri* is known to produce reuterin, a broad-spectrum antibiotic substance, able to inhibit the growth of pathogenic Gram-negative and Gram-positive bacteria, yeasts, fungi, and protozoa [111, 112].

4.2 Modes of action of Probiotics

There are different modes of action for probiotics. It has been extensively studied in the intestinal tract. Here it is a summary of potential modes of action regarding intestinal epithelial cells but could be extrapolated to other tissues and environments (Figure 6):

a: Blocking pathogen entry into the epithelial cell by providing a physical barrier, referred to as colonization resistance.

b: Blocking pathogen entry creating a mucus barrier by causing the release of mucus from goblet cells.

c: Other probiotics maintain intestinal permeability by increasing the intercellular integrity of apical tight junctions, for example, by upregulating the expression of zona-occludens 1 (a tight junction protein), or by preventing tight junction protein redistribution thereby stopping the passage of molecules into the *lamina propia*.

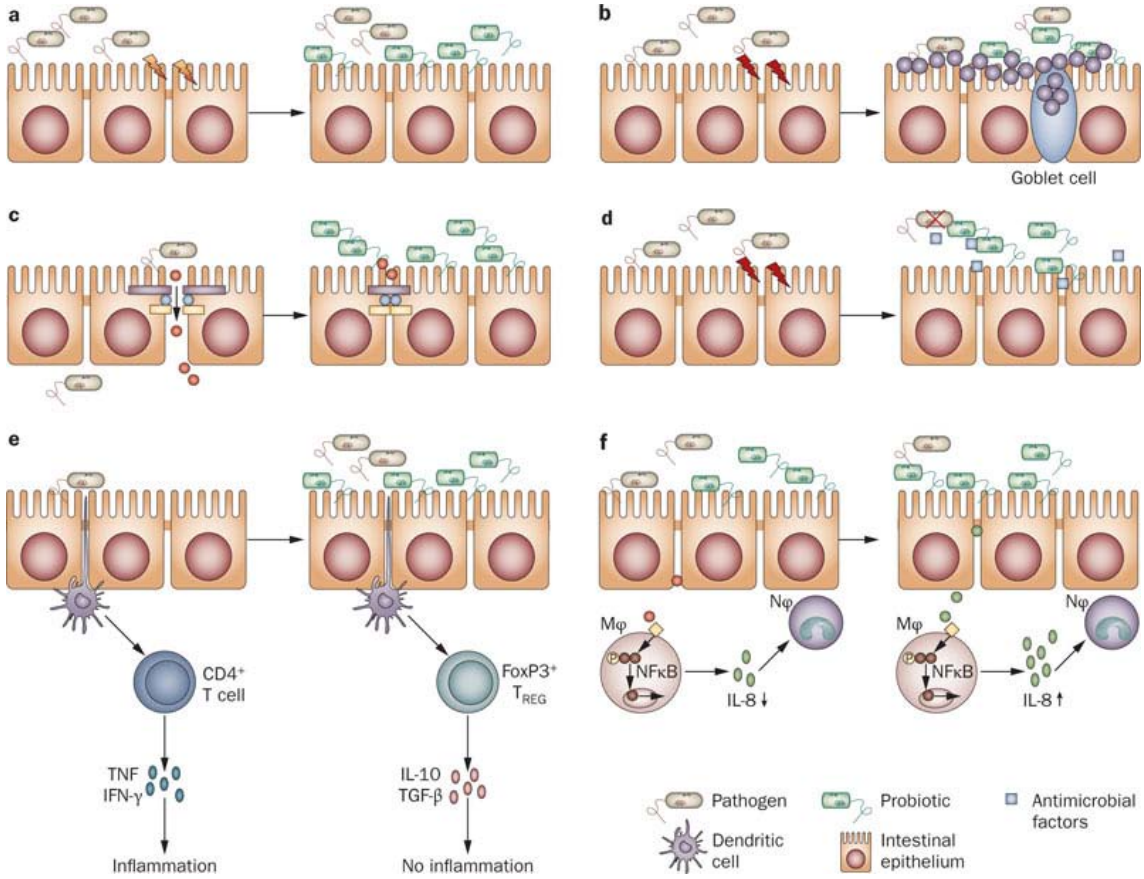
d: Some probiotic strains have been shown to produce antimicrobial factors.

e: Still other strains stimulate the innate immune system by signaling dendritic cells, which then travel to mesenteric lymph nodes and lead to the induction of TREG cells and the production of anti-inflammatory cytokines, including IL-10 and TGF-β.

f: Some probiotics (or their products) may also prevent or trigger (right-hand side) an innate immune response by initiating TNF production by epithelial cells and

inhibiting (or activitating) NFκB in macrophages and dampening (or priming) the host immune response by influencing the production of IL-8 and subsequent recruitment of neutrophils to sites of intestinal injury [113].

Figure 6: Potential mechanisms of action of probiotics. Probiotic organisms can provide a beneficial effect on intestinal epithelial cells in numerous ways. Abbreviations: Mϕ, macrophage; Nϕ, neutrophil; TREG cell, regulatory T cell. Reproduced from [113].



It seems that when lactic acid bacteria is applied in the vagina produces antimicrobial factors (d) and triggers innate immune response (f).

4.3 Future perspectives: Antibiotic resistance

Until now, research with probiotics has been mainly focused in diseases related with the gastrointestinal tract in animal production. New lines of research have started regarding respiratory, gut-brain axis, and uterine diseases. Antibiotic resistance is recognized worldwide among the top public health challenges facing the 21st century, and thus there is growing concern regarding the potential impact of extensive use of antibiotics in livestock.

Antimicrobial resistance is resistance of a microorganism to an antimicrobial drug that was originally effective for treatment of infections caused by it. Resistant microorganisms (including bacteria, fungi, viruses, and parasites) are able to withstand attacks by antimicrobial drugs, such as antibacterial drugs (e.g. antibiotics), antifungals, antivirals, and antimalarial, so that standard treatments become ineffective and infections persist, increasing the risk to spread to others.

The traditional antimicrobial treatment applied against metritis may lack efficacy, especially in cases of sustained inflammation [53] making metritis a possible target to be address with probiotics.

CHAPTER II

Objectives

The main objective of this thesis was to evaluate the potential of Lactic acid bacteria to reduce *Escherichia coli* infection and endometrial inflammation from bovine endometrium. The specific objectives were:

1. To evaluate the efficacy of four LAB strains at reducing *E. coli* infection and endometrial inflammation *in vitro*.
2. To evaluate the ability of different combinations of LAB strains at reducing infection and inflammation of the endometrium *in vitro*.
3. To evaluate *ex vivo* in endometrial explants the ability of a combination of LAB at reducing the inflammatory response after *E. coli* infection.
4. To study if LAB applied in the vagina affects inflammation and immunity in the endometrium.
5. To study the best approach to apply the LAB combination in dairy cows (vagina or uterus) and its incidence in metritis prevalence.
6. To study the influence of LAB at NEFA concentration, neutrophil activity, and its interaction.
7. To study if LAB applied in the uterus at post-partum is able to regulate general inflammatory status in metritic or healthy cows.

To achieve these objectives, four studies were conducted:

Study 1 **"***Potential of lactic acid bacteria at regulating Escherichia coli infection and inflammation of bovine endometrium"*

Study 2 *"A combination of Lactic Acid Bacteria Regulates Escherichia coli Infection and Inflammation of the Bovine Endometrium"*

Study 3 **"***Effects of intravaginal lactic acid bacteria on bovine endometrium: implications in uterine health"*

Study 4 **"***Lactic Acid Bacteria Combination Regulates Neutrophil Activity and Reduces Metritis Prevalence"*

CHAPTER III: Study 1

Potential of lactic acid bacteria at regulating *Escherichia coli* infection and inflammation of bovine endometrium

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This research has been published in:

Theriogenology, 2016; Vol 85

1. Introduction

Uterine function is often compromised in cattle by bacterial contamination and inflammation after calving [35, 36, 114]. On average, 20–40% of cattle develop acute clinical uterine disease within a week from parturition (metritis), 20% have a persistent clinical disease 3 weeks after calving (endometritis) and approximately 30% have a chronic subclinical inflammation of the uterus (subclinical endometritis) [33, 35, 36, 45, 114, 115]. Metritis is an inflammation of the uterus resulting in systemic signs of sickness, including fever, red-brown watery foul-smelling uterine discharge, dullness, inappetance, elevated heart rate, and low production of milk [53]. Endometritis is characterized by the presence of purulent (>50% pus) uterine discharge detectable in the vagina, or mucopurulent (approximately 50% pus, 50% mucus) discharge detectable in the vagina [33]. The economic impact of uterine diseases in cows is associated with infertility, increased culling rate due to failure to conceive, reduced milk production, and the increased cost of antibiotic treatment. The average economic cost of a single case of metritis has been estimated to be about 350€ [44, 45].

It is generally considered that bacterial contamination of the uterus, or at least exposure of the endometrium to bacterial lipopolysaccharide (LPS), along with the need for substantial tissue repair as part of involution, initiates inflammation of the endometrium and in occasions deeper layers of the uterus [47, 48]. However, the excessive and persistent inflammatory response occurring in some cases is attributable, in part, to reduced immune function before calving. Progesterone maintains uterine quiescence throughout gestation, but before parturition the influence of blood progesterone concentrations diminish rapidly. In several species, the reduction in the influence of progesterone is associated with an increase in inflammatory mediators, such as IL-1β, IL-6, IL-8, and TNF-α [49]. Also, stressors such as shortage of nutrient supply around the time of parturition may lead to increased inflammatory responses and risk of disease [116, 117].

Epithelial cells are efficiently sealed by tight junctions that separate and protect them from the external media. Tight junctions strictly seal the intracellular spaces and contribute to establish an efficient barrier against intracellular infections. A leaking

barrier can favor the passage of pathogens [118]. Claudins and occludins are transmembrane proteins present in the tight junctions that interact with the adjacent cell [119]. Pathogenic bacteria efficiently produce defects in the cell-cell contact, delocalizing proteins in the tight junctions [120].

Escherichia coli and *Arcanobacterium pyogenes* are the most prevalent bacteria isolated from the uterine lumen of cattle with uterine infections, followed by a range of anaerobic bacteria. *E. coli* is particularly prevalent in the first week postpartum and is associated with metritis. The traditional antimicrobial treatment applied may lack efficacy, especially in cases of sustained inflammation [53]. Moreover, antibiotic resistance is recognized worldwide among the top public health challenges facing the 21st century, and thus there is growing concern regarding the potential impact of extensive use of antibiotics in livestock.

Under normal conditions, the vaginal tract of a dairy cow is populated by a diversity of bacteria dominated mainly by lactic acid bacteria (LAB) [97]. It has been recently shown that LAB of the genera *Enterococcus, Lactobacillus,* and *Pediococcus* were present in the vaginal tracts of healthy and infected cows [100, 121]. Vaginal *Lactobacilli* inhibit the growth of genitourinary pathogenic microorganisms through mechanisms of competitive exclusion, stimulation of the host immune system, and production of specific antibacterial compounds such as acetic and lactic acids [101].

Although several investigators have suggested utilization of LAB as prophylactic or treatment interventions against uterine infections in cattle, to our knowledge there are no *in vitro* studies describing the potential of probiotics at regulating the endometrial inflammation and preventing infections, and there is only one recent study performing a first evaluation of LAB as a preventing strategy of postpartum uterine disease [121]. We hypothesized that the administration of adequate doses of some LAB would improve the inflammatory status of the endometrium and would reduce the ability of *E. coli* to cause infection. Thus, the aim of this study was to evaluate the potential of four LAB (*L. rhamnosus, P. acidilactici, L. sakei* and *L. reuteri*) to modulate inflammation and *E. coli* infection in bovine primary endometrial cultures.

2. Materials and methods

2.1 Bacterial strains and culture conditions

Pediococcus acidilactici (CECT 5915) and *Lactobacillus rhamnosus* (CECT 278) were purchased from CECT (Colección Española de Cultivos Tipo, CSIC Valencia, Spain), and *Lactobacillus reuteri* (DSM 20016) and *Lactobacillus sakei* (DSM 20100) were acquired at DSMZ (German collection of microorganisms and cell cultures, Leibniz, Germany). *Lactobacillus sakei* and *Lactobacillus reuteri* were used in this study as two probiotics with low adhrent capacity in the endometrium; whereas *Pediococcus acidilactici* and *Lactobacillus rhamnosus* were selected as two probiotics with moderate adherent capacity (++) following the criteria and methods previously described [122] (Table 1). Three doses of LAB referred as low, intermediate, and high were established considering any possible cytotoxic effect for the endometrial cells using a lactate dehydrogenase (LDH) activity assay kit (Sigma-Aldrich, St. Louis, USA) following the manufacturer's instructions. The doses showing the least LDH release and activity, and therefore with least cell damage, were selected to evaluate potential of LAB strains at modulating infection and inflammation in endometrial cultures. For *P. acidilactici* and *L. sakei*, the LAB:endometrial cells ratios of 1, 25, and 50 and 10, 25, and 50 were selected, respectively. When using these ratios, cell damage was not different from that measured in negative control cells (Table 2). In the case of *L. rhamnosus* and *L. reuteri*, LAB:endometrial cells ratios of 25, 50, 100 and 1, 2, and 100 were selected respectively because cell damage was, actually, lower than in negative control cells (Table 2). A swab sample was taken from the uterus of 4 different metritic cows to isolate pathogenic *E. coli*. Each swab was spread in blood agar, MacConkey agar, and chocolate agar. The final identification of the colonies was made by Vitek (Biomiéreux, Craponne, France). The *E. coli* selected was fimH positive (virulence factor) and hemolytic. Bacterial cultures were performed inoculating 1 mL of a glycerinate in 9 mL of Luria Bertrani (LB) media (10 g/L peptone, 5 g/L yeast extract and 10 g/L NaCL) for *E. coli* or Man, Rogosa, and Shape medium (MRS, Scharlau, Sentmenat, Spain) at 37ºC in static conditions overnight (O/N). Then, bacteria were centrifuged at 6000 x *g* for 15 min and resuspended at $1x10^8$ colony-forming units (CFU)/mL with DMEM (Gibco, NY, USA) supplemented with 8 µg/mL bovine insulin and 50 µg/mL hydrocortisone. *E. coli* CFU/mL was confirmed by serially diluting

and plating the cell extract on MacConkey (Scharlau, Sentmentat, Spain) agar media. Colonies were counted after an O/N growth at 37ºC.

Table 1. Biofilm formation capacity of the different lactic acid bacteria tested. The ability to form biofilms by LAB is indicated as a measure of absorbance (A490) and classified as weakly (+) or moderately (++) adherent (n = 8 for each bacteria).

LAB[a]	Absorbance	Adherence
L. sakei	0.653	+[b]
L. reuteri	0.977	+
P. acidilactici	1.455	++[c]
L. rhamnosus	1.742	++

[a] Lactic acid bacteria

[b] + = 0.5 < A490 < 1

[c] ++ = 1 < A490 < 2

Table 2. Dose selection of lactic acid bacteria. Average lactate deshydrogenase activity (LDH) ± SEM for each ratio (multiplicity of infection, MOI) of LAB:endometrial cells for low, intermediate, and high doses selected for *P. acidilactici*, *L. rhamnosus*, *L. Sakei*, and *L. reuteri.*

Sample	Dose category	LDH, mU/mL	*P*-value[a]
Negative control		13.08 ± 0.016	
P. acidilactici MOI=1	Low	12.90 ± 0.013	0.908
P. acidilactici MOI=2	Discarded	14.81 ± 0.014	0.087
P. acidilactici MOI=5	Discarded	15.65 ± 0.014	0.017
P. acidilactici MOI=10	Discarded	15.44 ± 0.016	0.036
P. acidilactici MOI=25	Intermediate	14.19 ± 0.016	0.265
P. acidilactici MOI=50	High	14.54 ± 0.016	0.172
P. acidilactici MOI=100	Discarded	18.18 ± 0.018	0.001
L. rhamnosus MOI=1	Discarded	16.92 ± 0.770	0.001
L. rhamnosus MOI=2	Discarded	14.47 ± 0.770	0.211
L. rhamnosus MOI=5	Discarded	11.88 ± 0.770	0.277
L. rhamnosus MOI=10	Discarded	15.36 ± 0.843	0.053
L. rhamnosus MOI=25	Low	1.94 ± 0.843	<0.0001
L. rhamnosus MOI=50	Intermediate	0.74 ± 0.843	<0.0001
L. rhamnosus MOI=100	High	0.18 ± 0.843	<0.0001
L. sakei MOI=1	Discarded	14.81 ± 0.177	0.617
L. sakei MOI=2	Discarded	14.65 ± 0.177	0.651
L. sakei MOI=5	Discarded	15.78 ± 0.177	0.133
L. sakei MOI=10	Low	11.72 ± 0.194	0.689
L. sakei MOI=25	Intermediate	13.70 ± 0.194	0.846
*L. sake*i MOI=50	High	11.88 ± 0.194	0.726
L. sakei MOI=100	Discarded	33.75 ± 0.194	0.001
L. reuteri MOI=1	Low	11.17 ± 0.028	0.088
L. reuteri MOI=2	Intermediate	12.97 ± 0.028	0.883
L. reuteri MOI=5	Discarded	13.57 ± 0.028	0.704
L. reuteri MOI=10	Discarded	15.25 ± 0.032	0.145

L. reuteri MOI=25	Discarded	14.05 ± 0.032	0.524
L. reuteri MOI=50	Discarded	13.20 ± 0.032	0.932
L. reuteri MOI=100	High	4.41 ± 0.0349	<0.0001

[a] Difference with respect to negative control (untreated sample)

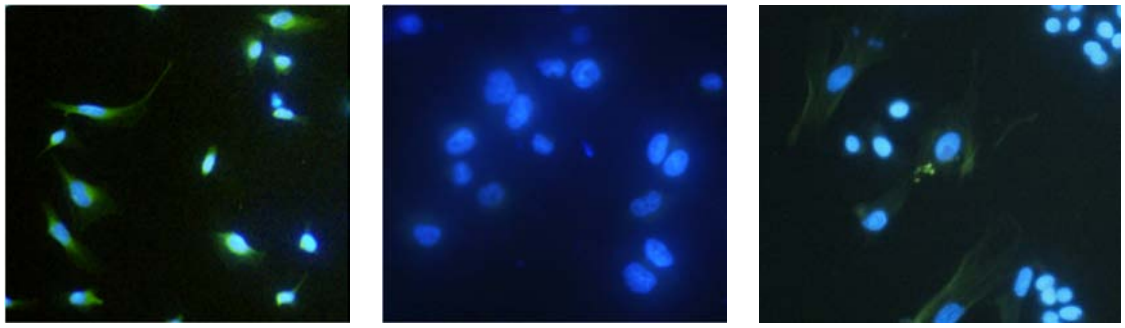
2.2 Endometrial primary culture

Endometrial tissue was obtained at a slaughterhouse from one Holstein post-pubertal non-pregnant cow with no evidence of genital disease or microbial infection and transported in chilled 40 mL of PBS with 100 µg/mL streptomycin, 100 U/mL penicillin and 2.5 µg/mL amphotericin B (PEF) to the laboratory. The whole endometrial tissue was collected (two horns). Then, tissue was cut in small pieces, discarding the myometrium, and incubated with 15mL of PBS supplemented with 1xPEF, 0.1 *mM* EDTA and 0.1 *mM* DTT for 10 min at 37ºC in 5% CO_2 at 200 rpm. Then the supernatant was removed and RPMI 1640 media (Gibco, NY, USA) with 0.25% collagenase type II (Gibco, NY, USA) was added and further incubated 15 min at 37ºC in 5% CO_2 at 200 rpm. Supernatant was collected and mixed with (1:1) RPMI 1640 media enriched with 0.02 mg/mL DNase I RNase free (Roche, Mannheim, Germany) for 30 min at RT. Cells present in the supernatant were recovered after centrifuging 5 min at 300 x *g*. This process was repeated 3 times from the collagenase II treatment. 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 EGF (epithelial growth factor, Sigma-Aldrich, St. Louis, USA). Cells were quantified by haemocytometer counting and incubated at 160,000 cells/cm^2 in culture flasks (Sarstedt, Nümbrecht, Germany). Epithelial cell phenotype was confirmed by immunofluorescence staining against anti-cytokeratin antibodies (monoclonal anti-cytokeratin pan antibody produced in mouse, Sigma, 1:500 dilution, St. Louis, USA) of about 90% of isolated cells (Figure 1) as previously described [123]. For the immunofluorescence, endometrial primary cells were grown on coverslips and fixed with 4% paraformaldehyde (diluted in PBS). HeLa cells were used as positive control and macrophage differentiated THP-1 cells as a negative control. Coverslips were blocked with 150 µL of PBS containing goat serum (0.05% v/v) and Triton 0.2%, for 30 min at room temperature. Primary antibody (monoclonal anti-cytokeratin pan antibody produced in mouse, Sigma, 1:500 dilution) was incubated in blocking buffer for 2 h at room temperature. After 3 washes with PBS,

secondary antibody (antimouse-FITC, Sigma, St. Luis, USA, 1:1000) was incubated for 1 h at room temperature. Lastly, cells were washed and dried coverslips mounted in glass slides using Fluoroprep (Biomerieux, Craponne, France) and observed by fluorescence microscopy.

Figure 1. Primary endometrial epithelial culture. Immunofluorescence of primary endometrial cultures with pan-cytokeratin. HeLa cells (a) as positive control, THP-1 macrophages (b) as negative control, and endometrial cultures (c).

A b c



2.3 Inflammation model of bovine endometrium

To establish an inflammation model of bovine endometrium, two different pro-inflammatory cytokines were used IL-1β and IL-6 (KingFisher, St. Paul, USA). Endometrial cells were incubated with DMEM medium supplemented with 8 µg/mL bovine insulin, 50 µg/mL hydrocortisone, in a 24-well plate at 80,000 cells/well O/N at 37ºC and 5% of CO_2. After an O/N cells reached a confluence of the 80%. Then 10 µg/µL of IL-1β or IL-6 were added per well. The cells were incubated for 1, 6, or 24 h and harvested with TRIzol (5 Prime, Gaithesburg, USA) to extract total RNA for further quantification of IL-8 and IL-1β gene expression as inflammation markers. The mRNA abundance of IL-8 increased upon time when the cells were pre-treated with IL-1β compared with their corresponding negative controls; whereas, cells treated with IL-6 did not show any increment of inflammation markers; on the contrary, the abundance of IL-8 mRNA diminished at 6 and 24 h when IL-6 was used to induce inflammation compared with the corresponding controls (Figure 2A). The inflammation caused by IL-1β treatment was also corroborated by the increased mRNA abundance of IL-1β after 6 and 24 h relative to control preparations (Figure 2B). The gene expression of IL-1β was also increased by IL-6 at 24 h

compared with the corresponding control. Thus, the treatment of endometrial cells with 10 µg/µL of IL-1β per well during 6 h was selected as an effective model to establish inflammation of bovine endometrial cells.

