# Using Intravaginal Probiotics to Lower the Incidence of Uterine Infections and Improve Reproductive Performance and Productivity of Dairy Cows in Dairy Farms in Alberta

by

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## Abstract

Uterine infections are the number one reason for culling of cows in Canada and elsewhere because are associated with high incidence of infertility. Five hundred and twenty-six dairy cows (426 Holstein and 100 Jersey cows) from 4 dairy farms in Alberta (three farms had Holstein dairy cows and one farm had Jersey cows) were assigned into 3 experimental groups. In this study we tested the effects of administrating a cocktail of lactic acid bacteria (LAB) in the vaginal tract of dairy cows on uterine health, reproductive performance, clinical diseases as well as milk production and composition. Overall, the results showed that the incidence rate of uterine infections by infusion of probiotics was decreased by 29%. The incidence of uterine infections in Farm A, C, and D (Holstein breed herds) decreased by 19%, 36%, and 21%, respectively. Interestingly, in Farm B (an organically managed Jersey herd) the incidence rate of uterine infections decreased by 50%. There was a difference in the incidence of uterine infections with regards to parity. Control cows that were administered skim milk (TRT1) and saline (TRT2) had higher odds of developing uterine infections compared to cows administered with probiotics (TRT3). The probiotic cocktail is composed of *Lactobacillus sakei* and two strains of *Pediococcus acidilactici* isolated from vaginal mucus of healthy pregnant Holstein cows. Results also showed that probiotics infused intravaginally lowered concentrations of glucose and cholesterol in the serum of both primiparous and multiparous cows diagnosed with uterine infections compared to TRT1 and TRT2 cows at +1 and +4 wks after parturition. In conclusion, intravaginal infusion of probiotics improved overall health status and increased milk production in multiparous dairy cows and modulated serum concentrations of glucose and cholesterol after parturition in dairy cows affected by uterine infections

**Implications:** Around 45% of dairy cows are affected by metritis and 15% of the total number of cows registered with DHI Canada are culled because of reproductive issues totaling more than $100 million every year in Canada. A product such as intravaginal probiotics able to lower uterine infections might improve their overall health, productivity, and profitability of Canadian dairy industry.

## Preface

The idea, project proposal, and experimental design were developed by my supervisor Dr. Burim N. Ametaj. Dr. Ametaj also supervised the training and conduction of the experiment, and development of databases for the experiment. He also supervised all the laboratory analysis, and outlining, writing, and editing of all the sections of this thesis. Dr. Andre Luiz Dias contributed to application of treatments, collection of samples, ultrasound imaging, and evaluation of clinical diseases for part of the experiment. Dr. Erkan Pehlivan assisted in sample collection during the whole experimental period. Ashley Egeydy assited with sample collection, laboratory analysis, and evaluation of clinical diseases. Suzanna M. Dunn provided assistance with laboratory analyses and collection of samples. The role of Dr. Michael G. Gänzle was in providing the probiotics and assistance in analysis of microbiota. I was responsible for the administration of treatments, ultrasound scanning, collection of samples and data, as well as for the major part of sample analyses in the laboratory. The statistical analyses and manuscript composition were my original work.

The research project, of which this thesis is a part, received research ethics approval from University of Alberta Animal Care and Use Committee for Livestock (Animal use protocol AUP#00001811) entitled Using intravaginal probiotics to lower the incidence of uterine infections and improve reproductive performance and productivity of dairy cows in dairy farms in Alberta.

## Dedication

I dedicate this thesis to my wife (Maria Jose) and kids (Valentina and Mateo), my parents (Maria E. and Gustavo), and brothers who supported me throughout this adventure. Thank you for your support, love, and unconditional encouragement.