2.4 *Escherichia coli* infection assays

Epithelial cells were plated at 80,000 cells/well in 24 well-plates with DMEM medium supplemented with 8 µg/mL bovine insulin, 50 µg/mL hydrocortisone, and kept O/N at 37ºC and 5% CO_2. When *E. coli* infection was evaluated in endometrium under inflammation, IL-1β (Figure 2) was added at 10 ug/µL 6 h previous to LAB administration. Cells were incubated (n = 6 for each dose) O/N with three doses of each LAB (high, intermediate, and low; (Table 1) and then challenged with $4x10^6$ CFU/well of *E. coli* for 6 h. Infection without LAB was used as an infection control and incubation with media instead of *E. coli* as a negative control. Cells were washed twice with chilled PBS and bacteria were released with 500 µL 0.9% NaCl/well. *E. coli* was quantified by serially diluting and plating the cell extract on MacConkey agar media. Colonies were counted after an O/N growth at 37ºC. Cell cultures used for gene expression analyses were washed twice with PBS and harvested with 500 µL of TriZol (5 Prime, Gaithesburg, USA) for RNA extraction.

2.5 Quantitative RT-PCR analyses

One microgram of RNA was retro-transcribed to cDNA using PrimeScript RT reagent kit (Takara, Shiga, Japan) following manufacturer's instructions. qPCR (iQ5 Termocycler, Bio-Rad, Hercules, USA) was performed for bovine *IL-8*, *IL-1β*, *TNF-α*, *IL-6* and *OCLN* using specific primers. The primers for all the genes were designed using the Primer Blast software (NCBI) or selected from other publications (Table 2). A total reaction volume of 20 µl was used, containing 50 ng of cDNA, 10 µl of SYBR Green Fluorescent (Bio-Rad, Hercules, USA), and the optimized primer concentration for each gene (Table 3). The qPCR reactions were cycled as follows: an initial denaturing step of 10 min at 95ºC, followed by 40 cycles of 10 s at 95ºC, 15 s at optimized annealing temperature for each gene, 30-60 s at 72ºC and a final extension of 10 min at 72ºC. Relative gene expression was calculated using the 2ΔCt method [124] with *GAPDH* as a reference gene control and

all the qPCR amplifications were performed in triplicate. *GAPDH* was chosen because it is stably expressed in a variety of cells [125], including uterine tissues [116, 126].

Table 3. Sequence, annealing temperature (At), concentration (µM), accession number or reference, and forward (Fw) and reverse (Rv) primers used for qPCR.

Gene	Fw	Rv	At, cº	uM	Accesion number or ref.
GAPDH	GCATCGTGGAGGGACTTATGA	GGGCCATCCACAGTCTTCTG	52ºC	0.125	NM_001034034
IL-8	TTGAGAGTGGACCACACTGC	TGCACCCAGTTTTCCTTGG	55ºC	0.5	[127, 128]
IL-1β	TGGGAGATGGAAACATCCAG	TTTATTGACTGCACGGGTGC	50ºC	0.3125	M37211
IL-6	GGCGGAGCCTTGCGTTAT	AACTGCTGTGCTTGCTTCAT	51.5ºC	0.5	[129]
TNFα	AACAGCCCTCTGGTTCAAAC	TCTTGATGGCAGACAGGATG	60ºC	0.5	M_173966
OCLN	ATCAACCCCGGTGCCGGAAG	GTGGTCTTGCTCTGCCCGCC	57ºC	0.5	NM_001082433

2.6 Statistical analysis

Prior to statistical analyses, data were transformed by log or root when necessary to achieve a normal distribution. Results herein are expressed as the means of non-transformed data ± SEM obtained with normalized data (except otherwise stated). The data were analyzed using an ANOVA (SAS Inst. Inc., Cary, NC). Treatment and infection were tested as fixed effects. Differences were considered significant when $P < 0.05$.

3. RESULTS

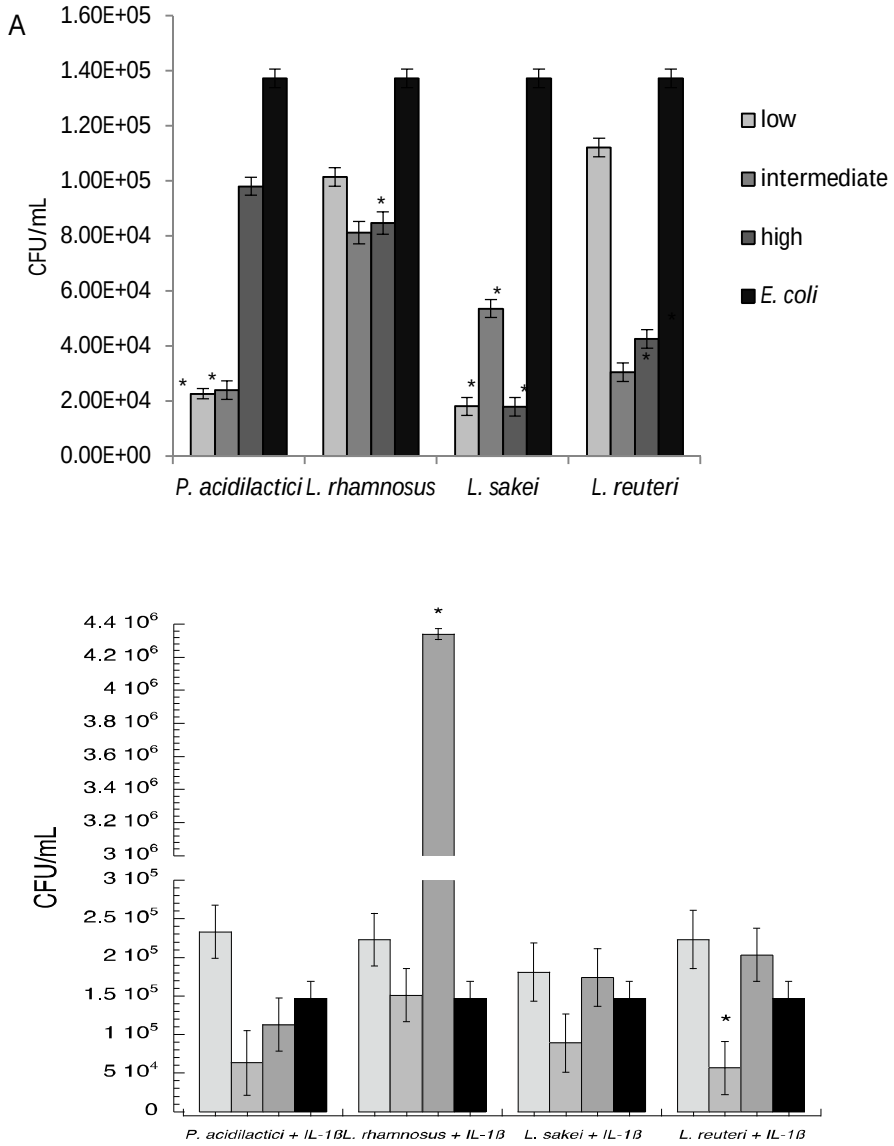
3.1 Infection of endometrial cells with *Escherichia coli*

The effect of the LAB species during an *E. coli* infection was evaluated. Some doses of the four LAB reduced ($P < 0.0001$) *E. coli* infection *in vitro* (Figure 3A). The *E. coli* infection was reduced up to 83.4% with the presence of *P. acidilactici* at the low dose and up to 82.5% with the intermediate dose. In the case of *L. rhamnosus,* only the high dose showed a significant reduction of *E. coli* infection (38.1%). The infection of *E. coli* was decreased by *L. sakei* at the three doses tested (86.8% for low, 60.8% for intermediate, and 86.9% for high, compared with control). Lastly, *E. coli* infection was decreased with the

intermediate (77.7%) and the high (69.0%) doses of *L. reuteri* relative to the negative control.

The probiotic potential and the modulatory effects of LAB in the endometrial cells against *E. coli* was different in the presence or absence of inflammation (Figure 3B). There was only a reduction of *E. coli* infection by the intermediate dose of *L. reuteri* (61.4%, $P < 0.0001$) and a 96.61% increment ($P < 0.0001$) of *E. coli* was found in the cells treated with the high dose *of L. rhamnosus*.

Figure 3. *E. coli* infection of the endometrium. Viable cell counts as CFU/mL of *E. coli* infection for each dose of LAB in an endometrium in the absence (a) or in the presence of inflammation (b) (n = 6 for each condition). Bars represent the means ± SEM for the different treatments. Bars with asterisk are different ($P < 0.0001$) from *E. coli*.

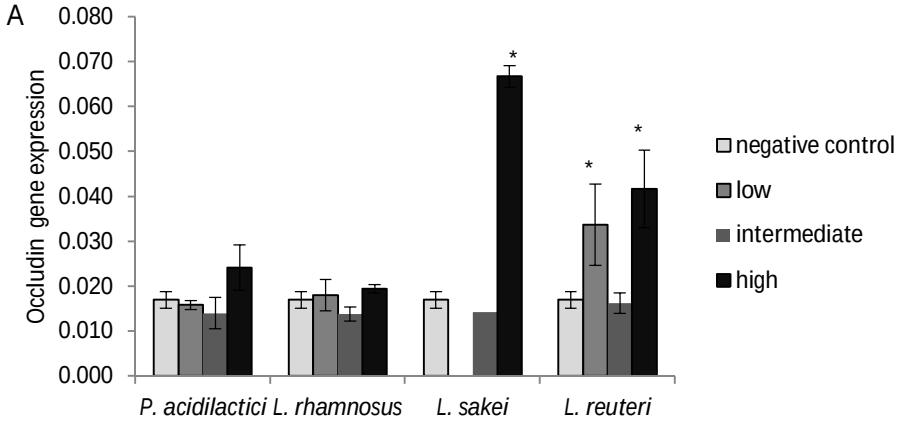


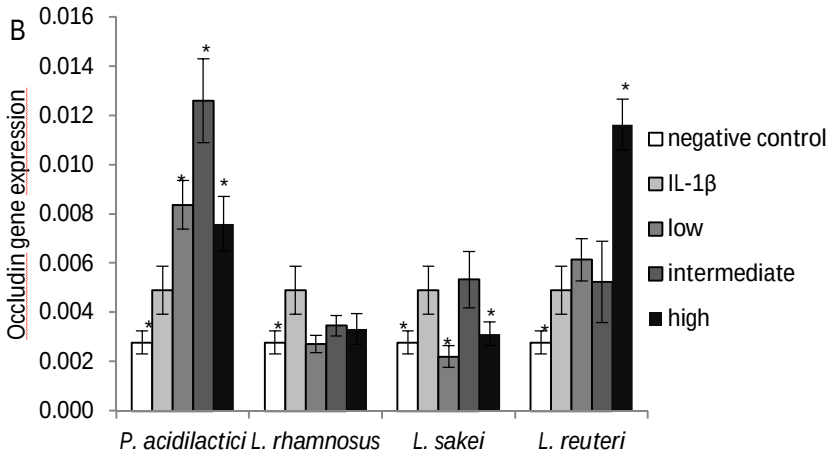
3.2 Lactic acid bacteria effects in tight junctions in endometrial cells

Occludin gene expression was analyzed as a marker of tight junction integrity. The mRNA abundance of occludin was affected ($P < 0.0001$) by LAB treatment (A). The gene expression of occludin increased 3.9 fold in the cells pre-treated with the high dose of *L. sakei* compared with the negative control cells (not treated with LAB). An increased gene expression was also observed with the low (2 fold) and high dose (2.47 fold) of *L. reuteri*. However, no differences were observed when *P. acidilactici* or *L. rhamnosus* were added to the cells.

The mRNA abundance of occludin by the endometrial cells in the presence of inflammation was greater than the one for the normal endometrial cells (negative control) and it was also affected by LAB treatment ($P < 0.0001$). When *P. acidilactici* was added to the cells at the low dose, compared with the control cells under inflammation (IL-1β), the gene expression of occludin was incremented 1.6 fold, 2.6 fold with the intermediate dose, and 1.6 fold with the high dose. *L. rhamnosus* did not exert any effect in the gene expression of occludin and the low dose of *L. sakei* reduced 2.5 fold the mRNA abundance of occludin compared with IL-1β control. Lastly, the high dose of *L. reuteri* increased the gene expression of occludin (2.4 fold) when compared with the inflammation elicited with IL-1β control.

Figure 4. Occludin expression. Relative units of gene expression of occludin in an endometrium in the absence (a) or in the presence of inflammation (b) (n = 6 for each condition). Bars represent means ± SE for the different treatments. Bars with asterisk are different ($P < 0.0001$) from negative control (a) or IL-1β (b).





3.3 Innate immunity

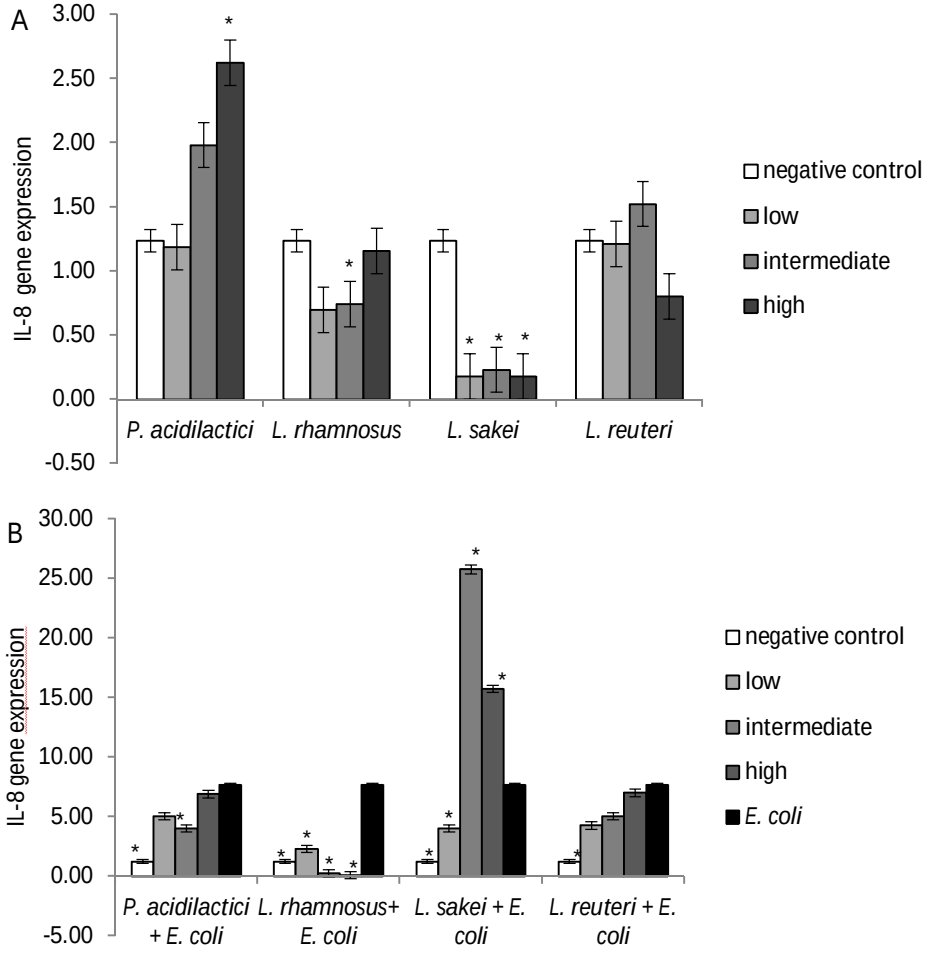
3.3.1 Infection model

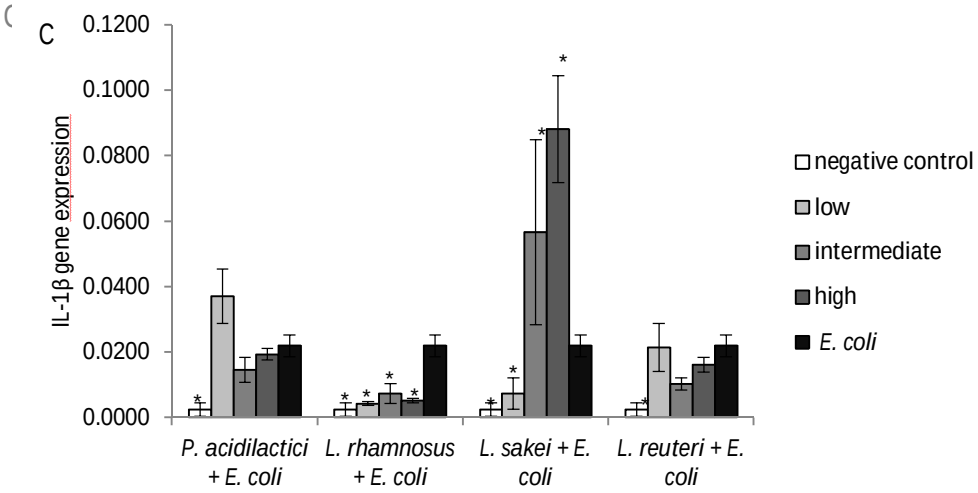
The expression of *IL-8, IL1-β, TNF-α* and *IL-6* were evaluated after the LAB treatment of endometrial cells and after the *E. coli* infection of either LAB-treated or not treated cells. Significant differences were observed in IL-8 and IL1-β mRNA abundance. The IL-8 gene expression was affected by LAB treatment ($P < 0.0001$) when the cells were not infected with *E. coli* (Figure 5A) and also ($P < 0.0001$) in the presence of infection (Figure 5B). When the cells were treated with the high dose of *P. acidilactici,* a 2.13-fold increment of IL-8 mRNA abundance was observed compared with the negative control. The intermediate dose of *L. rhamnosus* decreased the gene expression of IL-8 1.66 fold, and *L. sakei* lowered 5.35 fold the gene expression of IL-8 for all the tested doses when compared with the negative control (Figure 5A).

E. coli infection increased 6.23 fold the gene expression of IL-8 with respect to the negative control. When *E. coli* infected the cells, the presence of *P. acidilactici* at the intermediate dose downregulated the gene expression of IL-8 (1.91 fold).The presence of *L. rhamnosus* reduced the mRNA abundance of IL-8 in infected cells up to 3.34 fold with the low, 28.37 fold with the intermediate, and 85.1 fold with the high dose. When the infected cells were treated with *L. sakei,* the gene expression of IL-8 varied depending on the dose: with the low dose showing a 1.92-fold reduction, and the intermediate and high doses showing and increase in mRNA abundance of IL-8 (3.36 and 2.05 fold, respectively). No significant differences were obtained when *L. reuteri* was added to the infected cells (Figure 5B).

The gene expression of IL1-β was not affected (*P* =0.123, data not shown) by LAB treatment when endometrial cells were not infected by *E. coli*. However, the gene expression of IL1-β after *E. coli* infection was modified (*P* < 0.0001, Figure 5C) by LAB pre-treatment. The mRNA abundance of IL-1β by the endometrial cells infected with *E. coli* increased 9.57 fold respect to the negative control, but this increment was reduced when cells were treated with *L. rhamnosus* using either the low, intermediate, or high dose (5.24, 3.01, and 4.23 fold, respectively). However, when the intermediate and high doses of *L. sakei* were used, the gene expression of the IL-1β was increased (2.58 and 4.01 fold, respectively). Lastly, no differences in IL-1β mRNA abundance in infected cells were observed when cells were treated with *P. acidilactici* and *L. reuteri* before inducing the infection. No differences were observed in the gene expression of TNF-α and IL-6 (data not shown).

Figure 5. Endometrium innate response. Relative units of gene expression of IL-8 when the cells were treated with LAB in the absence (a) or in the presence of an *E. coli* infection (b). Expression of IL-1β (c) with infected cells (n = 6 for each condition). Bars represent means ± SE (a, c) or SEM (b) for the different treatments. SEM are based on transformed data; whereas SE correspond to un-transformed data. Bars with asterisk are different (*P* < 0.0001) from negative control (a) or *E. coli* (b, c).





3.3.2 Inflammation model

The gene expression of IL-8 in the inflammation model of endometrial cells (pre-treated with 10 µg/µL of IL-1β) was as well affected by LAB treatment with ($P < 0.0001$, Figure 6B) or without E. *coli* infection ($P < 0.0001$, Figure 6A). The pre-treatment of endometrial cells with IL-1β increased the mRNA abundance of IL-8 by 15 fold. In the presence of inflammation, when the endometrial cells were treated with *P. acidilactici,* the gene expression of IL-8 decreased when using the low (1.66 fold), intermediate (1.85 fold) or the high dose (1.73 fold). Also, the treatment with *L. rhamnosus* dropped the mRNA abundance of IL-8 about 2.05 fold with the low, 2.9 fold with the intermediate, and 2.27 fold with the highest dose. No differences were observed with *L. sakei* or *L. reuteri* (Figure 6A).

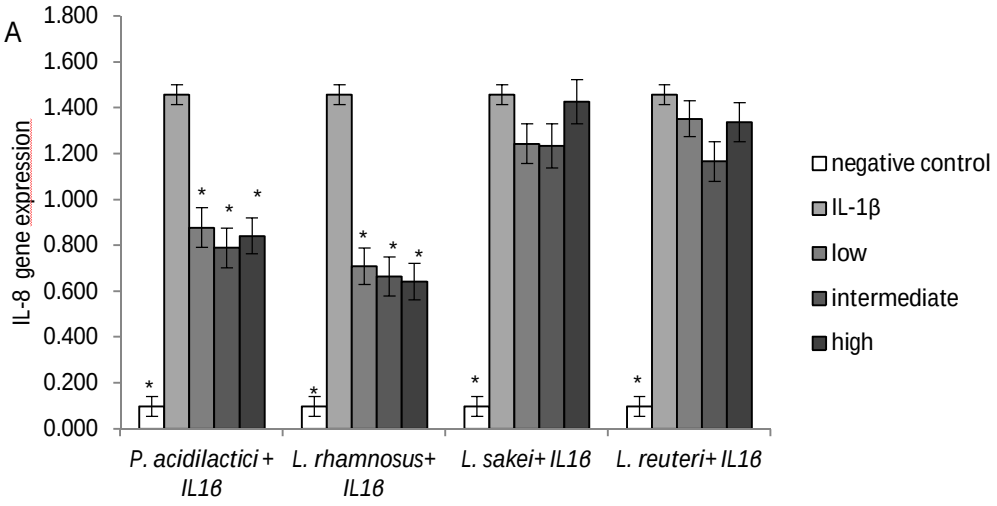
In the presence of inflammation, *E. coli* infection increased even more (2.27 fold) the mRNA abundance of IL-8, when compared with the non-infected endometrial cells. The gene expression of IL-8 of pre-inflammated and post-infected endometria was reduced up to 2.45 fold with the low and 2.63-fold with the intermediate dose of *P. acidilactici*. The intermediate dose of *L. reuteri* reduced up to 2.35 fold the gene expression of IL-8 when the cells were infected. On the contrary, the intermediate dose of *L. sakei* caused a 1.02-fold increase in the mRNA abundance of IL-8 compared with the *E. coli* infection along with the high dose (1.69 fold). Lastly, no significant differences were observed when the infected cells were treated with *L. rhamnosus* (Figure 6B).

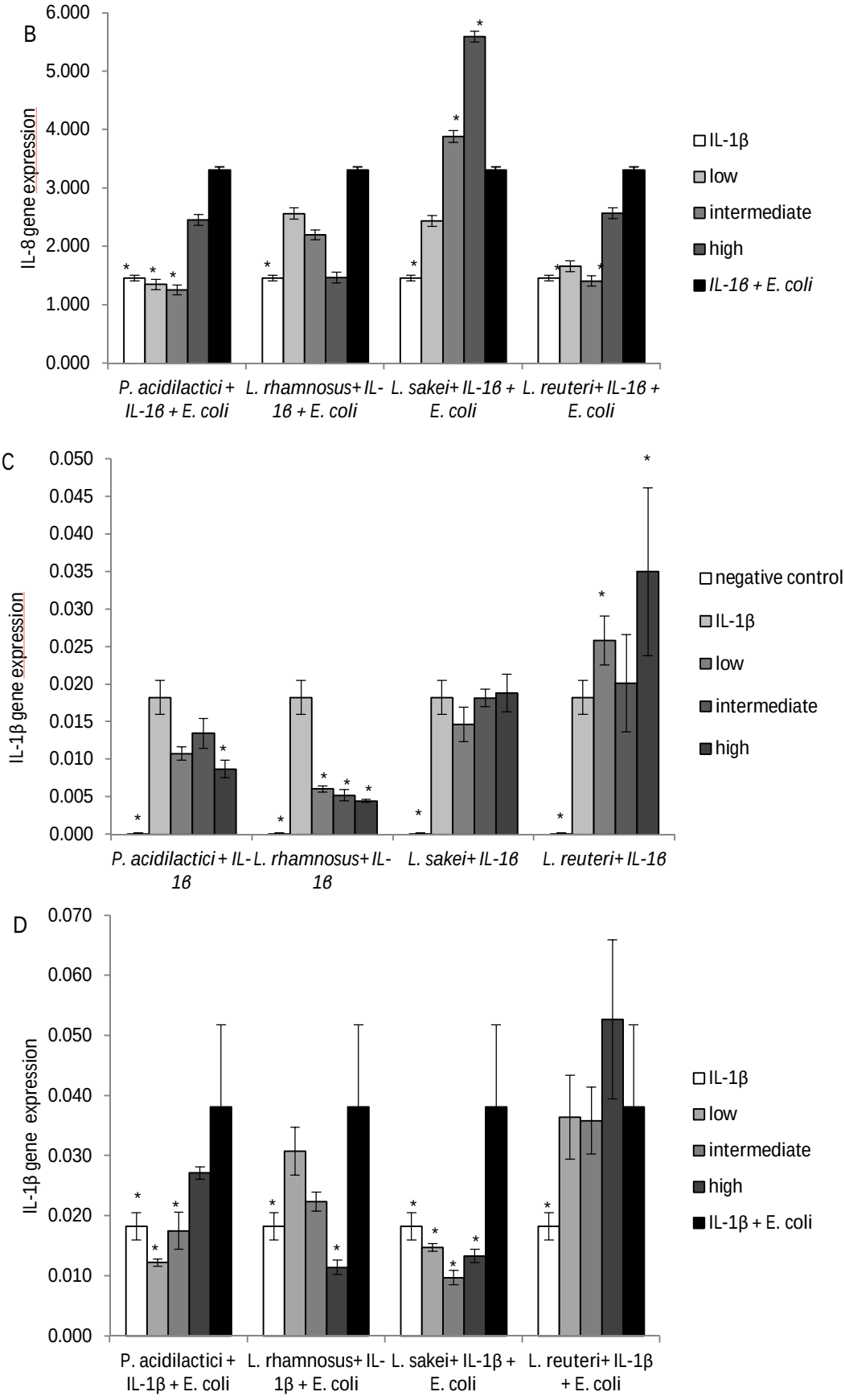
The gene expression of IL-1β in the endometrial cells in the presence of inflammation was affected ($P < 0.0001$) by LAB treatment both, when the cells were not infected (Figure

6C) and when the infection was induced (Figure 6D). The inflamed endometrial cells overexpressed IL-1β about 18 fold more than the negative control did. The high dose of *P. acidilactici* decreased 2 fold the gene expression of IL-1β in inflamed endometrial cells. Also the low, intermediate, and high doses of *L. rhamnosus* decreased IL-1β mRNA abundance in inflammated endometrial cells (3, 3.6, and 4.5 fold, respectively). No differences were observed with *L. sakei*. The low and the high doses of *L. reuteri* caused a 1.44 and 1.94-fold increase in the gene expression of IL-1β (Figure 6C).

E. coli infection increased the gene expression of IL-1β 2.11 fold compared with non-infected but inflammated cells. The mRNA abundance of IL-1β was reduced by the low and intermediate doses (3.17 and 2.24 fold, respectively) of *P. acidilactici*. A 3.45-fold reduction was also observed with the high dose of *L. rhamnosus*. *L. sakei* diminished (2.53 fold) the gene expression of IL-1β with the three tested doses. No changes were found when the cells were treated with *L.reuteri* (Figure 6D), and no differences were found in the gene expression of TNF-α and IL-6 (data not shown).

Figure 6. Innate response of endometrium in the inflammation model. Gene expression of IL-8 when the cells were treated with LAB in the absence (a) or in the presence of an *E. coli* infection (b). Expression of IL-1β with no infection (c) and with infected cells (d) (n = 6 for each condition). Bars represent means ± SEM (a, b) or SE (c, d) for the different treatments. SEM are based on transformed data; whereas SE correspond to un-transformed data. Bars with asterisk are different ($P < 0.0001$) from IL-1β (a, c) or IL-1β + *E. coli* (b, d).




B
IL-8 gene expression
6.000
5.000
4.000
3.000
2.000
1.000
0.000
IL-1β
low
intermediate
high
IL-1β + E. coli
P. acidilactici + IL-1β + E. coli
L. rhamnosus+ IL-1β + E. coli
L. sakei+ IL-1β + E. coli
L. reuteri+ IL-1β + E. coli
C
IL-1β gene expression
0.050
0.045
0.040
0.035
0.030
0.025
0.020
0.015
0.010
0.005
0.000
negative control
IL-1β
low
intermediate
high
P. acidilactici + IL-1β
L. rhamnosus+ IL-1β
L. sakei+ IL-1β
L. reuteri+ IL-1β
D
IL-1β gene expression
0.070
0.060
0.050
0.040
0.030
0.020
0.010
0.000
IL-1β
low
intermediate
high
IL-1β + E. coli
P. acidilactici + IL-1β + E. coli
L. rhamnosus+ IL-1β + E. coli
L. sakei+ IL-1β + E. coli
L. reuteri+ IL-1β + E. coli

4. DISCUSSION

Lactic acid bacteria are, under normal conditions, present in the vaginal tract of dairy cows [100]. It is known that vaginal *Lactobacilli* inhibit the growth of genitourinary pathogenic microorganisms through mechanisms of competitive exclusion of pathogens, stimulation of the host immune system, and production of specific antibacterial compounds [99, 100]. Also in the food industry, some microorganisms commonly associated with meat have proved to be antagonistic towards pathogenic bacteria; in particular *Lactobacillus sakei* has been proposed to enhance microbial safety of non-fermented meat products [109]. In this study, we have evaluated *in vitro* the potential of four LAB species at modulating, not only bacterial infection, but also tissular inflammation of the bovine endometrium, which are the two main processes causing uterine disease in the cow postpartum. The *E. coli* infection experiments (Figure 3) indicated that *P. acidilactici*, *L. sakei* and *L. reuteri* have a high potential at inhibiting *E. coli* infection in the endometrial cells. This capacity was not well correlated with the ability to form biofilms by the four LAB species (Table 2). Although *P. acidilactici* presented the highest capacity to form biofilms, it was equivalent to that of *L. rhamnosus,* which did not perfome well at inhibiting *E. coli* infection. Also, *L. sakei* showed a low adherence in the biofilm experiment, but was the most effective in inhibiting *E. coli* infection.

Tight junctions strictly seal the intracellular spaces and contribute establishing an efficient barrier against intracellular infections. A leaking barrier can favor pathogen and other antigen passage [118]. Occludins are transmembrane proteins that form part of tight junctions and interact with the adjacent cells. Pathogenic bacteria efficiently produce defects in cell to cell contact, delocalizing tight junction proteins [120, 130-132]. Hence, an increase in the expression of tight junction proteins could partially explain the ability of LAB to reduce *E. coli* infection. However, the gene expression of occludin (Figure 4) did not corroborate this hypothesis. Occludin was not always upregulated in the endometrium treated with *P. acidilactici*. It was ony up-regualted when an inflammation was present, which was when, interestingly, *P. acidilactici* did not reduce *E. coli* infection. Also, the increased expression of occludin was observed randomly in some doses of *L. reuteri* and *L. sakei* but not in all the cases associated with *E. coli* reduction. Other modes of action, such as a direct acidification of the medium, because of the metabolic activity

of LAB [99], or the production of antimicrobial compounds could account for a direct reduction of *E. coli* infection [97]. In this context, it is known that *Pediococci* produce antimicrobial compounds such as organic acids, hydrogen peroxide, and antimicrobial peptides including pediocin AcH/PA-1 [100], and *L. sakei 5*, used in the brewery industry, produces sakacin T and sakacin X, identified as IIb bacteriocins [133]. Although the mechanisms by which these organisms reduce infection are still unclear, but they could involve: 1. an ability to adhere to and populate the vaginal epithelium and mucin layer, 2. to inhibit pathogens from invading the tissue, 3. to reduce pathogen virulence, and 4. to modulate host defenses. For example, *L. sakei* may have the capacity to inhibit pathogens from adhering to the cells, and *L. rhamnosus* may be able to modulate host defenses [99, 134]. Other more complex mechanisms can aslo be involved, such as the production of toxins. For instance, some strains of *L. reuteri* are able to produce one or more molecules that inhibit the expression of toxins secreted by *Staphylococcus aureus* leading to an inhibition of the infection [111].

When the *E. coli* infection was combined with tissular inflammation, there was only a single dose of *L. reuteri*, among all tested LAB, that showed an ability to reduce *E. coli* infection. This corroborated what has previously been described in the field: uterine infection on a tissue with inflammation makes the eradication of the pathogen more difficult [135, 136] and reinforces the importance of exploring new strategies to prevent inflammation of the uterus during postpartum.

Several studies have indicated that *Lactobacillus* species exert a favorable influence in human health by decreasing the level of cytokines involved in inflammation [137]. It is known that the endometrium regulates the inflammatory response after an infection by synthesizing and releasing cytokines and chemokines. TNF-α and IL-1β stimulate the expression of chemokines such as IL-8 and molecules adhesive to the vascular endothelial cells, leading to neutrophile and monocyte chemoattraction [126], and IL-6 stimulates the expression of acute phase proteins [10, 89].

The ability to reduce the inflammation by the four LAB tested herein was also assessed (Figure 5 and 6). *L. sakei* reduced the gene expression of IL-8 in endometrial cells but, surprisingly, the high and intermediate doses increased the abundance of IL-8 and IL-1β

mRNA when the endometrial cells were infected with *E. coli*. On the contrary, the low dose of *L. sakei* diminished the gene expression of IL-8 and IL-1β independently of the presence of infection or inflammation. Altogether, these results indicate that depending on the dose used of *L. sakei*, the response of endometrial cells change dramatically, disqualifying this bacterium as a potential probiotic. Although *P. acidilactici* at high dose slightly increased the gene expression of IL-8 in the endometrial cells, under infection and inflammation, there was a consistent effect to reduce inflammation, mainly with the intermediate and low doses, which were also the ones most efficient at fighting *E. coli* infection. *L. rhamnosus* did not present any potential at inhibiting *E. coli* infection (there was only a moderate inhibition with the high dose), but it demonstrated a high potential to downregulate inflammation; mainly when there was an acute inflammation caused by infection. However, there was a dose-dependent response that led to a progressive downregulation of IL-8 and IL-1β gene expression as the dose of *L. rhamnosus* dose increased. Lastly, *L. reuteri* did not exert an immunomodulatory response despite its capacity to reduce *E. coli* infection, and depending on the dose used there was a potentiation of endometrial inflammation, which would be nocive to the animal.

5. CONCLUSIONS

In conclusion, some LAB have great potential to modulate endometrial infection and inflammation. *P. acidilactici* presented both, the ability to inhibit *E. coli* infection and also downregulating inflammation, mainly when intermediate and low doses were used. *L. rhamnosus* presented a great capacity to reduce inflammation in the endometrium, mostly in the presence of an acute inflammation caused by bacterial infection. Although *L. sakei* and *L. reuteri* are effective at preventing *E. coli* infection, they present a dose-variable response on tissular inflammation that could lead to negative consequences to the host. Further studies assessing the effect of combining different doses of LAB probiotics could be of relevant importance to establish an adequate balance between reducing pathogen infections and modulating the inflammation of the endometrium before *in vivo* studies could be conducted.

CHAPTER IV: Study 2

A combination of Lactic Acid Bacteria Regulates *Escherichia coli* Infection and Inflammation of the Bovine Endometrium

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This research has been accepted in:

Journal of Dairy Science, September 2016

1. Introduction

Uterine function is often compromised in cattle by bacterial contamination and inflammation after calving [35, 36, 114]. 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% [138], and is characterized by an enlarged uterus and a watery red-brown fluid uterine discharge, which has a fetid odor [34]. 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 [33, 35, 36, 45, 114, 115], and thus the problem may occur unnoticed. The economic impact 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€ [44, 45].

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 [68]. It is generally considered that bacterial infection fosters the recruitment of immune cells and leads to acute inflammation in the uterus [51]. This process coexists with post-partum 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 [139]. In this context, efforts to reduce uterine infection 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 [140].

The mechanisms of infection and immunity in the endometrium have mostly been studied *in vitro* [34, 51], whereas the characterization of uterine infection and insights into the pathogenesis of disease often stem from observational studies of spontaneous post-partum uterine infections *in vivo* [36, 45, 114]. 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 [89]. The study of tissue

explants collected from the endometrium *ex vivo* allows investigation of potential links between whole animal clinical signs and cellular function [141]. 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 it does not typically improve reproductive performance of the treated animals [97, 142], the use of probiotic strains of bacteria has been proposed as an alternative to prevent postpartum uterine infections and inflammation [107, 121]. In our recent work, various individual strains of LAB have been shown to protect against *E. coli* infection *in vitro* and reduce inflammation in epithelial cells [143]. The hypothesis of present work is that the combination of LAB strains tested in our previous work [143] 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 uterine inflammation caused by basal tissue inflammation, such as that associated with calving, or by acute factors such as *E. coli* infection.

2. Materials and methods

2.1 Bacterial strains and culture conditions

Pediococcus acidilactici (CECT 5915) and *Lactobacillus rhamnosus* (CECT 278) were purchased from CECT (Colección Española de Cultivos Tipo, CSIC Valencia, and 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 four 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 (Biomiéreux, Craponne, France). The *E. coli* selected was FimH positive (virulence factor) and hemolytic. Bacterial cultures were performed inoculating 1 mL of a glycerinate in 9 mL of Luria Bertani (LB) medium (10 g/L peptone, 5 g/L yeast extract and 10 g/L NaCl) for *E. coli* or Man, Rogosa, and Sharpe medium (MRS, Scharlau, Sentmenat, Spain) for *L. rhamnosus, P. acidilactici,* and *L. reuteri* at 37ºC in static conditions overnight (O/N). Then, bacteria were centrifuged at 6,000 x *g* for 15 min and resuspended at $1x10^8$ colony-forming units (CFU)/mL with DMEM (Gibco, NY, USA) supplemented with 8 µg/mL bovine insulin and 50 µg/mL hydrocortisone. *E. coli* CFU/mL was confirmed by serially diluting and plating into MacConkey (Scharlau, Sentmentat, Spain) agar media. Colonies were counted after an O/N growth at 37ºC.

2.2 Endometrial primary culture

Endometrial tissue was obtained at a slaughterhouse from post-pubertal non-pregnant 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. Then, tissue was cut into small pieces and incubated with PBS supplemented with 1xPEF, 0.1 mM EDTA, and 0.1 mM DTT for 10 min at 37ºC in 5% CO_2 at 200 rpm. Then, the supernatant was removed and RPMI 1640 medium (Gibco, NY, USA) with 0.25% collagenase (Gibco) was added and further incubated 15 min at 37ºC in 5% CO_2 at 200 rpm. Supernatant was collected and mixed (1:1) with RPMI 1640 media enriched with 0.02 mg/mL DNasa (Roche, Mannheim, Germany). Cells present in the supernatant were recovered after centrifuging 5 min at 300 x *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 (EGF; Sigma-Aldrich, St. Louis, USA). Hydrocortisone was included in the medium to foster cell growth [124]. Cells were quantified using a haemocytometer and incubated at 160,000 cells/cm^2 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 [143]. For the immunofluorescence, endometrial primary cells were grown in 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 µL of PBS containing goat serum (0.05% v/v) 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 1:500 (monoclonal anti-cytokeratin pan antibody produced in mouse; Sigma-Aldrich). After 3 washes with PBS, secondary antibody, an antimouse-FITC, was incubated for 1 h at room temperature at a dilution 1:1000 (Sigma-Aldrich). Lastly, cells were washed and dried coverslips mounted on glass slides using Fluoroprep and observed by fluorescence microscopy.

2.3 *Ex vivo* bovine endometrial explants

Segments of endometrial tissue were obtained at a slaughterhouse from 20 post-pubertal non-pregnant 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. The tissue was cut into 0.8 cm^2 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, NY, USA) supplemented with 10% FBS, 50 µg/mL streptomycin, 50 IU/mL penicillin, 2.5 µg/mL amphotericin B in 5% CO_2 at 37ºC. These experiments were repeated on 4 different days (each day involving five independent animals to reach a total of 20 cows) and using in each experiment 6 replicates/animal for each *ex vivo* treatment.

2.4 *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 µg/mL bovine insulin, 50 µg/mL hydrocortisone, and kept O/N at 37ºC and 5% CO_2. Cells were incubated with six 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 2 bacteria/endometrial cell, in the presence of basal inflammation). The basal inflammation was induced with 10 µg/µL of IL-1β as described elsewhere [143].Then, cells were challenged with $4x10^6$ 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-1β and medium. Cells were washed twice with chilled PBS and associated bacteria were released with 500 µL 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 an O/N growth at 37ºC. Cells used for gene expression analyses were washed twice with PBS and harvested with 500 µL of TriZol (5Prima, Gaithesburg, USA) for RNA extraction.

Table 1: Combinations of LAB used in this study. Ratio of each LAB corresponds to the number of LAB vs epithelial cells.

Combination	Ratio *L. rhamnosus*	Ratio *P. acidilactici*	Ratio *L. reuteri*
A	100	25	0
B	25	25	0
C	100	25	2
D	25	25	2

2.5 Ex vivo experiments: LAB treatment, inflammation and *E. coli* infection

Explants were incubated in six replicates O/N with the selected LAB combination and then challenged with $1.5x10^6$ 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-1β was added at 10 ng/well 1 h before LAB administration. In this case explants inflammated and infected were used as an infection control and, explants inflammated and treated with LAB were used as a negative control. Supernatant was collected for further Il-8, IL-1β, and IL-6 ELISA quantification.

2.6 Quantitative RT-PCR analysis

One microgram of RNA was retrotranscribed 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 µl was used, containing 50 ng of cDNA, 10 µl of SYBR Green Fluorescent (Bio-Rad, Hercules, CA, USA), and the optimized primer concentration for each gene (Table 2). The qPCR reactions were cycled as follows: an initial denaturing step of 10 min at 95ºC, followed by 40 cycles of 10 s at 95ºC, 15 s at the optimized annealing

temperature for each gene, 30 to 60 s at 72ºC and a final extension of 10 min at 72ºC. Relative gene expression was calculated using the Pfaffl method [144] 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 [116, 126]. Moreover, we checked that no differences in the expression of *GAPDH* were found among treatments.

Table 2: Sequence, annealing temperature (At), concentration (µM), amplicon size (bp) of forward (Fw) and reverse (Rv) primers used for qPCR and accession number or reference.

Gene	Fw	Rv	At, Cº	µM	Accession number or ref.
GAPDH	GCATCGTGGAGGGACTTATGA	GGGCCATCCACAGTCTTCTG	52	0.125	NM_001034034
CXCL8	TTGAGAGTGGACCACACTGC	TGCACCCAGTTTTCCTTGG	55	0.5	[145]
IL1B	TGGGAGATGGAAACATCCAG	TTTATTGACTGCACGGGTGC	50	0.3125	M37211

2.7 Enzyme-linked immunosorbent assay (ELISA)

Concentrations of IL-1β, IL-6 and IL-8 were measured in supernatants from explants by ELISA according to the manufacturers' instructions: Bovine IL-1β ELISA Kit (ESS0027; ThermoFisher Scientific, Cramlington, UK); Bovine IL-6 ELISA Kit (ESS0029; ThermoFisher Scientific, Cramlington, UK); 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 [141, 146].

2.8 Field emission scanning electron microscopy (FESEM)

Microdrops of *Lactobacillus rhamnosus, Pediococcus acidilactici,* and *Lactobacillus reuteri* cultures were directly deposited for 2 min onto silicon wafers (Ted Pella Inc., Redding, CA, USA), 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 1kV.

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% (v/v) glutaraldehyde

(Merck, Darmstadt, Germany) in phosphate buffer (PB) 0.1M at pH 7.2 (Sigma-Aldrich, Steinheim, Germany) for 2 h at room temperature, rinsed 4 times with PB, post-fixed with 1% (w/v) osmium tetraoxide (TAAB Lab., Reading, UK) in PB for 2 h at 4ºC, rinsed 4 times in PB, dehydrated in ethanol (Panreac, Barcelona, Spain), and dried with CO_2 in a critical point drying system (Bal-Tec AG, Bakzers, Liechtenstein). Samples were mounted in stubs and observed without coating with a Zeiss Merlin microscope under the same conditions as LAB samples.

A qualitative 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 cell surface, and cell debris.

2.9 Transmission electron microscopy (TEM)

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% (w/v) paraformaldehyde (TAAB Lab., Reading, UK) and 2.5% (v/v) glutaraldehyde (Merck, Darmstadt, Germany) in 0.1 M PB (Sigma-Aldrich, Steinheim, Germany), post-fixed for 2 h with 1% (w/v) osmium tetroxide (TAAB Lab., Reading, UK) containing 0.8% (w/v) potassium hexocyanoferrate (Sigma-Aldrich, Steinheim, Germany) in PB, sequentially dehydrated in acetone, embedded in Eponate 12™ resin (Ted Pella Inc., Redding, CA, USA), 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 80kV and equipped with a CCD Gatan ES1000W Erlangshen camera.

A semiquantitative analysis was performed on 10 ultrathin sections randomly chosen from three different areas per treatment. The parameters studied were presence of mucus on 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 showed higher size, round shape, non-homogeneous matrix electrodensity, and/or disorganized or disappeared cristae, necrotic cells showed non-homogeneous and disorganized cytosol and conserved nucleus (see representative images in Fig 8).

2.10 Statistical analysis

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 non-transformed data ± SEM obtained with normalized data (unless otherwise stated). Data were analyzed using a mixed-effects model (SAS Inst. Inc., Cary, NC, USA), 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., Cary, NC, USA), assigning a value from 0 (-) to 3 (+++) for each parameter. Differences were considered significant when $P < 0.05$.