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I sincerely thank:

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Eduardo Barahona, Edmonton, Canada

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## List of abbreviations

| | |
|---|---|
| ACTH | Adrenal corticotropin hormone |
| AMPs | Anti-microbial peptides |
| APC | Antigen presenting cells |
| BCR | B cell receptors |
| BCS | Body condition score |
| CD | Cytolysin |
| CE | Clinical endometritis |
| CM | Cervical mucus |
| CRH | Corticotropin releasing hormone |
| CVM | Cervicovaginal mucus |
| DAMPS | Damage-associated molecular patterns |
| DC | Dendritic cells |
| DIM | Days in milk |
| DMI | Dry matter intake |
| *E. coli* | *Eschericha coli* |
| EHEC | Enterohemorrhagic *E. coli* |
| EPEC | Enteropathogenic *E. coli* |
| ETEC | Enterotoxigenic E. *coli* |
| FAO | Food and Agriculture Organization of the United Nations |

| | |
|---|---|
| FRT | Female reproductive tract |
| IGF-1 | Insulin-like growth factor-1 |
| IgG | Immunoglobulin G |
| IL-1β | Interleukin-1 beta |
| LAB | Lactic acid bacteria |
| LPS | Lipopolysaccharide |
| NAPSs | Neutrophil-activating peptides |
| NEFA | Non-esterified fatty acids |
| NK | Natural killer cells |
| NLR | Nucleotide-like receptors |
| NOD | Nucleotide- binding oligomerization domain |
| NTEC | Necrotoxic E. *coli* |
| PAMPs | Pathogen-associated molecular patterns |
| PGE | Prostaglandin $E_2$ |
| $PGF^{2\alpha}$ | Prostaglandin $F_{2\alpha}$ |
| PLO | Pyolysin |
| PMN | Polymorphonuclear neutrophils |
| PRR | Pattern recognition receptor |
| SCE | Subclinical endometritis |
| sIgA | Secretory immunoglobulin A |
| TLR | Toll-like receptors |
| TNF- α | Tumor necrosis factor alpha |

# Chapter 1 Literature Review

## 1.1 Uterine Infections in dairy cows

### 1.1.1 Uterine infections and its consequence to the industry

Problems regarding cow diseases are encountered on Canadian farms operations every day. This makes the farms be less efficient, therefore, profitability margins decrease. Metabolic and infectious diseases are the major reasons for which dairy cows are being culled in farms (Can West DHI, 2015). According to Sheldon (2009) diseases, such as uterine infections, can affect almost half of dairy herds due to compromised immunity in postpartum dairy cows. It has been reported that around two weeks after calving, cows develop uterine diseases, such as metritis, due to the high numbers of pathogenic bacteria that ascend into the uterus. Consequently, 40% of the cows that do not clear unwanted pathogens efficiently will develop an endometritis (Williams et al., 2007). Uterine diseases in dairy cows can be classified as puerperal metritis, clinical metritis, clinical endometritis, and subclinical endometritis (Sheldon et al., 2006). This classification is based on the time that uterine infections occur with puerperal metritis immediately after calving, metritis up to three weeks after calving, endometritis more than three weeks postpartum.

According to the CanWest DHI and Valacta, culling rates for reproductive issues of dairy cows in 2016 were 17.2% out of 28.19% of the total culling rates. Therefore, understanding and lowering the incidence of uterine infections in dairy cows is one of the challenges of Alberta's cattle industry.

### 1.1.2 Economic implications of uterine diseases

Exact economic loss models due to uterine diseases in dairy cows are difficult to find, due to different treatments used, medication costs, prices of milk, and use of the discarded milk. The most recent economic model adapted by Overton and Fetrow (2008), estimated the loss in milk production, culling risk, and reproductive performance due to uterine infections. Adding up the estimations for the above mentioned and depending what antibiotic is used to treat the uterine infection, an estimate to treat a case of metritis in dairy cows can sum up to USD 386 per cow or CAD 478 (Rate: 1 Canadian dollar = US dollar 0.81). If we adapt the results of this model to treat a case of metritis in the 79,500 dairy cows in the province of Alberta, having a 22% of incidence rates for metritis in 4 farms in this experiment, the total cost of treating uterine infections would be approximately CAD 8,360,220 per year. If adapted to 956,900 dairy cows which is the total herd in Canada, and having the same incidence rates for metritis, the total cost of treating uterine infections would be approximately CAD 100,627,604 per year. Even though the exact cost is difficult to calculate, Overton and Fetrow (2008) model serves as a base to suggest that there are improvements to be done in prevention and early diagnosis of uterine diseases management activities, that will increase the number of calves and decrease the number of culling rates in Canadian farms.