3. Results

3.1 Selection of LAB combination in vitro

The best combination of LAB strains tested in our previous work [143] was selected *in vitro* taking into account its ability to reduce inflammation and *E. coli* infection.

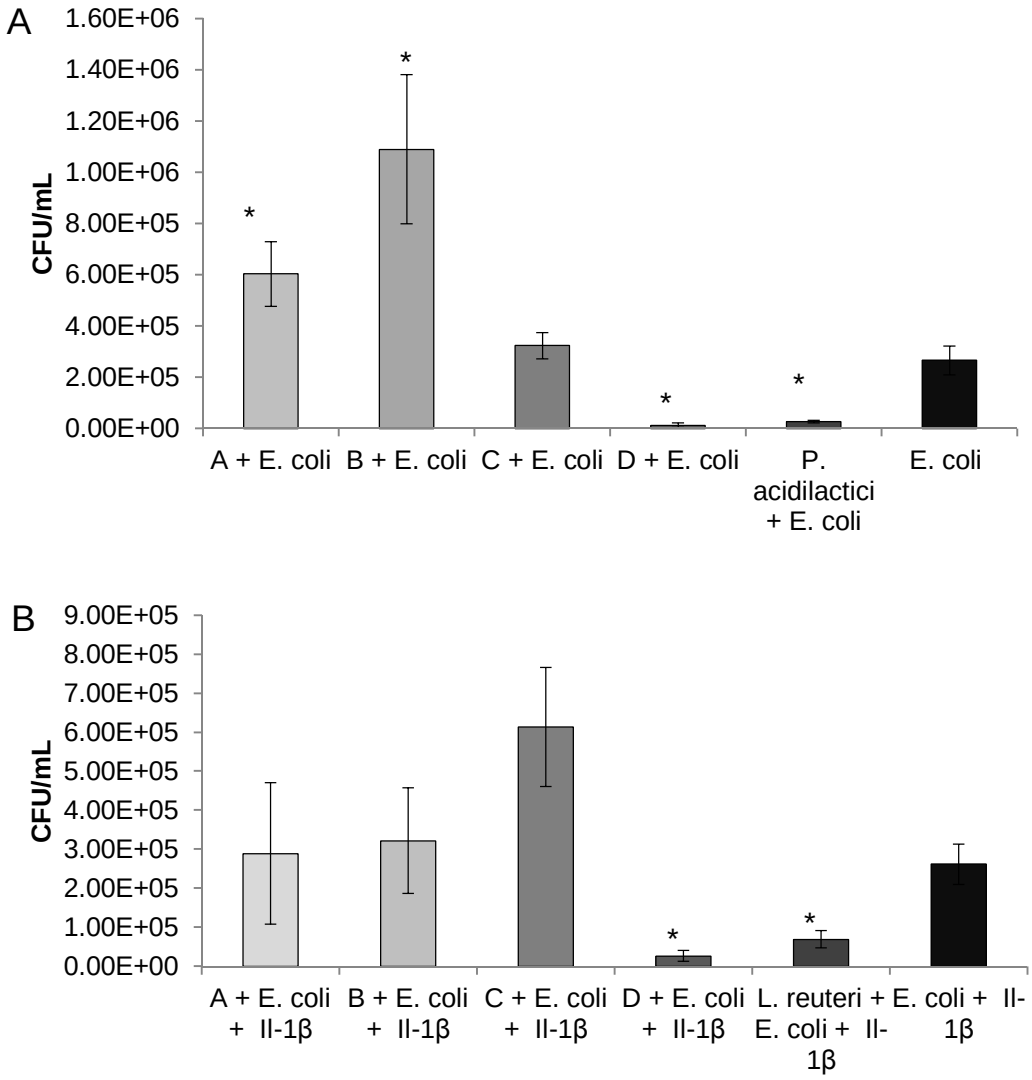
3.1.1 *E. coli* infection.

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%.

The probiotic potential and modulatory effects of the LAB combinations against endometrial *E. coli* infection were similar in the presence (Fig 1A) or absence of a basal inflammation (Fig 1B). Under basal inflammation, there was a reduction of *E. coli* infection

with LAB combination D (89.78%; P < 0.01) and no differences were found with other LAB combinations. LAB 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.

Figure 1: ***E. coli* infection of primary endometrium cultures**. Viable cell counts (CFU/mL) of *E. coli* for each combination of LAB in an endometrium in the absence (A) or in the presence of a basal inflammation (B). Bars represent means ± SE. Bars with an asterisk differ (P < 0.01) from *E. coli*.



3.1.2 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 non-treated endometrial cells. The abundance of *CXCL8* mRNA was affected (P < 0.01) by LAB

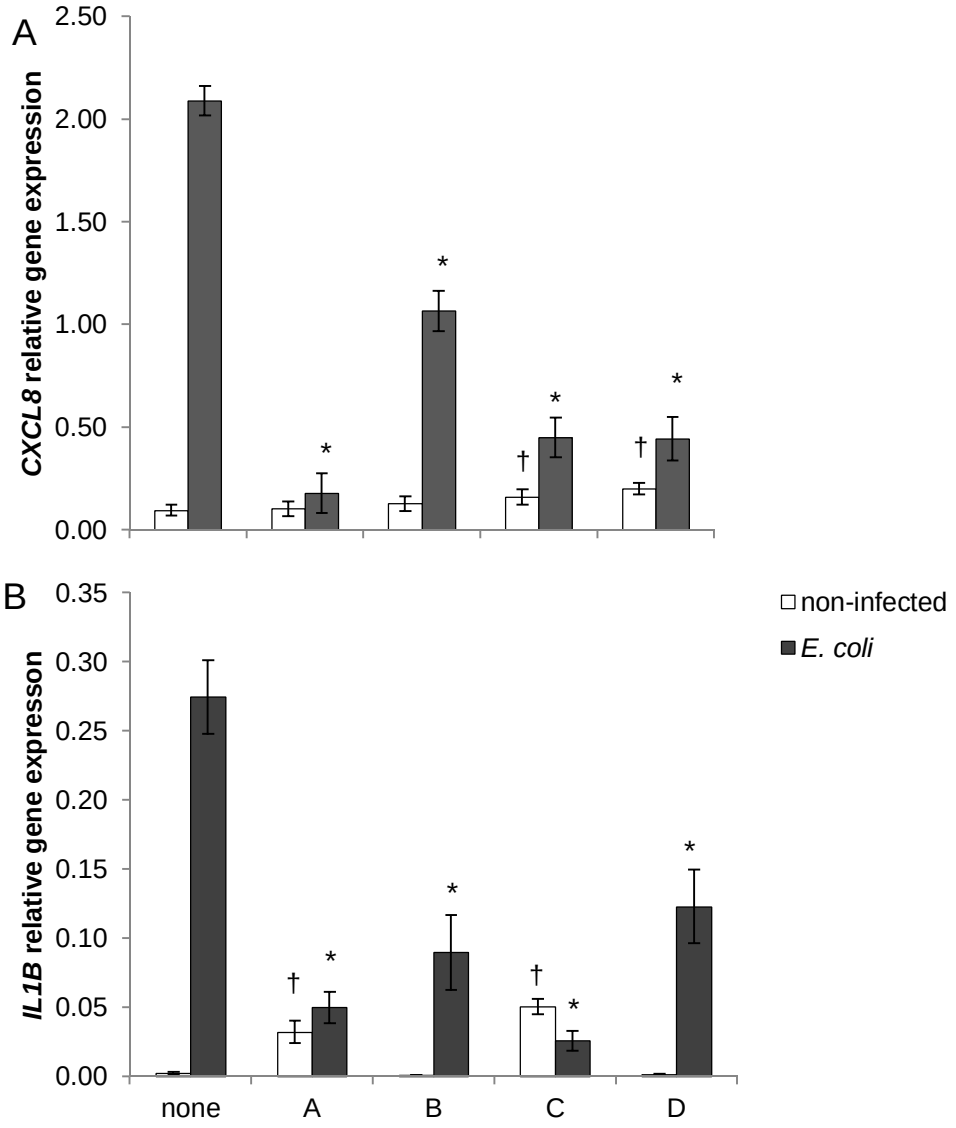
treatment when cells were not infected (Fig 2A, open bars) and also (P < 0.0001) in the presence of infection (Fig 2A, grey bars). Specifically, when cells were treated with the LAB combinations C and D, a 1.7- and a 2.1- fold increment in *CXCL8* expression in epithelial cells were observed, respectively, compared with non-treated 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 (Fig 2A, grey 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, 4.7 fold reduction, respectively; Fig 2A, grey bars).

The gene expression of *IL1B* was affected by LAB treatment (P < 0.0001) when cells were uninfected (Fig 2B, open bars) and also (P < 0.0001) in the presence of infection (Fig 2B, grey 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 (Fig 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; Fig 2B, open bars).

Figure 2: **Innate immune response in primary endometrial cultures under an acute inflammation.** Relative units of gene expression of *CXCL8* when cells were treated with LAB combinations in the absence (Fig 2A, open bars) or in the presence of an *E. coli* infection (Fig 2A, grey bars). Gene expression of *IL1B* in uninfected (Fig 2B, open bars) and infected (Fig 2B, grey bars) cells. Bars represent means ± SEM (Fig 2A) or SE (Fig 2B) for the different treatments. Bars with a cross differ (*P* < 0.01) from the negative control, whereas bars with an asterisk differ (*P* < 0.01) from *E. coli* control. Negative control and *E. coli* control differed at *P* < .0001.



3.1.3 Innate immunity under a basal and an acute inflammation.

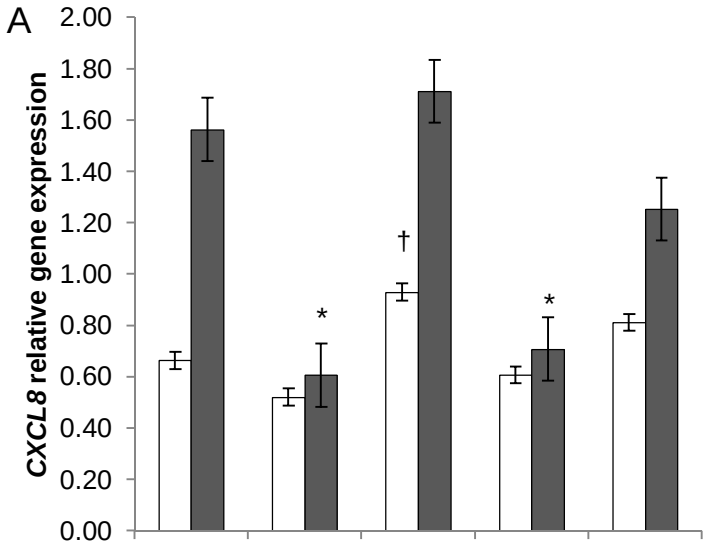
The gene expression of *CXCL8* in the basal tissue inflammation model of endometrial cells (cells pre-treated with 10 µg/µL of IL-1β) was affected by the different LAB combinations both in the presence ($P < 0.0001$; Fig 3A, grey bars) and in the absence of an acute inflammation caused by *E. coli* infection ($P < 0.0001$; Fig 3A, open bars). Pre-treatment of the endometrium with IL-1β 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 (Fig 3A, open bars).

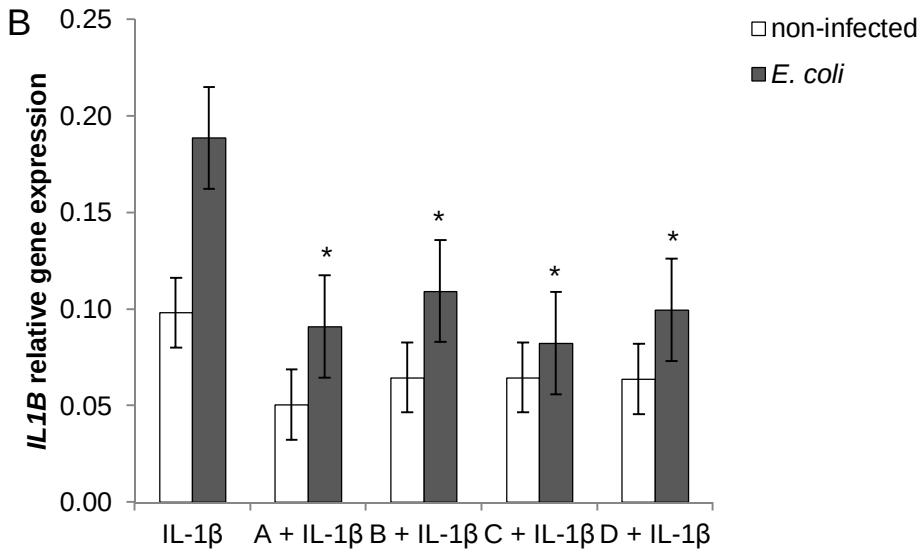
Infection with *E. coli* increased 2.35 fold the expression of *CXCL8* by epithelium under a basal inflammation. When cells were treated with LAB combinations A or C, a decreased

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 (Fig 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 (Fig 3B, grey bars; P < 0.0001). The inflammated endometrium expressed 49 fold more *IL1B* than the negative control. However, no differences were observed when the LAB treatments were applied (Fig 3B, open bars). *E. coli* infection (acute inflammation) raised the expression of *IL1B* 1.93 fold compared with 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 (Fig 3B, grey bars).

Figure 3: **Innate immune response of primary endometrial cultures under a total (acute and basal) inflammation**. Expression of *CXCL8* gene when cells were treated with LAB combinations in the absence (Fig 3A, open bar) or in the presence of an *E. coli* infection (Fig 3A, grey bars). Gene expression of *IL1B* in infected (Fig 3B, open bars and uninfected (Fig 3B, grey bars) cells. Bars represent means ± SEM for the different treatments. Bars with a cross differ ($P < 0.05$) from IL-1β, whereas bars with an asterisk differ ($P < 0.05$) from *E. coli* + IL-1β. Difference Negative control and *E. coli* control differed at $P < .0001$.





3.2 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).

3.2.1 Ex-vivo acute inflammation model.

The secretion of IL-8, IL-1β, and IL-6 was evaluated after an acute inflammation produced by E. coli infection of either LAB-treated or non-treated explants.

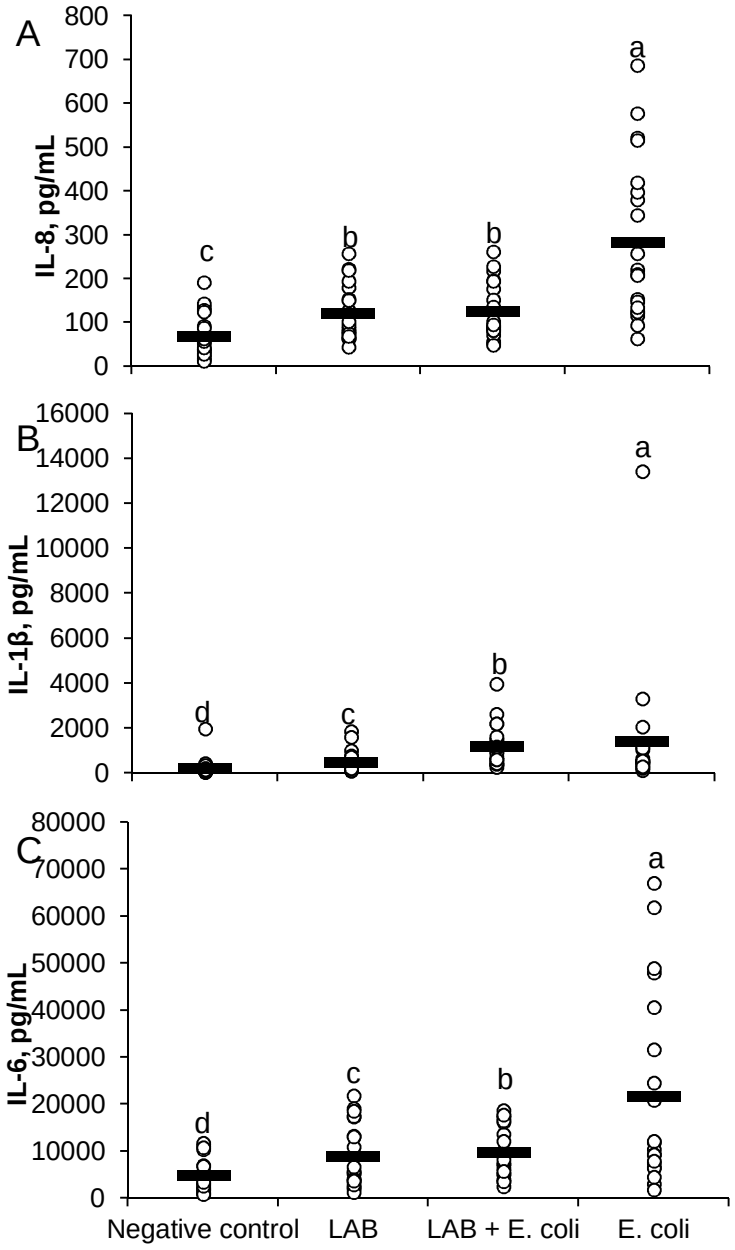
IL-8 secretion was affected ($P < 0.0001$) by *E. coli* infection and an interaction ($P < 0.0001$) between infection and treatment was also observed (Fig 4A). When the probiotic combination was applied to explants, IL-8 secretion increased 1.6 fold compared with the negative control. When infection was induced, the secretion of IL-8 increased 3.7 fold compared with 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-1β was affected by LAB treatment ($P < 0.0001$), infection ($P < 0.0001$), and the interaction between treatment and infection ($P < 0.0001$, Fig 4B). The probiotic combination increased the secretion of IL-1β by 2.1 fold compared with the negative control. When the explants were infected with *E. coli*, the secretion of IL-1β 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-1β 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 ($P < 0.0001$, Fig 4C). The probiotic combination increased the secretion of IL-6 1.8 fold compared with the negative control. When the explants were infected by *E. coli*, secretion of IL-6 increased 4.5 fold, whereas when explants were pre-treated with the probiotics, IL-6 secretion decreased 2.2 fold.

Figure 4: Innate immune response in endometrial explants treated *ex vivo* under an acute inflammation. Secretion (pg/mL) of IL-8 (Fig 4A), IL-1β (Fig 4B), and IL-6 (Fig 4C). Dots represent means for each treatment and cow, whereas lines represent the means for each treatment. Columns with different letters differ ($P < 0.0001$).



3.2.*2 Ex-vivo* total inflammation model.

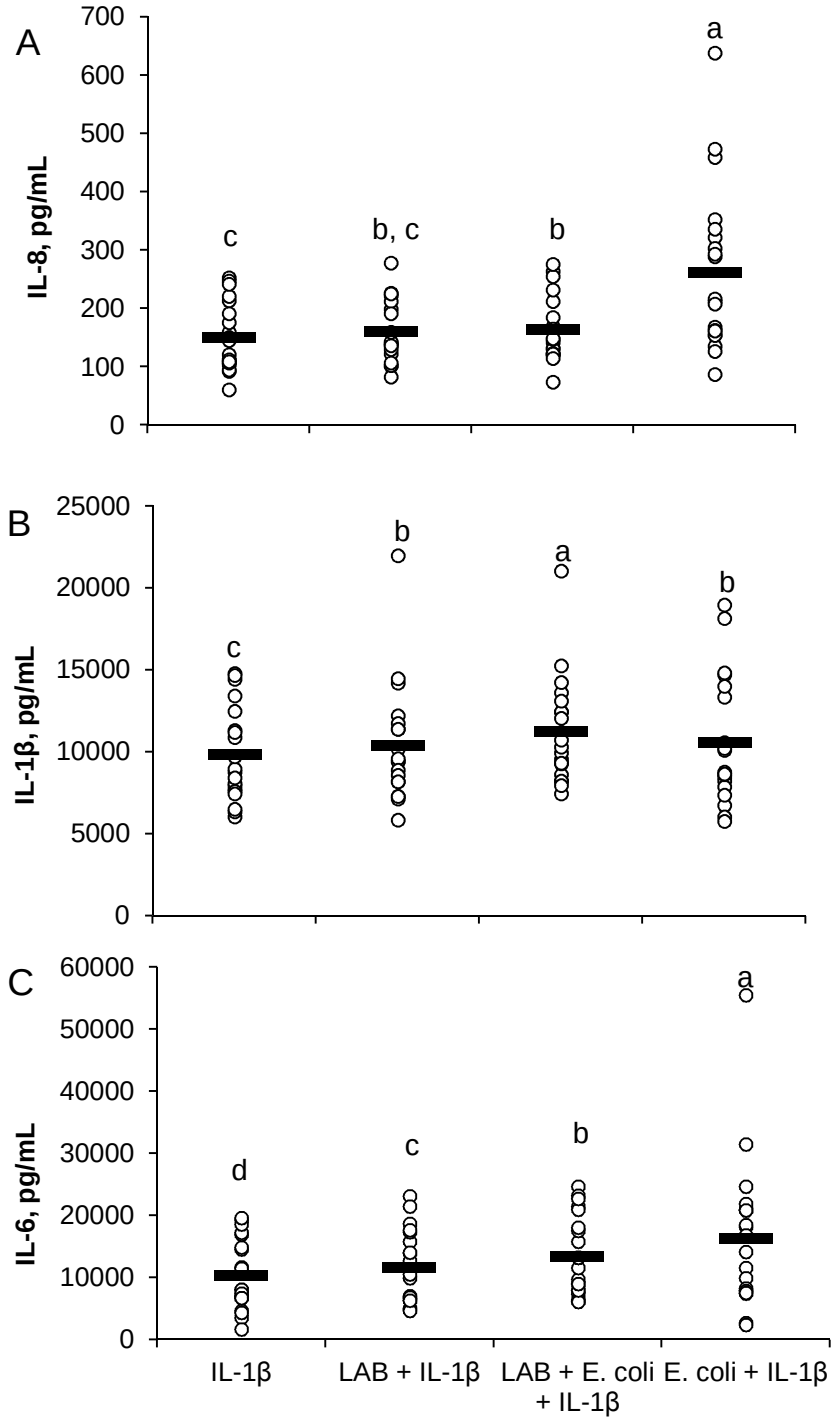
The secretion of IL-8, IL-1β, and IL-6 was evaluated in LAB-treated and untreated endometrial explants after inducing inflammation (basal + acute inflammation).

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

Secretion of IL-1β was affected by infection ($P < 0.0001$), and the interaction between treatment and infection ($P < 0.0005$, Fig 5B). *E. coli* infection increased the secretion of IL-1β 1.1 fold compared with the negative control. When the non-infected explants were treated with LAB, an increment of 1.04 fold was observed compared with 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-1β 1.1 fold compared with the infected explants.

Secretion of IL-6 was affected by infection ($P < 0.0001$), and the interaction between treatment and infection ($P < 0.0002$, Fig 5C). The LAB treatment increased the secretion of IL-6 1.1 fold compared with 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 (Fig 5A), IL-1β (Fig 5B), and IL-6 (Fig 5C).Dots represent means for each treatment and cow, whereas lines are the means for each treatment. Columns with different letters differ ($P < 0.0001$).