### 1.1.3 Physiology of the reproductive tract in dairy cows

The reproductive tract is composed of the ovaries, oviducts, uterus, cervix and vagina, considered as interconnected tubes, also important for transport of gametes to the point of fertilization, as well as the transport, development and implantation of the developing embryo (Carson et al., 1998). It is also composed of external organs known that facilitate copulation and are also physical barriers against external pathogens. The reproductive tract is supported by the

broad ligament, which acts as a suspensory tissue supporting the reproductive tissue from the dorsal wall into the abdominal cavity (Senger, 2009).

The ovaries are connected to the fallopian tubes or oviducts where fertilization of the oocytes take place. If the oocyte is fertilized, the developing zygote and embryo are transported into the uterus by means of ciliary action, where implantation will take place (Wood and Marshal, 1920; Senger, 2009). Fertilization is usually considered to occur as the oocyte passes from the ampulla to the isthmus (Senger, 2009). The posterior end of the uterus forms the only uterine opening known as the cervix also called the *os uteri*. The cervix will act as the first anatomical defense barrier preventing pathogens from entering the uterine lumen (Azawi, 2008). The cervix allows the path of the sperm during mating for fertilization and during pregnancy it will be filled up with mucus creating a cervical plug that would be denoted as a second mechanical barrier that prevents unwanted pathogen from ascending into the uterus. In preparation for parturition, during the days prior to calving the cervical plug is expulsed (Wood and Marshal, 1920; Senger, 2009).

The uterus is divided into three layers: the perimetrium, myometrium, and the endometrium (Budras, 2003). In the first two to three weeks after parturition, the uterus decreases in more than 80% of its pre-calving size; this process is very complex and managed by a variety of control structures that permits the process of uterine involution (Klingborg,1996). In a cow, the uterus plays various important roles and can take on many states: support gamete and embryo transport, recognize the presence of and support development of the pre-implantation embryo, facilitate placental development and attachment, and support fetal development until the time of parturition (Senger, 2009). Close to and after parturition secretions of corticotropin releasing hormone (CRH) and adrenal corticotropin hormone (ACTH) stimulate the production of fetal cortisol in the calf. The fetal cortisol will pass to the maternal side and initiate a cascade of events that lead to

parturition (Senger, 2009). As part of this cascade cortisol will change the endocrine balance from progesterone to estradiol and will enhance the production of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Post-partum, $PGF_{2\alpha}$ will trigger the involution and contractions of the uterus, causing the expulsion of uterine residues and fluids 3-6 weeks after parturition (Deutscher et al.,1980; Kindahl et al.,1999; Sheldon,2004).

Subsequently, regeneration of the endometrium will be completed around 25 days after calving if elimination of bacteria by the host was successful. While a return of estrogenic state will allow for a return to the estrous cycle and normal ovarian cyclical activity is expected if the first dominant follicle is formed around 12 days after parturition (Savio et al., 1990; Beam and Butler, 1997; Sheldon et al., 2008).

### 1.1.4 Bacterial contamination

The vulva, vagina and cervix are anatomical barriers that prevent pathogens ascend to the uterus in postpartum dairy cows (Källerö, 2010). After parturition, the reproductive tract is compromised due to the dilated state of the cervix that allows bacteria to ascend from the external environment into the uterine lumen, which if persisting, can cause clinical diseases that might lead to subfertility and/or infertility. Lower conception rates, increased number of days opened, and increased culling rates performance are associated to bacterial uterine infections (Sheldon and Dobson, 2004; LeBlanc et al., 2002). Bacteria found in the uterus have been reported to lower ovarian follicular growth and function (Sheldon et al., 2004). A study conducted by Williams et al., (2007) supported this by providing evidence that ovarian folliculogensis was disrupted by bacterial contamination, meaning that growth and function of the first dominant follicle was

suppressed. Furthermore, uterine infections caused by bacteria will also result in decreased estrous detection, therefore lower conception rates (Sheldon, 2017).

In human's, bacteria that ascend from the vagina are the most common pathogens causing uterine infections (Racicot et al., 2013). A healthy uterus will clear pathogenic bacteria and keep it in its normal sterile state by means of defense mechanisms during the first four to six weeks after parturition. Furthermore, elimination of bacteria depends on various factors, such as uterine involution, uterine contractions, innate and adaptive immunity, and regeneration of the endometrium (Källerö, 2010). Ease in calving, retention of placentas, twins, and environments not proper sanitized can lead to high risks factors for uterine infections on cattle (Erb et al., 1980; Lewis, 1997a; Sheldon et al., 2008; LeBlanc et al., 2011; Onyango et al., 2014).