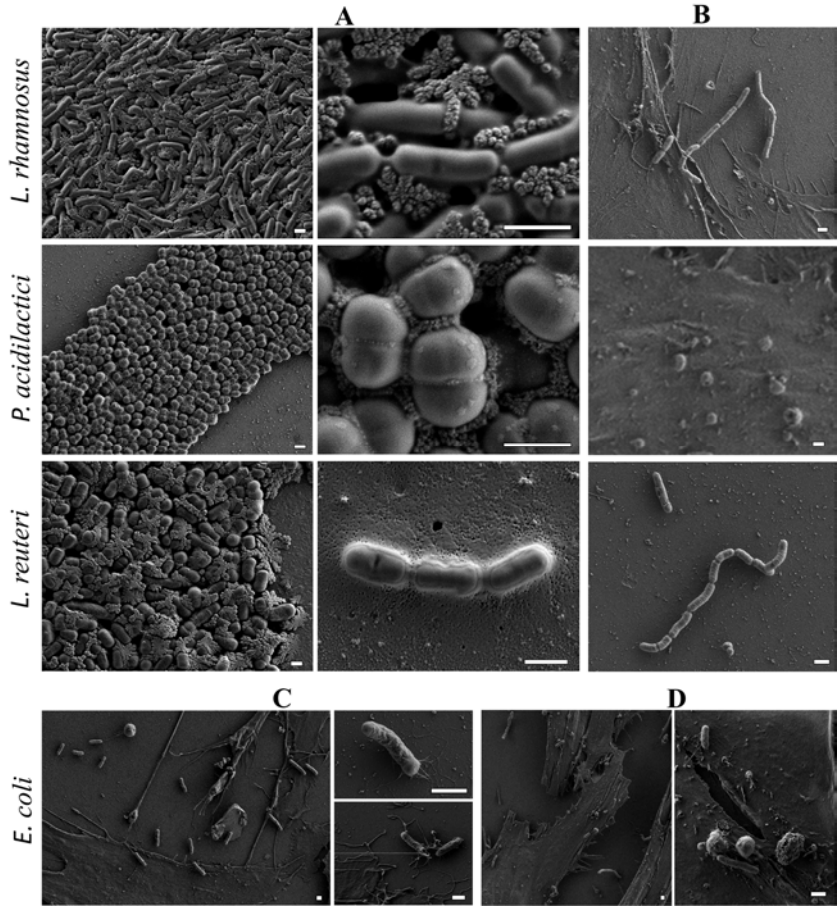


3.3 FESEM

The study of LAB by FESEM showed bacteria with similar size and shape in each culture and also after incubation with primary endometrial epithelia (Fig 6 A-B). Moreover, *E. coli* bacteria were also observed in contact with epithelial cells either incubated alone or with the LAB combination D (Fig 6 C-D). *E. 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 (Fig 6 C-D). *E. coli* abundance was lower in samples treated with LAB than in those infected with *E. coli* alone ($P < 0.05$).

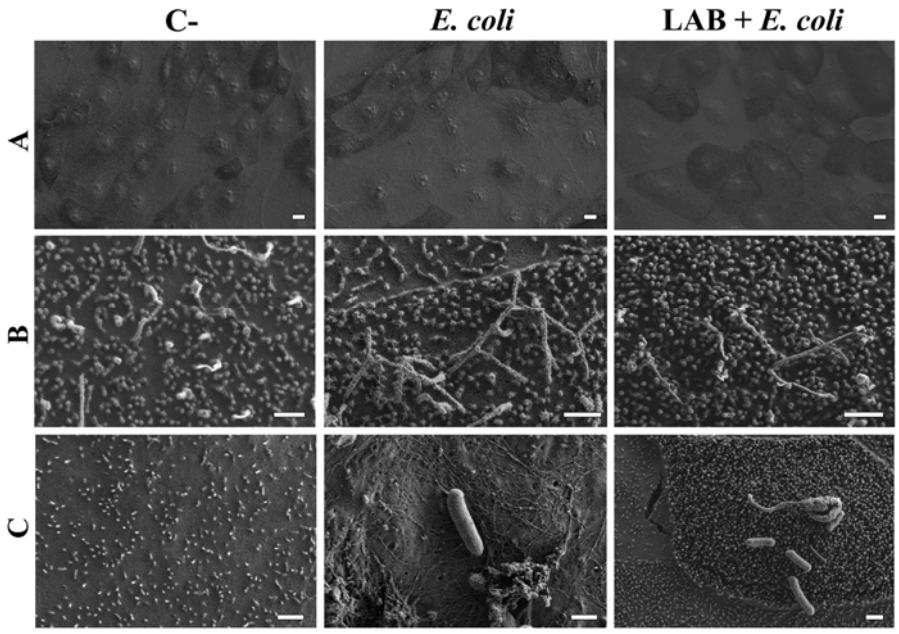
Figure 6: Representative images of LAB and *E. coli*. General view and detail of LAB cultures (Fig 6A). Detail of LAB incubated with epithelial cells (Fig 6B). General view and details of *E. coli* incubated alone (Fig 6C) or with LAB combination D (Fig 6D; *L. rhamnosus*, *P. acidilactici*, *L. reuteri*). Bars size: 1 µm.



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 in were observed on the surface of epithelial cells (Fig 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.*

Figure 7: Representative images of epithelial cells of negative control, *E. coli* and LAB with *E. coli*. General view of epithelial cell surface showing similar aspect in the 3 treatments (Fig 7A). Detail of cell surface of the 3 treatments showing microvilli longer and more abundant in epithelia

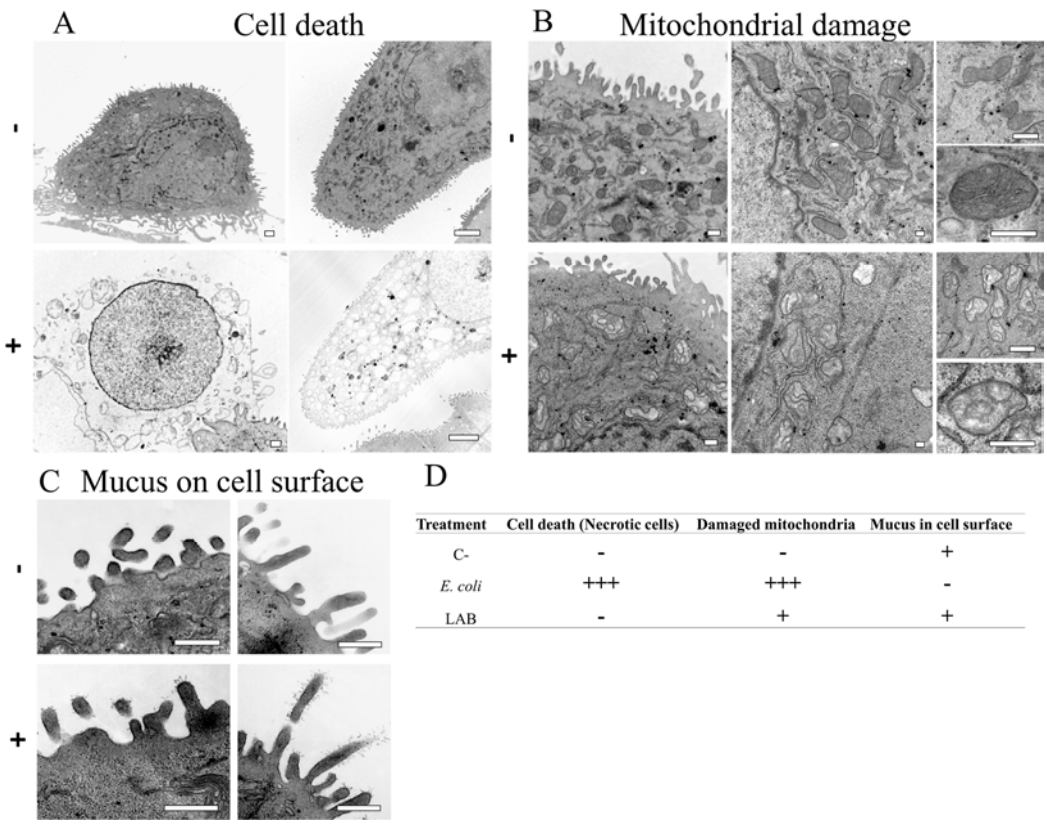
treated with *E. coli* alone and *E. coli* with LAB (Fig 7B) than with control cells. Detail of *E. coli* bacilli (rod-shape bacterium) with cells covered with cell debris (misshapen rests of degraded organelles and other cell components) or detail of *E. coli* with LAB associated to cells with a normal aspect (Fig 7C). Bars size for general view: 5 µm, for the rest: 1 µm.



3.4 TEM

Quantitative data by TEM clearly illustrated the highest incidence of necrosis in among all samples infected with *E. coli*, indicating a clear harmful effect of this pathogen in our model ($P = 0.07$, Fig 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.

Figure 8: Representative images of the semiquantitative assessment of ultrastructure. Healthy (-) and necrotic (+) cells (Fig 8A). Bars size: 2 µm. Healthy (-) and damaged (+) mitochondria (Fig 8B). Bars size: 0.5 µm. Absence (-) or presence (+) of mucus on cell surface (Fig 8C). Bars size: 0.5 µm. A semiquantitative assessment for all treatments ranging from – (without alteration) to +++ (high alteration) was assigned for each parameter and treatment used for statistical comparisons (Fig 8D).



Treatment	Cell death (Necrotic cells)	Damaged mitochondria	Mucus in cell surface
C-	-	-	+
E. coli	+++	+++	-
LAB	-	+	+

4. 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 [147, 148]. The use of tissue explants gives 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.

The LAB combinations tested herein were selected from the results obtained during former experiments [143]. 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 was able to inhibit *E. coli* infection and down-regulate total inflammation, *L. rhamnosus* ratio 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 [143].

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 by *L. rhamnosus* ratio 25, *P. acidilactici* ratio 25, and *L. reuteri* ratio 2 (Fig 1 and Fig 2). *L. 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. Although the importance of the role 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 [143]. 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 reduced most efficiently the *E. coli* infection (Fig 1), but it also led to the greatest reduction of acute and basal inflammation (Fig 2 and 3). Probably, in the case of the combination D applied to epithelial cultures, the obtained reduction of CFU of *E. coli* (Figure 1) was the main cause behind the decreased expression of pro-inflammatory 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 non-specific phagocytosis by neutrophils [53]. Parturition in dairy cattle is associated with impairment of polymorphonuclear leukocytes phagocytosis and with a decrease in the ability to fight bacterial infections [88]. A decrease in neutrophils' activity during the periparturient period has been suggested to influence the occurrence of uterine infections in both cows [149, 150] and buffalos [151]. The phagocytic capacity of neutrophils remains high throughout the peripartum period but their killing capacity is impaired [152].

There are several studies that demonstrate the capacity of *L. rhamnosus* to activate neutrophils [153, 154]. Fish receiving a feed supplemented with *P. acidilactici* also presented elevated proportions of circulating neutrophils [155] and *L. reuteri* recruited neutrophils against pneumovirus in mice [156].

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 (Fig 4 and 5) could be responsible of the recruitment of neutrophils. This, in combination with the previously demonstrated ability of LAB for reducing *E. coli* CFU, would protect the tissue against *E. coli* infection and the 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 (Fig 4 and 5). It is likely that the LAB probiotics prepared the tissue pre-infection to efficiently respond against pathogens and avoid a high inflammatory feedback that could be deleterious for the uterus.

FESEM images (Fig 6 and 7) demonstrated that there was no direct interaction between the different strains, or between bacteria and endometrial cells, and that there was no formation of biofilms. Probably the presence of LAB may have reduced, by competition mechanisms, the direct interaction of *E. coli* with the epithelial cells. These ultrastructural analyses also suggested 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) [157]. In this context, Pediocin PA, a bacteriocin produced by *Pediococcus acidilactici,* has been described to inactivate *Listeria* and other food-pathogens [158]. Also, a recent study showed that increased secretion of MUC3 mucin leads to reduced adhesion of enteropathogenic *E. coli* and enterohemorrhagic *E. coli* strains [159, 160]. It has also been proved that the bacterial exopolysaccharide reuteran, synthesized by *L. reuteri*, reduces enterotoxigenic *E. coli* colonization of piglet jejunal epithelial cells [161]. 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 [162, 163]. In general, the loss of epithelial cells increases the risk of infections, inflammation, and other deleterious

effects in the tissues [162]. LAB treatment reduced mitochondrial damage compared with *E. coli* infected cells (Fig 8). Our ultrastructural findings are relevant because mitochondrial damage and cell death compromise the tridimensional organization of epithelial cells and hampers their barrier function, crucial for endometrium homeostasis [164]. These results complemented our biochemical data, since intracellular signaling pathways of inflammation participate actively in tissue homeostasis [165] and may induce morphological changes [166].

5. Conclusions

In conclusion, a LAB combination consisting of *L. rhamnosus* ratio 25, *P. acidilactici* ratio 25, and *L. reuteri* ratio 2 presented 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-1β, 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 pro-inflammatory cytokine production and necrosis) consequence 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.

6. Acknowledgments

We are indebted to the Servei de Cultius i Producció d'Anticossos i Citometria and the Servei de Microscopia at the Autonomous University of Barcelona (Barcelona, Spain). The authors thank the Department of Ruminant Production of IRTA for helpful technical assistance and discussions.

CHAPTER V: Study 3

Effects of intravaginal lactic acid bacteria on bovine endometrium: implications in uterine health

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Submitted to Theriogenology, August 2016

1. Introduction

Infection and subsequent inflammation of the bovine uterus compromise uterine health and hamper reproductive efficiency in dairy cows [167]. The onset of milk production leads to a dramatic increase in energy requirement of dairy cows which cannot be compensated by feed intake resulting in a negative energy balance, which can continue up to about 3 mo of lactation difficulting the resolution of peripartum disease [168]. Moreover, the recovery process for the uterus after parturition requires tissue repair, regeneration of the endometrial epithelium, and clearance of bacteria that contaminate the uterus around parturition. In this context, both a basal tissue inflammation (due to post-parturm uterine involution) and an acute inflammation (caused by bacterial infections) may coexist in the uterus. Whether bacteria that reach the endometrium will ultimately cause disease depends on the balance between the classic triad of the virulence of the microbes, the effectiveness of the host defense systems, and the environment [169].

Escherichia coli, *Trueparella pyogenes*, and *Fusobacterium necrophorum* are considered important etiological agents of uterine diseases. *E. coli* is particularly prevalent in the first week postpartum and is commonly associated with metritis. Some authors have demonstrated that *E. coli* isolated from cows with metritis were adherent and invasive to endometrial epithelial and stromal cells, and able to induce inflammation through TLR-4 in a mouse model [51, 56]. Infection in the uterus is accompanied by systemic fluctuations of inflammatory cytokines such as IL-8, IL-1β and IL-6 [170].

It is hypothesized that reproductive tract diseases represent a failure of the immune system to switch sufficiently fast from a heightened state of function for postpartum clearance of bacteria and tissue debris to a down-regulated state necessary for maintenance of pregnancy [171]. Probiotics are live microorganisms that, when administrated in adequate amounts, confer a health benefit to the host. They have been shown to exert a wide range of effects, such as altering intestinal microbiota or enhancing the immune system [172]. In our recent work we have demonstrated the effectiveness of a combination of probiotics (*Lactobacillus rhamnosus, Pedioccocus acidilactici,* and

Lactobacillus reuteri) at reducing inflammation and *E. coli* infection in epithelial endometrial cells and tissue [143].

The objective of this study was to evaluate the effect of LAB probiotic combination, administered intravaginally to dairy cows, on the innate immunity of the endometrium and on the ability of endometrial tissue to withstand further *ex vivo E. coli* infection or inflammation challenges.

2. Materials and methods

2.1 Animals and experimental design

This experiment was conducted in the facilities of Institut de Recerca i Tecnologia Agroalimentaries (IRTA) at Monells (Spain). All experimental procedures were approved by the IRTA Animal Care Committee. Twenty lactating Holstein cows at the end of stage 2 of the estrous cycle were enrolled to this study for a 3-wk period. At the end of the 3 wk cows were culled. Cows were enrolled during stage 2 of the estrous cycle to guarantee they would be in the stage II and I at the end of the treatment. Stage I and II were chosen due to its similarity with post-partum cows in progesterone concentration [141]. Cows were randomly assigned to either 2 doses of LAB per wk (**LAB**); or 2 doses of carrier (sterile sodium chloride 0.9%) (**CTRL**). The LAB treatment consisted of a mixture of *Lactobacillus rhamnosus* CECT 278 (Colección Española de Cultivos Tipo, Valencia, Spain), *Pediococcus acidilactici* CECT 5915, and *Lactobacillus reuteri* DSM 20016 (German collection of microorganisms and cell cultures, Leibniz, Germany) with a final cell count of $4.5 \cdot 10^{10}$cfu/dose and a relationship among the 3 probiotics of 12:12:1, respectively. LAB mixture was made fresh every day. The LAB or carrier was infused into the vaginal tract with sterile 5-mL syringes (Terumo, Germany) and deposited in the vagina just before the entrance to the cervix. Aseptic procedures were maintained during LAB administration. All uteri were collected at the slaughterhouse and transported to the lab at 4ºC. Vaginal and endometrial swabs (HydraFlock, Guilford, USA) were taken from all animals and stored at -80ºC until further qPCR analysis or kept at 4ºC to perform bacterial cell counts.

2.2 Blood samples and lab analysis

Two blood samples from each cow were collected from the coccygeal vein 1 d before culling using 10-mL vacutainer tubes with $EDTAK_2$ (BD Vacutainer Systems, Plymouth, UK). Blood samples were centrifuged at 3500 rpm at 4ºC for 15 min. Serum and plasma samples were collected and stored at -20ºC until further analysis.

Concentrations of progesterone were measured in plasma using a commercially available kit (PROG-EASIA Kit, DIAsource ImmunoAssays S.A.,Belgium) with a sensitivity of 0.8ng/mL.

2.3 Bacterial strains and culture conditions

Pediococcus acidilactici (CECT 5915) and *Lactobacillus rhamnosus* (CECT 278) were purchased from CECT (Colección Española de Cultivos Tipo, CSIC Valencia, Spain), and *Lactobacillus reuteri* (DSM 20016) was acquired at DSMZ (German collection of microorganisms and cell cultures, Leibniz, Germany). A swab sample was taken from the uterus of 4 metritic cows (not part of the 20 cows enrolled in this study) to isolate pathogenic *E. coli*. Each swab was spread in blood agar, MacConkey agar, and chocolate agar. The final identification of the colonies was made by Vitek (Biomiéreux, Craponne, France). The *E. coli* selected was FimH positive (virulence factor) and hemolytic. Bacterial cultures were performed inoculating 1 mL of a glycerinate in 9 mL of Luria Bertrani (LB) media (10 g/L peptone, 5 g/L yeast extract and 10 g/L NaCL) for *E. coli* or MRS (Scharlau, Sentmenat, Spain) at 37ºC in static conditions overnight (O/N). Then, media were centrifuged at 6000 x *g* for 15 min, and resuspended in 1 mL of sterile NaCl 0.9%. To prepare the LAB treatment, $2.4 \cdot 10^{10}$ cfu of *L. rhamnosus*, $2.4 \cdot 10^{10}$ cfu of *P. acidilactici*, and $1.93 \cdot 10^{9}$ cfu of *L. reuteri* were resuspended in 2 mL of NaCl 0.9%.

2.4 *Escherichia coli* infection of *ex vivo* explants

Segments of endometrial tissue were obtained at the slaughterhouse from the 20 cows 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. The tissue was cut into 0.8 cm^2 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 media (Gibco, NY, USA) supplemented with 10%

FBS, 50 µg/mL streptomycin, 50 IU/mL penicillin, 2.5 µg/mL amphotericin B in 5% CO_2 at 37ºC. Endometrial explants were plated in 24 well-plates and kept O/N at 37°C and 5% CO_2. Explants were challenged with 1.5x10^6 CFU/well of *E. coli* O/N. Incubation with media instead of *E. coli* was used as a negative control. When *E. coli* infection was evaluated in endometrium under a basal inflammation, IL-1β was added at 10 ng/mL 1h before inducing the infection with *E. coli*. Each treatment was performed by sixtiplicate. Supernatant was collected for further Il-8, IL-1β, and IL-6 analysis. Tissue was collected for the analysis of gene expression by RT-PCR.

2.5 DNA extraction swabs.

Total DNA was extracted from the swabs using Power Soil DNA Isolation kit (MOBIO, Carlsbad, USA) following the manufacturer's instructions. The amount of DNA was quantified using Nanodrop. *Lactobacillus* quantification was assessed by qPCR (Table 1).

Table 1: Sequence, annealing temperature (At), concentration (µM), amplicon size (bp) of the forward (Fw) and reverse (Rv) primers used for qPCR and accession number or reference.

Gene	Fw	Rv	At, Cº	µM	Accession number
GAPDH	GCATCGTGGAGGGACTTATGA	GGGCCATCCACAGTCTTCTG	52	0.125	NM_001034034
CXCL8	TTGAGAGTGGACCACACTGC	TGCACCCAGTTTTCCTTGG	55	0.5	[127, 128]
IL1B	TGGGAGATGGAAACATCCAG	TTTATTGACTGCACGGGTGC	50	0.3125	M37211
IL6	GGGCTCCCATGATTGTGGTA	GTGTGCCCAGTGGACAGGTT	51.5	0.5	NM_173923.2
TNFa	AACAGCCCTCTGGTTCAAAC	TCTTGATGGCAGACAGGATG	60	0.5	AF011926.1
NFkB	GGGCTCCCATGATTGTGGTA	GTGTGCCCAGTGGACAGGTT	51.5	0.5	NM_001076409.1
TLR4	TCAGAAACCTCCGCTACCTTG	TTCTGAAAAGAGTTGCCTGCC	52.1	0.5	NM_174198.6
IGF1	CCTGGATTTCTTTTTGCCTCAT	GGTGAAGGCGAGCAAGCA	50	0.25	NM_001077828.1
PGHS2	AGGTGTATGTATGAGTGTAGGA	GTGCTGGGCAAAGAATGCAA	50	0.25	NM 174445.2
MUC1	CATTGCCCTGGTTGTGTGTC	ACCATTGCCTGCAGAAACCT	54	0.25	NM_174115.2
B-defensins	GGTCACAAGTGGCAGAGGAT	TGGTTGAAGAACTTCAGGGC	55	0.5	[173]
EMMPRIN	GGTCACCATCATCTTCATCTA	AGAGCCTATGTCTTCATCATC	48	0.25	NM_001075371.2
IGFBP2	CTGGAGCACCTCTACTCCCT	CCGGTGTTAGGGTTCACACA	54	0.5	NM_174555.1
Lactobacillus	GCAGCAGTAGGGAATCTTCCA	GCATTYCACCGCTACACATG	60	0.25	_

2.6 Swab culture conditions

Vaginal swabs were cultured in MRS (Scharlau, Sentmenat, Spain) and MacConkey agar (Scharlau, Sentmenat, Spain) plates and kept in static conditions at 37ºC. Colonies were counted after an O/N.