### 1.1.5 Uterine bacterial core

The uterus in its normal state is considered to be sterile (Sheldon et al., 2004; Dubuc, 2011). Diversity of bacteria dominated mainly by *Enterobacteriaceae* were found on vaginal tracts of dairy cows; additionally, *Lactobacillus* spp. and *Propionibacter* spp. were found in microbiota although in lower numbers (Otero et al.,1999; Machado et al., 2012). Other studies also reported presence of bacilli, staphylococci, and lactic acid bacteria of the genera *Enterococcus*, *Lactobacillus*, and *Pediococcus* in uterine microbiota of healthy and infected cows. Interestingly, same studies demonstrated that *E. coli* and *T. pyogenes* where also found in both infected and healthy uterus of dairy cows shortly after calving (Wang et al., 2013; Knudsen et al., 2014). *A. pyogenes*, *E. coli*, *F. necrophorum*, and *P. melaninogenicus* are known to cause uterine infections in postpartum dairy cows (Griffin et al., 1974; Ruder et al., 1981; Bonnette et al., 1991). Although many studies have been performed to classify pathogens that may cause uterine infections, to our best knowledge, none have a complete overview of the microbiota in the reproductive tract of dairy

cows. Interestingly, in the vaginal microbiota of women, primates and rats the genus *Lactobacillu*s is dominant (Herthelius et al., 1989).

Anaerobic bacteria such as *Bacteroidetes, Fusobacteria*, *Helcococcus*, *Filifactor*, *Porphyromonas*, *Peptoniphilus*, *Peptostreptococcus*, *Campylobacter*, and *Prevotella* were found in the uterus of cows infected with metritis and are considered to contribute to the development of reproductive infections, especially bacterial strains of the phyla *Bacteroidetes* and *Fusobacteria*. Bacteria associated with uterine health such as *Candidatus Blochmannia*, *Escherichia*, *Sneathia*, and *Pedobacter* were also found in the uterine fluids (Jeon et al., 2015). Other studies conducted by Santos and Bicalho (2012) report similar microbiome in healthy and infected cows, the phyla are mainly composed of *Bacteroidetes, Fusobacteria, Firmicutes, Proteobacteria,* and *Tenericutes.* In addition to these bacteria, sequences from *Spirochaetes, Synergistetes*, and *Actinobacteria* appear less frequently.

It is believed that fecal and vaginal contaminants are one of the main agents that cause reproductive infections, residues of these contaminants were detected in the uterine fluids. Although *E. Coli* was found in the bacterial core community in both healthy cows and cows with uterine infections, they were found in much greater numbers in infected cows.

*Arcanobacterium pyogenes* is a Gram-positive (G+) bacterium found in mucus of reproductive, gastrointestinal and respiratory tracts in cattle (Machado et al., 2012). Immune cells of the host are affected by *A. pyogenes*, expression of fimbriae and extracellular matrix binding proteins cause haemolysis and cytolytic factors that oversee the destruction of the cells, therefore alter cytokine expression in the host (Jost et al., 2005). It is also known that *A. pyogenes* secretes pyolysin (PLO), cholesterol-dependent cytolysin (CDC) which can cause death to immune cells by binding cholesterol in cell membranes of eukaryotic cells forming pores and resulting in the

death of the cell (Jost et al., 1999). Additionally, *A. pyogenes* has the ability to survive the engulfment of macrophages and secrete neuraminidase which reduces mucous viscosity cooperating with pathogens to attach easily to the host (Jost and Billignton, 2005).