2.7 Retrotranscription and quantitative PCR (qPCR).

Total RNA was extracted from endometrial explants using Trizol ® (ThermoFisher Scientific, Cramlington) following manufacturer's instruction and quantified using Nanodrop. The RNA was retrotranscrived to DNA using iScript cDNA synthesis kit (Bio-Rad, California, USA). Reactions of qPCR were performed using iQ5 Termocycler (Bio-Rad, California, USA) and qPCR conditions for each set of primers were optimized (Table 1). The specificity of the amplification was evaluated by the single band identification at the expected molecular weight in DNA agarose gel and a single peak in the qPCR melting curves. The efficiency was calculated by amplifying serial 1/10 dilutions of each gene amplicon. A standard curve at Ct versus log was plotted to obtain the efficiency, which is calculated using the formula $10^{1/slope}$, with an acceptable range of 1.8-2.2 [124]. A total reaction volume of 20 μL was used, containing 50 ng of cDNA, 10 μL of SYBR Green Fluorescent (Bio-Rad), and the optimized gene concentration for each gene (Table 1). The qPCR reactions 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 optimized annealing temperature for each gene, 30 s at 72°C and a final extension of 10 min at 72°C. Relative gene expression was calculated using the Pfaffl method [144] with *GAPDH* as a reference control gene. All qPCR assays were performed in triplicate. *GAPDH* was chosen because it is stably expressed in uterine tissues [116, 126].

2.8 Enzyme-linked immunosorbent assay (ELISA)

Concentrations of IL-1β, IL-6 and IL-8 were measured in the supernatants of explants by ELISA according to the manufacturers' instructions: Bovine IL-1β ELISA Kit (ESS0027; ThermoFisher Scientific, Cramlington, UK); Bovine IL-6 ELISA Kit (ESS0029; ThermoFisher Scientific, Cramlington, UK); Human CXCL8/IL-8 DuoSet (DY208, Vitro S.A., Spain). The Human CXCL8/IL-8 DuoSet has previously been validated for the measurement of bovine IL-8 [141, 146].

2.9 Statistical analysis

Previous to statistical analyses, data were log- or square root-transformed when necessary to achieve a normal distribution. Results herein are expressed as the means of

non-transformed data ± SEM obtained with normalized data (except otherwise stated). Data were analyzed using an ANOVA (SAS Inst. Inc.; Cary, NC, USA), considering treatment and infection as fixed effects and animal as a random effect. *E. coli* presence in vaginal swabs was analyzed using a Chi Square. Differences were considered significant when $P < 0.05$.

3. Results

3.1 Blood progesterone

Blood progesterone concentrations confirmed that 89.7% of the cows (17/19) were in the stage I or II of the estrous cycle [141] [174] (data not shown). Two cows were in stage III, but the results were analyzed together.

3.2 Innate immunity of the endometrium

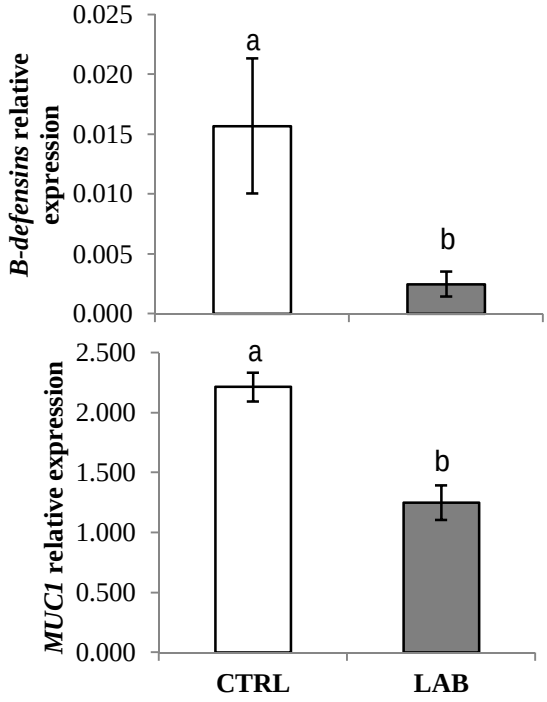
The expression of 10 genes considered markers of inflammation and innate immunity of endometrium [175-178] was evaluated. The endometrium of LAB-treated cows *in vivo* presented differences in expression levels of *B-defensins* and *MUC1* (Figure 1) but not in other innate immunity genes analyzed, in comparison with CTRL cows (not treated) (Table 2). In the case of *B-defensins*, gene expression was reduced 8 fold ($P < 0.05$) compared with the CTRL cows (Figure 1). The same pattern was observed for *MUC1*, which was reduced 1.77 fold ($P < 0.05$) in comparison with CTRL cows (Figure 1). The response of the endometrial explants treated *ex vivo* was performed as a positive control for the potential direct LAB effects on the endometrium (Table 2). The abundance of *CXCL8, IL1B, IL6*, and *TNFa* was affected ($P < 0.05$) by the direct LAB treatment *ex vivo*, whereas no differences were observed between groups in the expression of the other genes. Applying LAB *ex vivo* increased the expression of *CXCL8* 4.03 fold, *IL1B* 2.51 fold, *IL6* 2 fold, and *TNFa* 3.33 fold compared with the other groups.

Table 2: Gene expression in endometrial explants. Relative units of gene expression (mean ± SEM) for different groups of explants. Values with different letters differ ($P < 0.05$).

Animal treatment	CTRL	LAB	-
Ex vivo treatment	-	-	LAB
Name	Not treated	Treated	CTRL +
CXCL8	1.45 ± 0.230^{b}	1.21 ± 0.281^{b}	5.85 ± 0.288^{a}
IL1B	0.53 ± 0.187^{b}	0.36 ± 0.210^{b}	1.33 ± 0.193^{a}

IL6	0.186 ± 0.045[a,b]	0.23 ± 0.078b[b]	0.46 ± 0.114[a]
TNFa	0.003 ± 0.0011[b]	0.002 ± 0.0006[b]	0.010 ± 0.0042[a]
NFkB	0.22 ± 0.069	0.16 ± 0.065	0.33 ± 0.065
TLR4	0.05 ± 0.033	0.03 ± 0.033	0.07 ± 0.033
IGF1	0.03 ± 0.213	0.04 ± 0.213	0.03 ± 0.213
PGHS2	0.02 ± 0.020	0.01 ± 0.019	0.08 ± 0.025
EMMPRIN	0.186 ± 0.159	0.192 ± 0.142	0.46 ± 0.142

Figure 1: Explants *B-defensins* and *MUC1* expression. Relative units of gene expression of *B-defensins* and *MUC1*. Bars represent mean ± SEM for the different groups. Bars with different letters differ (P < 0.05).



3.3 *Ex vivo E. coli* infection

The resistance of the endometrium to *E. coli* infection was evaluated *ex vivo* using endometrium explants from CTRL and LAB cows. The infection was monitored through the production of IL-8, IL-1β, and IL-6 biomarkers [179]. The secretion of IL-8, IL-1β, and IL-6 was affected ($P < 0.0001$) by *E. coli* infection (Table 3). When the explants were infected, the secretion of IL-8 increased 1.30 fold compared with the uninfected explants. In the case of IL-1β concentration, infected explants expressed 6.29 fold more protein than the uninfected explants. Lastly, infected explants had 3.36 fold more concentration of IL-6 than uninfected explants. No differences were observed between treatments (Table 3) regarding the secretion of IL-8, IL-1β, or IL-6.

On the other hand, the response of endometrium from cows was also analyzed after *ex vivo* inflammatory stimulus (treatment with 10µg/µL of IL-1β) that mimicked the basal inflammation at calving. The concentration of markers IL-8, IL-1β, and IL-6 in the inflammation model was evaluated in endometrial explants before and after *E. coli* infection.

Table 3: Effect of *E. coli* infection in IL-8, IL-1β, and IL-6 concentration on endometrial explants.

	Infection		Treatment	
Protein	**Not infected**	**Infected**	**CTRL**	**LAB**
IL-8	60.80 ± 0.057[b]	79.42 ± 0.052[a]	66.04 ± 0.073	74.19 ± 0.072
IL-1β	207.76 ± 0.084[b]	1306.40 ± 0.085[a]	812.64 ± 0.102	701.52 ± 0.099
IL-6	4616.45 ± 0.066[b]	15515 ± 0.067[a]	8411.45 ± 0.085	11720 ± 0.081

[a-b] Means within a row with different subscripts differ ($P < 0.005$).

The amount of IL-8, and IL-6 was affected ($P < 0.0001$) by infection (Table 4), but there were no differences in either treatment with LAB or interaction between treatment with infection. Infected explants secreted 1.43 fold more IL-8, and 1.70 fold more IL-1β than not infected explants. No differences were found with IL-1β.

Table 4: Effect of *E. coli* infection on IL-8, IL-1β, and IL-6 concentration on pre-inflammated endometrial explants.

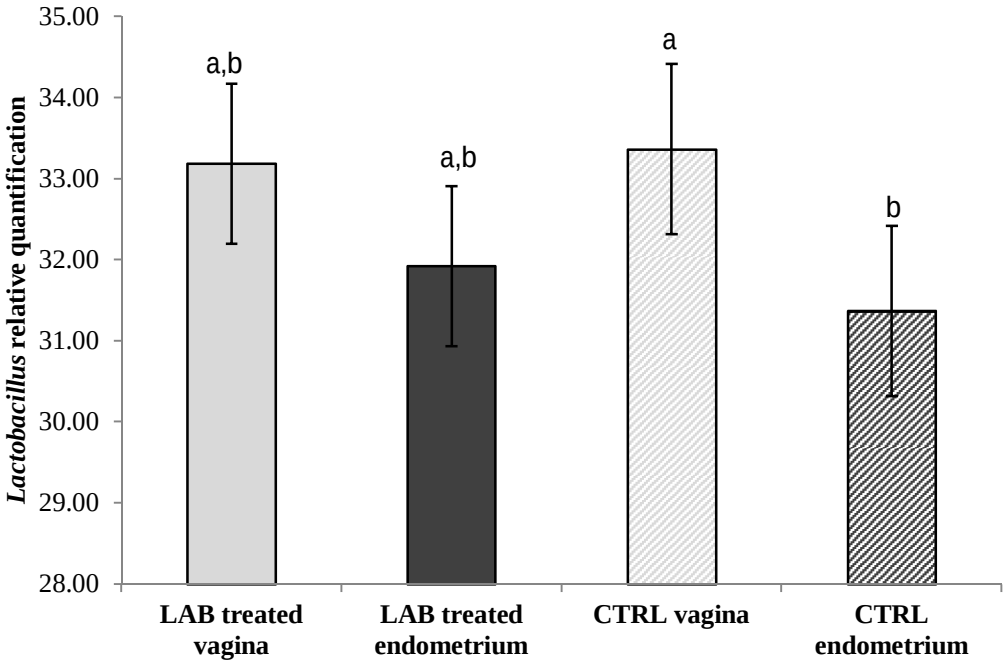
	Infection		Treatment	
Protein	**Not infected**	**Infected**	**CTRL**	**LAB**
IL-8	69.31 ± 0.047[b]	99.65 ± 0.047[a]	92.41 ± 0.060	76.54 ± 0.057
IL-1β	13528 ± 0.044	14904 ± 0.044	14379 ± 0.055	14107 ± 0.053
IL-6	13769 ± 0.070[b]	23476 ± 0.070[a]	18806 ± 0.080	18439 ± 0.078

[a-b] Means within a row with different subscripts differ ($P < 0.005$)

3.4 Vaginal *E. coli* and *Lactobacillus* spreading

The concentration of *Lactobacillus* was evaluated from vaginal and endometrial swabs from each cow. The amount of *Lactobacillus* was affected by tissue ($P < 0.05$), but not by treatment or the interaction between treatment and tissue (Figure 2).

Figure 2: *Lactobacillus* concentration**.** Reverse relative units of *Lactobacillus*. Bars represent mean ± SEM for the different groups. Bars with different letters differ ($P < 0.05$).



The amount of *Lactobacillus* observed in the vagina of the control cows compared with the endometrium was significantly bigger (1.06 fold, $P = 0.036$). No differences were observed in the LAB treated cows. On the other hand, the results from the vaginal swab cultures shown a tendency to have more presence of *E. coli* in CTRL cows than in the LAB-treated cows ($P = 0.088$).

4. Discussion

As uterine inflammation occurs in all cows during the post-calving uterine involution, the factors responsible for failure to resolve the endometrial inflammation at the start of the breeding period are critical. The scale of the inflammatory response to microbes is dependent on multiple factors, including microbial virulence effectors, host tissue factors, and on the regulation of intracellular signaling pathways associated with innate immunity [180]. Furthermore, this inflammated uterus seems to be more susceptible to *E. coli* infections. Thus, understanding which factors are involved in this process will help to diminish *E. coli* infections responsible for metritis and the inflammation associated with calving.

Pathologic or acute inflammation appears to differ from the physiologic or what we call basal inflammation in severity, duration, and the time postpartum in which it is more pronounced. It is accepted that the same inflammatory effectors are involved in disease

and in physiologic inflammation, but that the difference lies in the magnitude, regulation, duration, and effectiveness of the response [10]. Our models allow to study the LAB treatment and further endometrium performance in different situations: acute inflammation (infection), basal (tissue inflammation induced by IL-1β) and total inflammation (combining both models). These models are based on combining a LAB *in vivo* treatment with an *ex vivo* challenge ensuring a more controlled experimental environment without compromising ethical issues. When we compared the results of both basal and total inflammation (acute and basal inflammation) (Tables 3 and 4, respectively) we observed an increment in the concentration of all the cytokines. Specifically, the concentration of IL-8 was increased 1.25 fold, IL-1β 3.75 fold, and IL-6 1.51 fold in the total inflammation model (Table 4) compared with the acute inflammation model (Table 3). These results are in agreement with our previous results [181].

The comparison of the expression of pro-inflammatory cytokines in the endometrium of CTRL (not-treated) animals and LAB-treated animals revealed no differences. However in order to check that *Lactobacillus* was able to modulate some of these cytokines in the endometrium, an *ex vivo* LAB treatment was included in the experiments (what we called positive control) (Table 2, Figure 1). In this experiment we observed that *CXCL8, IL1B, IL6,* and *TNFa* were over-expressed in the LAB-*ex vivo* treatments compared with the other 2 groups, only treated *in vivo* (4.03, 2.5, 2, and 3.33 fold, respectively). This confirms that LAB treatment *in vivo* does not affect the endometrium because none of those markers were modified. That could be explained by the absence of differences in *Lactobacillus* counts in the endometrium of LAB-treated or CTRL cows (Figure 2), suggesting that the LAB treatment applied in the vagina did not reach the endometrium.

It is known that some probiotics (or their derivative products or metabolites) may prevent or trigger an innate immune response by initiating TNF-α production by epithelial cells, inhibiting or activating NF-κB and priming or dampening the host immune response by influencing the production of IL-8 and subsequent recruitment of neutrophils [182]. A previous study [183] reported that a LAB treatment composed by *Lactobacillus suntoryeus* HY7801, *Bifidobacterium longum* HY130504, *Lactobacillus plantarum* AK8-3, and *L. acidophilus* A101, inhibited TLR-4 linked NF-κB activation. Consistent with the NF-

κB results observed herein, no differences in *TLR4* expression were observed in that study. In our experiments, no differences were observed in the gene expression of *NFkB* indicating that 1) this may not be the route of immune modulation used by the LAB combination used herein, or 2) the sampling time was not adequate to observe differences in *NFkB* or *TLR4*.

Prostaglandin G/H synthases (PGHS2) is an enzyme involved in the conversion of arachidonic acid into PGH2, a common precursor of various forms of prostaglandins (PG) including PGE2 and PGF2α. The downstream enzyme PGE synthase (PGES) catalyzes the conversion of PGH2 to PGE2, which regulates the production of various cytokines including TNF-α and IL-6 [184]. However, no differences were observed between treatments or the positive control (Table 2). That again, lack of differences may be due to a delayed sampling time as differences in *TNFa* and *IL6* were observed. Postpartum dairy cows enter a period of negative energy balance associated with low circulating IGF-1, during which the uterus must undergo extensive repair following calving. IGF-1 is vital for proliferation, differentiation and steroidogenesis of granulose cells. It is also known that low levels of IGF-1 may cause delay in resumption of estrous cyclicity [185]. Multiparous cows with a low nadir in circulating IGF-1 in the first 2 wk postpartum subsequently fail to conceive. Fertility is strongly linked to animal's health around calving. The postpartum uterus is exposed to the prevailing metabolic environment within the animal, including IGF-1 [178]. A previous study reported that giving an oral combination of *Lactobacillus acidophilus, L. rhamnosus,* and *Lactobacillus casei* to human volunteers was able to regulate *IGF1* in the mucosa [186]. In our study, LAB was not able to regulate *IGF1* expression, at least when no infection was involved.

Extracellular matrix metalloproteinase inducer regulates several biological functions involving the modulation of cell behaviors via cell-cell and cell-matrix interactions, is also highly associated with endometrial remodeling, and it has been detected in the luminal and glandular epithelium and stroma [176]. No differences were observed between treatments.

The principal antimicrobial peptides expressed in the bovine endometrium include β-defensin and its transcripts are most abundant in the face of microbial challenge [89].

Several studies have found that tracheal antimicrobial peptide (TAP) is up-regulated in diseased endometrium, or in cytology samples collected from the endometrium. TAP is, a β-defensin produced by mucosal epithelial cells [60]. Our results showed that cows treated with LAB *in vivo* expressed less *B-defensins* than CTRL cows (Figure 1). Also, differences were observed (Figure 1) in *MUC1* (an inducible innate immune effector and an important component of the first line of defense against bacterial invasion of epithelial surfaces [175]). A decrease of *MUC1* expression of 1.77 fold was found in LAB treated cows compared with not treated and positive control. That seems to indicate that although the LAB applied in the vagina was not able to reach the endometrium, it may regulate the vagina environment as demonstrated by the amount of vaginal *E. coli* found in the swabs, where there was a tendency towards lower *E. coli* counts in cows treated *in vivo* with LAB compared with CTRL. This effect might reduce the bacterial load reaching the uterus, consequently, reducing the expression of *B-defensins* nor *MUC1.* This findings correlate with our results and leads to think that β-defensins may be use as a marker of uterine infection.

5. Conclusion

The vaginal treatment with a combination of LAB based on *L. rhamnosus, P. acidilactici,* and *L. reuteri* does not reach the endometrium and does not modulate inflammation and tissue remodeling genes. However LAB-treated cows showed a lower expression of B-*defensins* and *MUC1* in the endometrium, two indicators of uterine infection, which may be explained by a regulation of pathogenic environment in the vaginal tract by the action of LAB and a consequent reduction of pathogenic bacteria reaching the endometrium.

CHAPTER VI: Study 4

Lactic Acid Bacteria Combination Regulates Neutrophil Activity and Reduces Metritis Prevalence

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1. Introduction

Acute metritis is an inflammation of the uterus due to bacterial infection occurring the first 21 days after calving (more prevalent during the first 10 DIM). The main characteristics are systemic signs of sickness, including fever over 39.5°C, red-brown watery foul-smelling vaginal discharge, dullness, inappetance, elevated heart rate, and low milk production (Sheldon et al. 2006). Damage to the uterine tissue during parturition leads to inflammation that likely contributes to the systemic condition [76] This inflammation found in the uterus comes from the need for substantial tissue repair as part of post-partum involution, recruiting immune cells [51]. When microorganisms reach the uterus, the resistance to disease depends on the balance between the host defense systems, the virulence of the microbes, and the environment [89].

The periparturient dairy cow has nutritional requirements that exceed its dietary intake potential. This leads to a state of negative energy balance (NEB), during which blood glucose decreases and body tissue reserves are mobilized to provide more energy, hardening the resolution of peripartum diseases [187, 188]. Cows in greater degree of NEB have higher values of non-esterified fatty acids (NEFA) in blood, that at the same time are associated with lower feed intake [46, 150].

Parturition in dairy cattle is associated with impairment of neutrophil phagocytosis and oxidative burst activity [189] therefore with a decrease in the ability to fight bacterial infections. Other authors demonstrated *in vitro* that an increase of NEFA in blood tended to desensitize neutrophil oxidative burst [188], being the specific fagocytosis by neutrophils the most important component of uterine defense after bacterial infections [16].

The traditional antimicrobial treatment applied for metritis may lack efficacy and it does not typically improve the reproductive performance on the treated animals [142]. That along with the recommendations from the WHO to reduce antibiotic treatments leads to investigate alternative therapies. The use of probiotic strains has been proposed as an alternative to prevent postpartum uterine infections and inflammation. Deng et al had demonstrated that some lactic acid bacteria (LAB) strains are able to reduce uterine disease prevalence in dairy cows [107]. Our recent work, have demonstrated that other

LAB strains are also able to modulate uterine inflammation *in vitro* and *ex vivo* [143, 181] and when administered *in vivo* intra-vaginally the LAB does not reach the endometrium but regulates vaginal environment restricting the amount of bacteria reaching the uterus [190].

2. Materials and methods

2.1 Animals and experimental design:

This experiment was conducted in the facilities of Dairy Research and Educational Centre from University of British Columbia (UBC) at Agassiz, Canada. All experimental procedures were approved by the UBC Animal Care Committee. One hundred thirty-five cows were enrolled 3 wk before calving and randomly assigned to treatments to insure similar frequencies for parity and previous illness in all treatment groups. The treatment groups were: (1) two intravaginal doses of lactic acid bacteria (LAB) per wk during 3 wk pre-calving (**Vaginal**); (2) 1 intra-uterine dose 1 d after calving (**Endometrial**); and (3) no intervention (**Control**). The LAB treatment dose was a mixture of *Lactobacillus rhamnosus* CECT 278 (Colección Española de Cultivos Tipo, Valencia, Spain), *Pediococcus acidilactici* CECT 5915, and *Lactobacillus reuteri* DSM 20016 (German collection of microorganisms and cell cultures, Leibniz, Germany) with a final cell count of $4.5 \cdot 10^{10}$ CFU/dose and a proportion of 25/25/2, respectively. LAB treatment was made fresh every day. Intravaginal LAB treatments were applied with a plastic cannula, previously cleaned with water and 70% ethanol, deposited in the vagina just before the entrance of the cervix. For the endometrial treatment, LAB was applied with a sterile syringe directly inside the uterus. Aseptic procedures were maintained during LAB administration. A small sample from the endometrium was collected through a non-surgical procedure (at day 1, 3, and 6 after calving) from 15 cows from the endometrial treatment, and 15 cows from the control. Randomly selected cows received local anesthesia (epidural) using standard procedure indicated by the farm veterinary. Tissue collected was submerged in 500μL of RNA later (Thermo Fiser Scientific, Cramlington, UK) and kept O/N at 4°C. Then RNA later was removed and the tissue stored at -80°C until further analysis.

2.2 Clinical observations and measurements:

All cows were monitored clinically at 1, 3, and 6 DIM for metritis. Rectal temperature were measured twice and fever was declared when it was greater than 39.5°C. Retained placenta was declared if a cow did not expel the placenta within 24 hours after parturition. Cows with retained placenta were removed from the study as were treated with antibiotics. A metritic case was diagnosed if the cow had reddish brown vaginal discharge with fetid odor, or more than 50% pus, together with fever at DIM 6. To check the discharge the Metricheck device, a soft rubber hemisphere connected to a stainless steel rod, was inserted into the vagina. Vaginal discharge was evaluated after retracting the device caudally [191]

2.3 Sampling and laboratory analysis:

Blood samples were collected from the coccygeal vein at d -14, -10, -7, -4, +1, +3, +6, and +14 related to calving after being milked with 10 mL vacutainer tubes with EDTA (BD Vacutainer Sytems, Plymuth, UK). Blood samples were processed for neutrophil RNA isolation or centrifuged at 1573 x g at 5°C for 15 min to separate the plasma. Two aliquots for sample were stored at -20°C until further analysis. Concentrations of Non-esterified fatty acids (NEFA) were measured in plasma using a commercially available kit (NEFA standard solution, NEFA-HR2 Color A and B, and NEFA-HR2 Solvent A and B, Wako Diagnostics, Mountain View, USA).

2.4 Bacterial strains and culture conditions:

Pediococcus acidilactici (CECT 5915) and *Lactobacillus rhamnosus* (CECT 278) were purchased from CECT (Colección Española de Cultivos Tipo, CSIC Valencia, Spain), and *Lactobacillus reuteri* (DSM 20016) was acquired at DSMZ (German collection of microorganisms and cell cultures, Leibniz, Germany). Bacterial cultures were performed inoculating 1 mL of a glycerinate in 45 mL of Man, Rogosa, and Sharpe medium (MRS, Scharlau, Sentmenat, Spain) for *L. rhamnosus*, and *P. acidilactici*; and in 20 mL of MRS medium for *L. reuteri* at 37ºC in static conditions for 2 days. Then bacteria was centrifuged at 6000 x g for 15 min and resuspended with sterile NaCl with a final concentration of $4.5 \cdot 10^{10}$ CFU/dose. Each dose had a ratio of 25/25/2 for *L. rhamnosus, P. acidilactici,* and *L. reuteri,* respectively.

2.5 Neutrophil isolation:

Neutrophils were isolated from 20 mL of blood based on the procedures of Carlson and Kaneko (1973). Specifically, blood was centrifuged at 1000 x g for 45 min at 4°C, and then plasma, buffy coat, and two-thirds of the red blood pack were removed. Red blood cells in the remaining sample were lysed by adding 12 mL of hypotonic solution (10.6 mM Na_2HPO_4, and 2.7 mM NaH_2PO_4) and mixing for 90 s. Then, 6 mL of 4°C hypertonic solution (10.6 mM Na_2HPO_4, 2.7 mM Na_2PO_4, and 430 mM NaCl) was added, and tubes were mixed to restore instonocity. Samples were centrifuged at 800 x g for 5 min at 4°C. The supernatant was discarded and the pellet was washed with 10 mL of Hank's Balanced Salt Solution (HBSS, Sigma-Aldrich, St. Louis, USA). Then samples were centrifuged again at 800 x g for 5 min at 4°C and the pellet was washed with 10 mL of HBSS. Samples were centrifuged at the same conditions and the pellet was resuspended with 1 mL of HBSS and poured to 1.5 mL microcentrifuge tubes. The tubes were centrifuged at 9600 x g for 15 min at 4°C, the supernatant was pipetted off, and 500 μL of TRIzol reagent (5Prima, Gaithesburg, USA) was added. Samples were stored at -20°C until RNA extraction.

2.6 Retrotranscription and quantitative PCR (qPCR):

Total RNA was extracted from neutrophil isolations using TRIzol Reagent (5Prima, Gaithesburg, USA) following manufacturer's instructions and quantified using Nanodrop instrument (Thermo Fisher Scientific, Cramlington, UK). The RNA was retrotranscrived to DNA using iScript cDna Synthesis kit (Bio-Rad, California, USA). Reactions of qPCR were performed using CFX384 Touch Real-Time PCR Detection System (Bio-Rad) and qPCR conditions for each set of primers were optimized (Table 1). The specificity of the amplification was evaluated by the single band identification at the expected molecular weight in DNA agarose gel and a single peak in the qPCR melting curves. The efficiency was calculated by amplifying serial 1/10 dilutions of each gene amplicon. A standard curve at Ct versus log was plotted to obtain the efficiency, which is calculated using the formula $10^{1/slope}$, with an acceptable range of 1.8-2.2 [124]. A total reaction volume of 20 μL was used, containing 50 ng of cDNA, 10 μL of SYBER Green Fluoresecent (Bio-rad), and the optimized gene concentration for each gene (Table 1). The qPCR reactions were cycled as follows: an initial desnaturalizing step of 10 min at 95°C, followed by 40 cycles

of 10 s at 95°C, 15 s at optimized annealing temperature for each gene, 30 s at 72°C and a final extension of 10 min at 72°C. Relative gene expression was calculated using the Pfaffl method [144] with *GAPDH* and *RPS9* as reference gene controls and all the qPCR were performed in triplicate. *GAPDH* and *RPS9* were chosen because they are stably expressed in a variety of cells, including uterine tissues [87, 116].

Table 1: Sequence, annealing temperatures (At), concentration (µM), amplicon size (bp) of forward (Fw) and reverse (Rv) primers used for qPCR and accession number.

Gene	Fw	Rv	At, C°	µm	bp	Accession number
GAPDH	GCATCGTGGAGGGACTTATGA	GGGCCATCCACAGTCTTCTG	52	0.125	67	NM_001034034.2
RPS9	CCTCGACCAAGAGCTGAAG	CCTCCAGACCTCACGTTTGTTC	57	0.125	63	NM_001101152.2
SELL	ACGGGAAAAAAGGATTACTATGGA	GCCTATAGTTGCATATGTATCAAATTTTCA	51	0.25	144	NM_174182.1
NCF1	TCCTCAACTTCTTCAAGGTGCG	CAGCGTTGTTCTTGCCATCTTT	53	0.5	107	NM_174119.4
SOD1	GGCTGTACCAGTGCAGGTCC	GCTGTCACATTGCCCAGGT	55.8	0.25	100	NM_174615.2
TNFaR	GTGCAGTGCGTGTGTTTGTGTC	ATCTTCGCAACCACTGCCTTG	55.2	0.5	109	NM_174674.2

2.7 NanoString® nCounter Assay:

Gene expression from biopsy samples was measured on the NanoString nCounter Analysis System (NanoString Technologies, Seattle, WA, USA). The system measures the relative abundance of each mRNA transcript of interest using a multiplex hybridization assay as digital readouts of fluorescent barcode probes that are hybridized to each transcript. An nCounter CodeSet (NanoString Technologies) containing a biotinylated capture prove for 100 genes and 4 reference genes, but only 4 genes were analyzed for this experiment (Table 2) and reporter proves attached to a color barcode tags according to the nCounter™ code-set design was hybridized in solution to 200ng of total RNA for 18 h at 67ºC according to the manufacturer's instructions.

Table 2: Accession number, position and target sequence.

Gene	Accession Number	Position	Target sequence
ACTB	NM_173979.3	56-155	CGCCTTCGCCGCCGGTCGACACCGCAACCAGTTCGCCATGGATGATGATATTGCTGCGCTC GTGGTCGACAACGGCTCCGGCATGTGCAAGGCCGGCTTC
GAPDH	NM_001034034.1	213-312	TGATTCCACCCACGGCAAGTTCAACGGCACAGTCAAGGCAGAGAACGGGAAGCTCGTCATC AATGGAAAGGCCATCACCATCTTCCAGGAGCGAGATCCT
RPL19	NM_001040516.1	193-292	GCCTGTGACTGTCCATTCCCGGGCTCGATGCCGGAAAAACACCTTGGCTCGCCGGAAAGGC AGGCATATGGGTATAGGTAAGCGAAAGGGTACTGCCAAT
PGK1	NM_001034299.1	317-416	CTGATGGTGTCCCCATGCCTGATAAGTACTCCTTGCAGCCAGTTGCTGTAGAACTCAAATCT CTGCTGGGCAAGGATGTTTTGTTCTTGAAGGACTGTGT
CXCL8	NM_173925.2	40-139	CAGAAGAAACCTGACAAAAAGCCTCTTGTTCAATATGACTTCCAAGCTGGCTGTTGCTCTCT TGGCAGCTTTCCTGCTCTCTGCAGCTCTGTGTGAAGCT
IL1B	NM_174093.1	331-430	TGACCTGAGGAGCATCCTTTCATTCATCTTTGAAGAAGAGCCTGTCATCTTCGAAACGTCCT CCGACGAGTTTCTGTGTGACGCACCCGTGCAGTCAATA
IL6	NM_173923.2	320-419	CAAAAATGGAGGAAAAGGACGGATGCTTCCAATCTGGGTTCAATCAGGCGATTTGCTTGAT CAGAACCACTGCTGGTCTTCTGGAGTATCAGATATACCT
TNFa	NM_173966.2	1207-1306	TTCGCAACATTCCTTGAGAAGATCTCACCTAGAACTTGACATGCGTGGACTTCAACTCTCCCT TCCTGCCAATGTTTCCAGACTCCCCTGAGGTGGGAAG

2.8 Bioinformatics:

To obtain gene expression data from the NanoString nCounter assay, filtering of samples using quality control (QC) criteria was performed according to the manufacturer's recommendations. Row counts of QC-passed samples were normalized using 4 reference genes as internal controls (*GAPDH, ACTB, RPL19,* and *PGK-1*).

2.9 Statistical analysis:

Previous to statistical analysis, data were transformed by log or root when necessary to achieve a normal distribution. Results herein are expresses as the means of non-transformed data ± SEM obtained with normalized data (except otherwise stated). Neutrophils, NEFA and NanoString data were analyzed using an ANOVA (SAS Inst. Inc., Cary, NC, USA), considering treatment, day, as fixed effects, parity as a block effect and animal as a random effect. Differences were considered significant when $P < 0.05$. Metritis prevalence was analyzed using a Chi-square comparing each treatment against the control cows.

3. Results

3.1 Metritis prevalence:

The results of clinical observations for metritis are presented in Figure 1. Data showed that cows under vaginal treatment had lower prevalence of metritis compared with cows belonging to the control group ($P < 0.05$) while the endometrial treatment did not show any difference. Vaginal treatment lowered the prevalence rate of metritis by 58% compared with the CTRL group, showing an incidence of 13% whereas the control group had a 30% incidence of metritis. Primiparous cows (heifers) showed greater metritis prevalence than multiparous cows ($P < 0.05$, Table 3) but no interaction with treatment was observed.

Figure 1: Metritis prevalence. Percentage of metritis cases in each treatment. Different letters differ ($P < 0.05$).

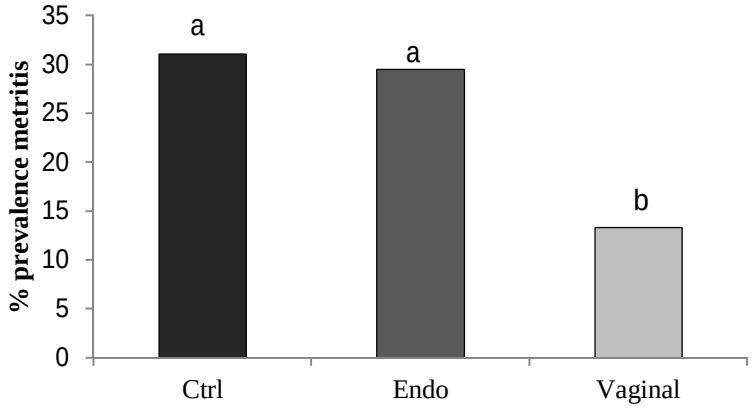


Table 3: Prevalence of metritis by parity, % (case/total).

	Metritis		
Parity	**No**	**Yes**	***P* value**
Heifers	63.65% (28/44)	36.36% (16/44)	
Cows	81.82% (72/88)	12.12% (72/88)	0.02

3.2 Neutrophil relative gene expression:

The expression of *SELL, NCF1, SOD1,* and *TNFaR* genes was used as a marker of neutrophil killing activity. Treatment was affecting the expression of *SELL* ($P < 0.005$), *NCF1* ($P < 0.05$), *SOD1* ($P < 0.05$), and *TNFaR* ($P < 0.05$, Table 4). Endometrial treatment reduced

gene expression of all the genes compared with the control, while vaginal treatment reduced *SELL* and *TNFaR* expression and a tendency was also observed with *NCF1.*

Table 4: Neutrophil gene expression by treatment.

	Treatment			
Gene	**Ctrl**	**Endo**	**Vaginal**	**p-value**
SELL	4.92 ± 0.093[a]	2.18 ± 0.092[b]	2.86 ± 0.092[b]	0.0022
NCF1	14.21 ± 0.012[a]	6.81 ± 0.012[b]	9.05 ± 0.12[a,b]	0.0137
SOD1	9.27 ± 0.097[a]	5.72 ± 0.095[b]	7.83 ± 0.098[a]	0.0135
TNFaR	1.67 ± 0.089[a]	0.99 ± 0.086[b]	1.37 ± 0.088[b]	0.0354

[a-b] Means within a row with different subscripts differ ($P < 0.05$)

Metritis also affected the expression of *TNFaR* ($P < 0.05$, Table 5). Cows with metritis expressed more *TNFaR* and tended to express more *SELL*. Furthermore, day of sampling tended to affect the expression of *NCF1* ($P = 0.069$, data not shown), down-regulating its expression at day 3 compared with day -10 and 1.

Table 5: Neutrophil gene expression by disease.

	Metritis		
Gene	**No**	**Yes**	**p-value**
SELL	2.60 ± 0.075[a]	4.02 ± 0.075[a]	0.0795
NCF1	10.01 ± 1.803[a]	9.97 ± 1.821[a]	0.3293
SOD1	5.69 ± 0.079[a]	9.67 ± 0.080[a]	0.3738
TNFaR	1.01 ± 0.072[b]	1.67 ± 0.072[a]	0.041

[a-b] Means within a row with different subscripts differ ($P < 0.05$)

No interaction has been observed between day and treatment, treatment and metritis, or day and metritis.

3.3 NEFA:

NEFA concentration was affected by day ($P < 0.0001$), and parity ($P < 0.0255$) but no by treatment ($P = 0.4457$). Interactions between treatment and parity ($P = 0.0062$, Fig. 2A), and parity and day ($P = 0.0048$, Fig. 2B) were significant whereas no relationship between

treatment and day ($P = 0.477$) was observed. Endometrial treatment in multiparous cows significantly increased NEFAs in blood compared with the rest of the treatments (with multiparous or primiparous cows) except primiparous control cows, at least 1.29 fold. NEFA concentration in blood was 1.55 fold lower before calving (day -10) than after calving in primiparous cows whereas NEFAs concentration in blood raised 2.03 fold after calving in multiparous cows. No differences were observed between heifers and cows before calving, but after calving, multiparous cows had at least 1.20 fold more NEFAs in blood than primiparous cows. No differences were observed between metritic and healthy cows (data not shown).

Figure 2: NEFA by treatment and parity (A), by parity and day (B). Bars represent mean ± SE. Different letters differ ($P < 0.05$).

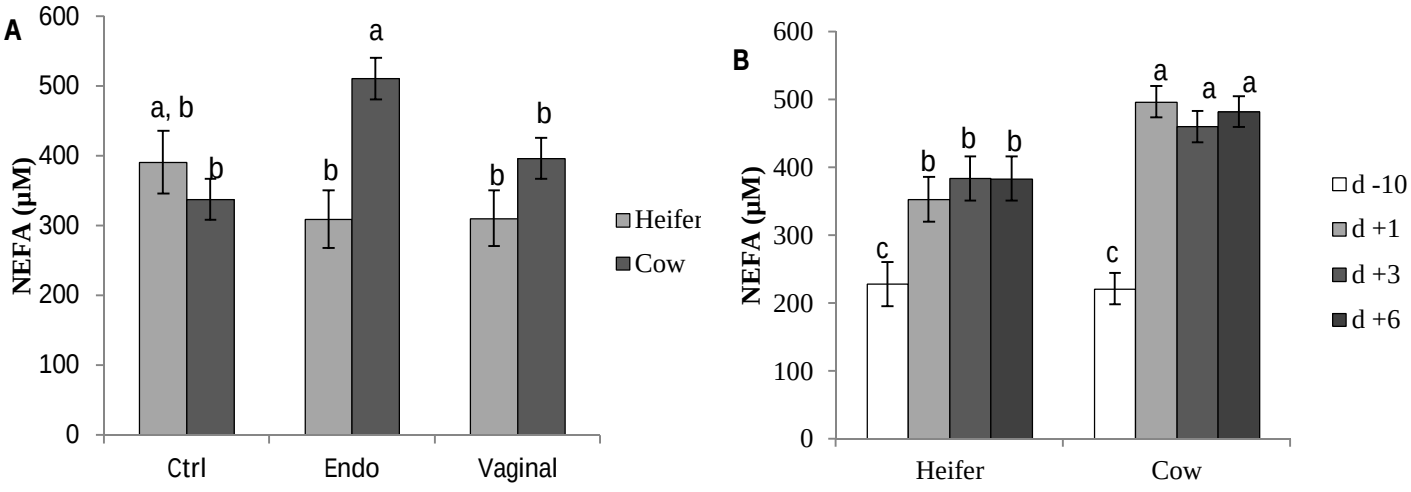


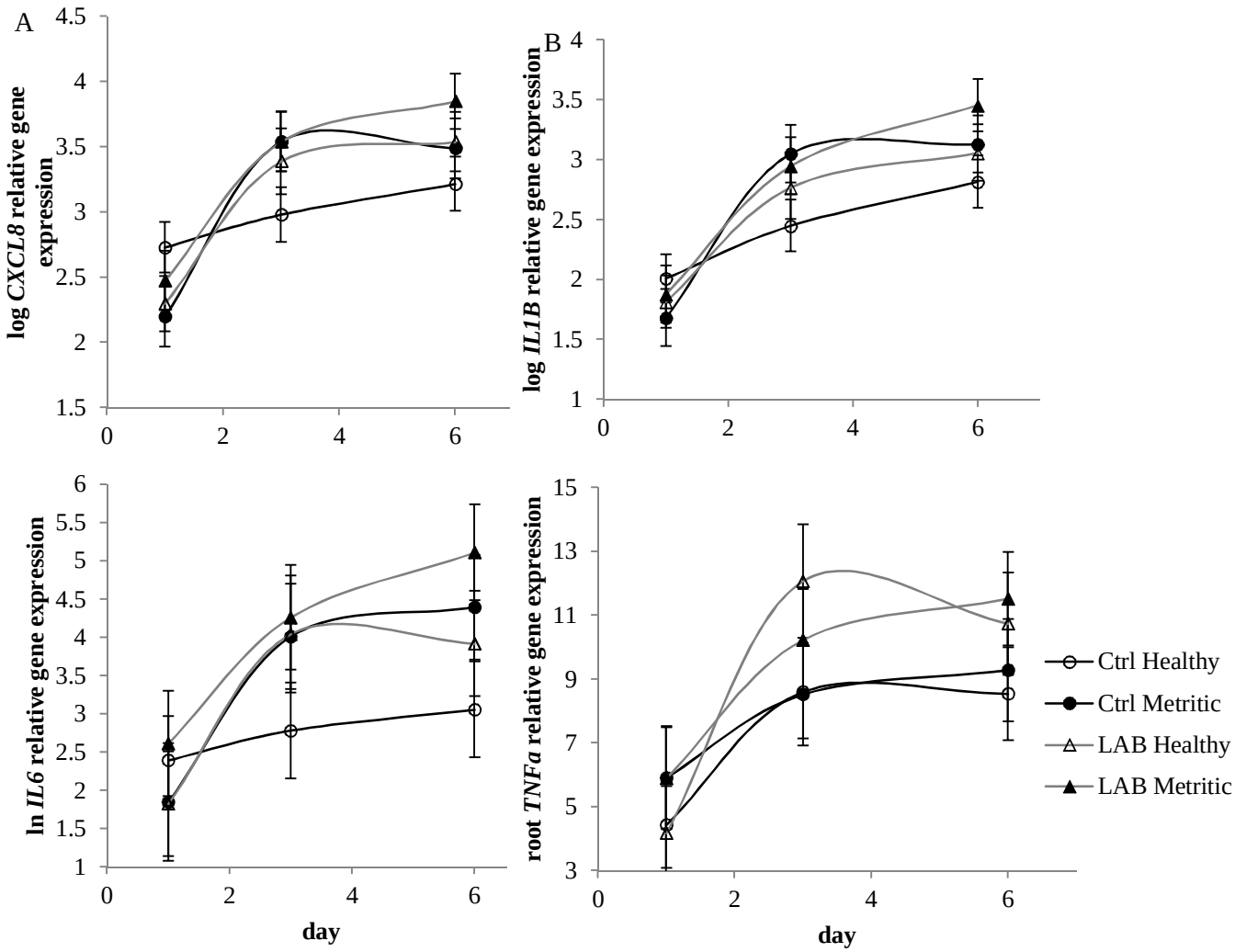
Table 6: Correlations between concentration of NEFA (µM) in blood and neutrophil relative gene expression.

	NEFA	
Neutrophil	r2	*P* value
SELL	0.024	0.096
NCF1	0.043	0.026
SOD1	0.015	0.19
TNFaR	0.009	0.32

3.4 Pro-inflammatory markers:

The gene expression of the pro-inflammatory cytokines *CXCL8, IL1B, IL6,* and *TNFa* was evaluated as markers of inflammation in the endometrium. The gene expression of this cytokines was affected by time ($P < 0.0001$), but not by treatment or metritis. No significant interactions were found between treatment and metritis, metritis and time, time and treatment. The triple interaction treatment*metritis*time was not observed neither (Figure 3).

Figure 3: Innate immune response from endometrial biopsies taken from healthy (white spots) and metritic (black spots) cows from the CTRL (black line with circumferences) and ENDO (grey line with triangles) treatment. Expression of *CXCL8* gene over time (Fig 3A), *IL1B* (Fig 3B), IL6 (Fig 3C), and TNFa (Fig 3D). Points represent means ± SEM for different conditions.



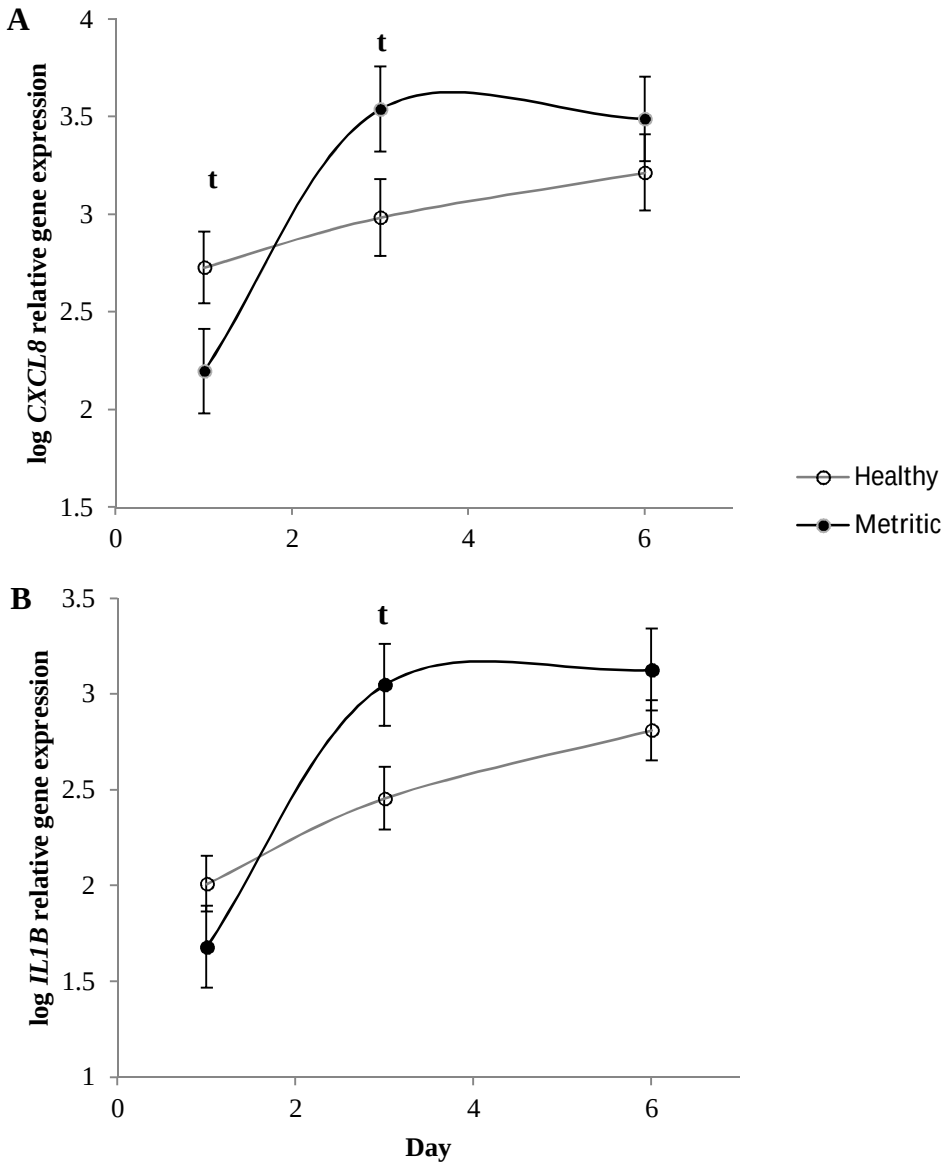
As treatment was not affecting the expression of pro-inflammatory profile, a second analysis was performed to evaluate differences in gene expression between metritic and

healthy cows, then removing treated cows (endometrial treatment) and only using those belonging to the control group.

The gene expression of *CXCL8* was affected by day ($P < 0.0001$), and there was an interaction of metritis and day ($P < 0.05$, Figure 4A). Healthy cows tended to express more *CXCL8* at day 1 (2.30 fold) whereas metritic cows tended to increase its expression by 2.45 fold at day 3 compared with healthy cows.

The *IL1B* gene expression was affected by day ($P < 0.0001$) and there was an interaction between day and metritis ($P < 0.05$, Figure 4B). Metritic cows tended to increase *IL1B* gene expression at day 3 by 3.05 fold compared with healthy cows. No differences were found at day 1 or day 6.

Figure 4: Innate immune response from endometrial biopsies taken from healthy (grey line) and metritic cows (black line) from the CTRL treatment. Expression of *CXCL8* gene over time (Fig 4A), and *IL1B* (Fig 4B). Points represent means ± SEM for different conditions. Points with t differ ($P < 0.1$) between healthy and metritic cows.



The gene expression of *IL6,* and *TNFa* was affected by day ($P < 0.05$), but no differences were observed between healthy and metritic cows.

4. Discussion

Previous work by other authors had proved in the past that intra-vaginal administration of some LAB strains was able to reduce uterine infections in dairy cows [107]. The main objective of this study was to evaluate the capacity of another LAB strains combination, previously proved to reduce *E. coli* infection *in vitro* and *ex vivo* [143] to modulate inflammation and reduce metritis prevalence after intra-vaginal or intra-uterine administration. The intra-vaginal application of LAB twice per week during the three weeks before calving (with a media of 5 doses/cow) was able to reduce up to 58% (P = 0.04, Figure 1) metritis prevalence in this group of cows (vaginal group) compared with the control cows. Previous work demonstrated that cows treated intra-vaginally with this combination of LAB tended to have lesser presence of *E.* coli in the vagina than not-treated cows (P = 0.088, unpublished data). These findings could explain partially the results observed herein. On the other hand, the intra-uterine application of the same dose of LAB did not show any differences in metritis prevalence compared with the control group (P =0.872, Figure 1). The idea behind this treatment was to study not only the effect on metritis prevalence but also if a more direct application of LAB to the endometrium would be able to down-regulate the post-calving inflammation associated with infection and involution processes.

In this study, neutrophil activity was quantified by gene expression as part of the inflammatory response. It is known that neutrophils are a primary source of immune response in the uterus, being the neutrophil activity associated with risk of metritis [150]. Some authors [192] have stated that desirable response should be a prompt, substantial flux of neutrophils into the uterus after calving. On the other hand, an excessive immune response could be counter-productive as impairs uterine or ovarian function [10, 193]. In our case, we had observed that metritic cows tended to express more *SELL (*l-selectin, P = 0.079, Table 4), a protein that is expressed on the neutrophil surface and promotes neutrophil infiltration into the infected tissue, than healthy cows. Furthermore, metritic cows also expressed more *TNFaR* (the TNF-α receptor that is involved in regulating

neutrophil function) than healthy cows (*P* = 0.041, Table 5). When we compare LAB treated cows with not-treated cows, we realized that the vaginal and endometrial treatment down-regulated the expression of all genes studied as markers of neutrophil activity (Table 4). Specifically, the expression of *SELL* and *TNFaR* was reduced along with the expression of *NCF1* and *SOD*, both involved in the respiratory burst. In the vaginal treatment the decrease of neutrophil activity probably could be related to the reduction of pathogenic bacteria in the vagina and the less incidence of infection in the uterus. No differences by LAB endometrial treatment were observed nor with *CXCL8*, nor with the other cytokines *IL1B, IL-6,* and *TNFa* expression levels (Figure 3). In the endometrial treatment this was an unexpected result because in previous *in vitro* and *ex vivo* studies, the direct treatment of endometrium with LAB modulated the levels of those cytokines [181]. The endometrial treatment produced some effects because there was a clear reduction of neutrophils activity but probably the number of administration was not enough to produce a sustained effect to modulate neither the proinflammatory cytokines expression nor the metritis incidence.

The differences between a pathological and physiological inflammation is not well understood and depends on the severity, timing and duration of inflammation [10]. It is widely accepted that all post-partum cows are in an inflammatory state [76] and that damage to uterine tissue during calving leads to inflammation that likely contributes to the systemic condition. In this study, when the profile of pro-inflammatory cytokines was analyzed only in control animals the results showed that metritic cows had impaired expression of *CXCL8* at day one compared with the controls, but at day 3 it is reversed, *CXCL8* and *IL1B* are over-expressed in those metritic cows compared with the controls (Figure 4). It seems that those cows with a less active immune system are the ones more sensible to the bacterial infection (that is present to almost all dairy cows during the 2-3 weeks after calving [194]) causing metritis at the end, as the immune systems reacts more slowly.

Hammon et al., 2006, found that cows with puerperal metritis had significantly higher NEFA levels pre-partum than healthy cows. In this study no differences in NEFA concentration in blood between healthy and metritic cows were observed (*P* =0.183). That may be due to different criteria to diagnose metritis, while we had diagnose metritis

when temperature > 39.5ºC and cows had vaginal purulent discharge at day 6, they classified cows as metritic if purulent discharge was observed between day 0 and 14 after parturition, regardless of presence of fever. It has been observed an increase of NEFA in multiparous cows in the endometrial treatment. This raising in NEFA concentration may be explained due to a bigger NEB in this group of cows. However no differences in milk production at 60 DIM were observed between treatments ($P = 0.5488$, data not shown), and no differences in body weight were observed either ($P = 0.956$, data not shown). As cows in the endometrial treatment modified neutrophil expression (Table 4) that may indicate that LAB is affecting energetic metabolism directly or indirectly. It has been described in the literature that high peripartum blood NEFA concentration is associated with impaired blood neutrophil functions [150] and proliferation [188]. Herein we have not found any correlation between NEFA and the expression of some genes related to neutrophils activity but we cannot discard other relationships with total neutrophil activity, not measured here. It is possible that with less neutrophil activity, the endometrium is recruiting more immune cells necessary for the involution processes, increasing the negative energy balance of the animal, but no total immune cell count was performed in this study. No differences in NEFA concentration was observed between metritic and healthy cows, but as expected pre-calving and post-calving NEFA concentration varied ($P < 0.001$, Figure 2b) being samples taken 10 days before parturition different from the ones taken after calving [195].

5. Conclusions

The LAB vaginal treatment reduced more than a half the metritis prevalence in dairy cows compared with control animals whereas the LAB endometrial treatment did not alter the metritis incidence. Both treatments reduced neutrophil activity but did not modify pro-inflammatory cytokines. This combination of LAB appears to be a promising strategy to prevent uterine infections by pre-calving intra-vaginal administration.

CHAPTER VII

General Discussion

The goal of this thesis was to identify possible preventive or/and therapeutic strategies against metritis in dairy cows.

Metritis accounts for the infection of the uterus during the first 21 days (more prevalent during the first 10 DIM) after calving. In this infection, pathogenic strains of *E. coli* play a crucial role because they have been identified as the first bacteria involved in the infectious processes [34]. Metritic infections and the consequent acute inflammation coexist with the physiological inflammatory status due to uterus involution after calving. Aims to reduce infection and associated acute inflammation are of extremely importance since they will reduce the total inflammatory status and the negative consequences such as tissue damage. Moreover, in the literature, it is exposed the need to find new strategies to fight infections without using antibiotics. The first reason is because often the antibiotic is not enough to fight against uterine infections [53]. In addition, free antibiotic use had led to an increment in antibiotic resistances, both in animals and humans, and in consequence its antimicrobial activity has been compromised. In this context, finding new antimicrobial alternatives is one of the top public health challenges in the 21st century. Antibiotics had already been banned as growth promoters in animal feed in the European Union and everything seems to indicate that a reduction in antibiotic administration as a preventive strategy against infections will be announced soon.

One of the most used strategies to fight infections in the gut is the use of probiotics with interesting results [113]. In the case of uterine infections, metritis specifically, not much research had been done regarding the use of probiotics.

In **chapter III**, four different Lactic acid bacteria (LAB) strains were selected from the literature to be tested as possible probiotics against uterine *Escherichia coli* infection. The chosen strains were *Lactobacillus rhamnosus, Pediococcus acidilactici, Lactobacillus reuteri,* and *Lactobacillus sakei*. All had been used as probiotics in the gastrointestinal tract or in the food industry. To be more specific, it is known the capacity of *Pediococci* to produce antimicrobial compounds such as organic acids or antimicrobial peptides such as pediocin AcH/PA-1 [100], and that *L. reuteri* produces reuterin, a broad-spectrum antibiotic substance able to inhibit pathogenic bacteria [111]. *L. rhamnous* is able to

protect the human urogenital tract by excreting biosurfactants to inhibit the adhesion of pathogens and it is extremely adherent to uroepithelial and vaginal cells [104], whereas *L. sakei* produces sakacin T and X, identified as bacteriocins [133]. It has been demonstrated by other authors that *Lactobacillus* are present in the vagina of healthy and infected cows [100], but more predominantly in the case of healthy cows [97]. That leaded to the hypothesis that *Lactobacillus* may be able to block *E. coli* infection and reduce the associated physiological (basal) inflammation due to uterus involution after calving. To prove that hypothesis, 4 different doses of each strain where tested against metritic *E. coli* infection in endometrial epithelial primary cell culture. Also, the potential of such strains was tested at modulating the physiological (basal) inflammation of the endometrium that may coexist with *E. coli* infections. Hence, two *in vitro*/*ex vivo* models were used: acute inflammation (induced by *E. coli* infection) and total inflammation model (basal inflammation combined with *E. coli* infection). The doses able to reduce *E. coli* infection and/or reduce the pro-inflammatory markers (*CXCL8* and *IL1B*) *in vitro* were selected for further experimentation. Interestingly, the different doses of each strain had different behavior, making the results very dose dependent. *P. acidilactici* was chosen due to its capacity to reduce *E. coli* infection when the cells did not have basal inflammation, *L. rhamnosus* because it was able to reduce inflammation markers (under basal and acute inflammation) and, *L. reuteri*¸ because was the only bacterium able to block *E. coli* infection in the total inflammation model. *L. sakei* was discarded even if it was the strain with better results fighting *E. coli* infection because, contrary to what it was expected, it increased the expression of pro-inflammatory cytokines. On the other hand, other authors found *L. sakei* a good candidate to be used as a prophylactic treatment against uterine infections, along with different strains of *P. acidilactici* [121].

In **chapter IV**, endometrial epithelial cells were analyzed with electron microscopy, both scanning and transmission. Those analysis gave us valuable information of how *E. coli* was acting, as well as the probiotic strains. In any case biofilms were observed at the used doses despite the ability of *P. acidilactici* and *L. rhamnosus* to form them demonstrated in **chapter III**. Furthermore, no direct interaction between the LAB strains and *E. coli* were observed. TEM analysis pointed out higher necrosis incidence in cells infected with *E. coli* but not treated with probiotics, whereas in samples pre-treated with LAB, necrosis

incidence was reduced. Again, more mitochondrial damage was found in not treated cells than in treated cells.

In **chapter IV**, the analysis of different combinations of selected LAB and doses were performed to finally find a synergic action that could efficiently reduce metritis in dairy cows. The combination composed by *L. rhamnosus*/ *P. acidilactici* / *L. reuteri* with a ratio of 12/12/1 showed to be the most efficient reducing *E. coli* infection and inflammation compared with the other combinations or to the strains alone that performed better *in vitro*. The selected LAB combination reduced 2.16 fold *E. coli* infection without basal inflammation compared with *P. acidilactici* alone, while again, the combination was able to reduce more *E. coli* infection in the total inflammation model than *L. reuteri* alone (2.59 fold). Furthermore, the combination led to a greater reduction of acute and basal inflammation.

Using endometrial tissue in *ex vivo* experiments instead of just endometrial epithelial cells was the logical step in order to better understand the effect of the LAB combination regulating infection and inflammation. The generation of cell cultures (*in vitro* model) modifies the tissue architecture and the spatial arrangement of cells, which may alter their normal function [136, 148]. That difficulty is overcomed using tissue explants because they are composed by multiple cell types rather than just epithelial or specialized immune cells, allowing the study of overall inflammatory response to pathogens [141].

When the LAB combination was applied to endometrial tissue, the same pattern was observed, the pro-inflammatory cytokines IL-8, IL-1β, and IL-6 were reduced when the probiotics were applied compared with *E. coli* infection alone. However, the decrease of the concentration of IL-8 and IL-1β from infected tissue treated with probiotics or not, was lower than the *in vitro* model. That could be explained, first, because the methodology to quantify those inflammatory markers were different. While in the *in vitro* model we were quantifying the gene expression of the markers, in the *ex vivo* model we were quantifying protein concentration. The methodology was different because the levels of protein in the supernatant of the *in vitro* model were not detectable by ELISA. Secondly, applying the LAB combination directly to the cells instead of tissue facilitates a more direct and homogenous contact between the bacteria and the cells, increasing the

stimulation of the innate response. Furthermore, the different types of cells present in the tissue react different to the LAB combination than only epithelial cells.

It has been described that impairment of polymorphonuclear leukocytes phagocytosis and a decrease in the ability to fight bacterial infections are associated with parturition in dairy cattle [88]. The most important component of uterine defense is the non-specific phagocytosis by neutrophils [53]. It is common knowledge that the immune system is inhibited before calving [53, 88], so a decrease in neutrophil activity during the periparturient period may decrease the ability to fight bacterial infections in cows [150]. In **chapter VI**, differences in neutrophil activity were observed between metritic and healthy cows. To be more specific, metritic cows tended to express more *l-selectin* (*SELL*), and expressed more *TNFaR* than healthy cows. During inflammation, *SELL* expressed in the neutrophils surface promotes neutrophil infiltration into infected tissue [196]. Once the neutrophils arrive at the site of infection, toll-like receptors (TLR) expressed on neutrophils surface recognize and bind pathogens. Neutrophils phagocyte pathogens and then destroy them by mechanisms including respiratory burst. Components of respiratory burst pathways include neutrophil cytosolic factor (*NCF1*, a component of the NADPH oxidase enzyme complex) and superoxide dismutase (*SOD*) [197]. Cytokines, such as TNF-α and IL-1, produced by epithelium and immune cells, can bind to receptors on neutrophils to regulate neutrophil function [196]. However, cows treated *in* vivo with the intra-uterine LAB combination at 1 day after calving reduced the gene expression of neutrophil activity markers compared with control cows but no interaction between LAB treatment and disease was observed. In the case of the intra-vaginal treatment, based in the LAB combination applied during 3 weeks pre-calving, a reduction of *SELL* and *TNFaR*, and a tendency to decrease *NCF1* gene expression was observed compared with control group. The drop in neutrophil activity in the vaginal treatment may be interpreted as a result in the diminution of metritis prevalence (58% reduction compared with control cows). Less metritis means less *E. coli* infection, and then a reduction in neutrophil activity. That correlates with the fact that metritic cows express more *SELL* and *TNFaR* than healthy cows. No interaction between disease and vaginal treatment was observed probably because only 6 cows were diagnosed with metritis in the vaginal group, not having enough statistical power. Some authors have stated that the killing capacity of

neutrophils is impaired through the peripartum period [152], which may be explaining the lack of differences in the gene expression of *NCF1* and *SOD1* between healthy and metritic cows.

Contrary to what it was expected after the results obtained *in vitro* and *ex vivo* (**chapters III** and **IV**), no differences in pro-inflammatory markers were found in the biopsies from cows treated *in vivo* with intra-uterine LAB treatment compared with control cows (**chapter VI**). The idea behind this treatment was to study not only the effect on metritis prevalence (which was not modified) but also if a direct application of LAB to the endometrium would be able to down-regulate the post-calving inflammation associated with infection and involution processes. We were expecting to see a decrease in the pro-inflammatory markers, especially in the case of *CXCL8*. *CXCL8* induces chemotaxis principally in neutrophils, causing them to migrate towards the site of infection. However, no differences were observed. Interestingly, when the biopsies from control cows were analyzed alone, metritic cows tended to express less *CXCL8* than healthy cows at d +1 after calving. Same pattern was observed with *IL1B* although it was not statistically significant. These results seem to indicate that considering that bacteria is present in the vagina of all cows after calving [194], the animals with the immune system more impaired are the ones who would likely be infected.

It has been described in the literature that cows with metritis have higher levels of non-esterified fatty acids (NEFA) in blood than healthy cows one week before calving [150]. NEFA are produced under a state of negative energy balance (NEB), characteristic in the periparturient period of the cow, where blood glucose decreases and body tissue reserves are mobilized to provide more energy. In the study explained in **chapter VI** no differences were observed between healthy and sick animals. As stated in the discussion of that chapter, that may be due to different criteria diagnosing metritis (other authors diagnose puerperal metritis while we diagnose acute metritis). Curiously, an increase of NEFA in blood in multiparous cows (cows with more than one lactation) treated with intrauterine LAB has been found. It is difficult to explain the reason of this increment as no differences in body weight loss were observed either. Cows with bigger NEB lose more weight, that at the same time increases NEFA in blood. Furthermore, no differences in milk production (which could explain more energetic requirements) were found between

those cows and the others. It is possible that with less neutrophil activity (reduced by the intrauterine treatment), the endometrium is recruiting more immune cells necessary for the involution processes, increasing the negative energy balance of the animal.

In **chapter VI** it is proved that the LAB combination is able to reduce metritis prevalence when the treatment is applied in the vagina but not if it is applied in the endometrium. Those results combined with the fact explained in **chapter V** that the LAB combination applied in the vagina does not reach the endometrium leads to conclude that the probiotics are regulating the vaginal environment blocking *E. coli* infection. This will correlate with the findings from Ametaj group, where even using another combination of probiotics, they reduce metritis prevalence as well [107]. Moreover, the study in **chapter V** demonstrated that cows treated intra-vaginally with this combination of LAB tended to have lesser presence of *E. coli* in the vagina and lesser expression of *Bdefensins* and *Muc1* (markers of infection) in the endometrium than not-treated cows.

In general, the results of this thesis, have demonstrated that the use of LAB in specific combination of strains and doses, allows the reduction of metritis prevalence in dairy cows and opens a new preventive strategy to improve fertility in cattle, greatly affected by uterine infections. Finally, this strategy also contributes to decrease and control the use of antibiotics in animal production, paving a safest and more sustainable future.

CHAPTER VIII

Conclusions

1. *Pediococcus acidilactici* inhibits *Escherichia coli* infection and downregulates inflammation in bovine endometrial epithelial cells.
2. *Lactobacillus rhamnosus* reduces inflammation mostly in the presence of acute inflammation caused by bacterial infection in bovine endometrial epithelial cells.
3. *Lactobacillus reuteri* inhibits *Escherichia coli* infection in bovine endometrial epithelium in the presence of basal inflammaton.
4. The best combination to modulate *E. coli* infection and inflammatory status in endometrium *in vitro* is composed by *Lactobacillus rhamnosus/ Pediococcus acidilactici/ Lactobacillus reuteri* with a ratio of 12/12/1.
5. The selected LAB combination *Lactobacillus rhamnosus/ Pediococcus acidilactici/ Lactobacillus reuteri* (at the ratio of 12/12/1), improve the control of infection and inflammation in comparison with the isolated strains.
6. The LAB combination reduces the concentration of acute and total inflammation markers *ex vivo* compared with control tissue.
7. The *in vivo* treatment of cows three weeks pre-calving with LAB combination at $4.5 \cdot 10^{10}$ CFU/dose in the vagina, twice per week, does not reach the endometrium.
8. There is no modulation of inflammatory genes at endometrium level when LAB is applied in the vagina or in the uterus.
9. LAB applied in the vagina three weeks pre-calving decreases the amount of *E. coli* in the endometrium and tends to reduce *E. coli* in the vaginal tract.
10. LAB applied in the vagina three weeks pre-calving decreases the expression of *Bdefensins* and *MUC1* (markers of infection) in the endometrium.
11. The preventive treatment of dairy cows with intra-vaginal LAB combination reduces metritis prevalence by 58% compared with control cows.
12. LAB combination treatment applied in the vagina or in the endometrium are able to reduce the neutrophil.
13. Neutrophil activity and NEFA concentration in blood of post-calving cows are not related.

14. LAB combination reduces necrosis and mitochondrial damage in endometrial epithelial cells caused by *E. coli* infection *in vitro*.
15. LAB combination does not create biofilms in endometrial epithelial cells *in vitro*.
16. There is no direct interaction between LAB combination and *E. coli in vitro*

CHAPTER IX

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