*E. coli* is a Gram-negative (G-) bacteria mostly found in greater numbers in cows presenting uterine infections (Wang et al, 2013; Deng et al., 2014). Interestingly, *E. coli* has been found to be prevalent in the first week after parturition increasing the gene expression of Interleukin 1 beta (IL-1β) (Genís et al., 2016). Data reported by Williams et al. (2007) stated that after parturition *E. coli* infections conditions the proper environment for *A. pyogenes* to proliferate uterine infections in dairy cattle. A study conducted by Sheldon et al. (2010) supports the idea stated above, that *E. coli* isolated from uterine lumen of dairy cows 2 weeks after parturition worsen via synergistic actions of *A. pyogenes,* chronic uterine infections in dairy cattle. It should be mentioned that in three different studies conducted by DebRoy and Maddox (2001), Wang et al. (2013), Deng et al. (2014), reported enteropathogenic *E. coli* (EPEC), enterotoxigenic E. *coli* (ETEC), enterohemorrhagic E. *coli* (EHEC), and necrotoxic E. *coli* (NTEC) found in reproductive tracts of various animals.

*Fusobacterium necrophorum* is a Gram-negative (G-) anaerobic bacterium usually found in mucous membranes which secretes leukotoxin and produces cytotoxic effects against white blood cells (Wright et al., 2012). Additionally, a secretion of ecotin protein which has been known to be a potent anticoagulant inhibit neutrophil elastase which attack bacteria at sites of inflammation and constrain plasma kallikrein, a component in the clotting cascade (Wright et al., 2012). Several leukocytes especially PMNs cells, monocytes, macrophages, and dendritic cells are affected by the leukotoxin secreted by this pathogen and is believed to be the main virulence factor of this species (Tan et al., 1994; Tadepalli et al., 2008). In addition, *F. necrophorum* is categorized into two

subspecies: *F. necrophorum* subsp. *necrophorum* (*Fnn*) now known as biovar A and *F. necrophorum* subsp. *funduliforme* (*Fnf*) now known as biovar B (Shinjo et al., 1991). *F. necrophorum* has been categorized as a commensal of the gastrointestinal, respiratory and genitourinary tracts of animals (Narayanan et al., 1997; Tadepalli et al., 2008). *F. necrophorum's* outer cell is composed of an endotoxic lipopolysaccharide (LPS) which is a bacterial membrane composed of a polysaccharide chain, known as the O-antigen, and consisting of Lipid A which is known to be toxic (Caroff and Karibian, 2003). Gram-negative bacteria can release their outer membrane vesicles that include LPS, therefore, a release of endotoxin is incorporated into the host (Mashburn-Warren et al., 2008; Eckel and Ametaj, 2016).

## 1.2 Uterine Infections in postpartum dairy cows

### 1.2.1 Metritis

It has been known that metritis is a huge concern for dairy farmers worldwide. Affecting 20 to 40% of the herd, metritis negatively affects productivity, survival and welfare of dairy cows, due to the lack of preventive diagnostic and ineffective treatments (Sheldon al., 2008). Uterine infections can be evaluated by the character and odor of the vaginal mucus and can be scored between 0, clear translucent mucus; 1, clear mucus containing flecks of white pus; 2, exudate containing less than 50% white or cream pus; 3, exudate containing more than 50% white, cream or bloody pus (Sheldon et al., 2006). Tools such as speculum (LeBlancet al., 2002), the Metricheck device (McDougall et al., 2006), collection of the mucus with a pipette using a syringe (Deng et al., 2014) or globed hand are used to evaluate uterine discharges.

Metritis is known as an inflammation of the uterus causing different signs of health such as: uterine foul-smelling discharges (usually accompanied by blood), fever, decrease in milk

production, and the presence of signs of toxemia (LeBlanc, 2008). Metritis causes inflammation of the uterine wall occurring in the first 21 days in milk (DIM) but usually occurring in the first 10-14 days after calving. Potential pathogens associated with metritis such as *E. coli*, *A. pyogenes*, *F. necrophorum*, *P. melaninogenica* and *Proteus* species have been found in the uterus of postpartum dairy cows. (Sheldon et al., 2002; Williams et al., 2007).

Metritis can cause irreversible changes in the reproductive tract of a cow and cause diseases that damage the endometrium causing infertility, due to the high concentrations of bacteria. Cows with metritis are known to have higher concentrations of LPS and prostaglandin $E_2$ (PGE) but not $PGF_{2\alpha}$, enhancing prolonged luteal phases and decreasing the regulating inflammatory responses in the endometrium (Williams et al., 2007).

Meanwhile Azawi (2008), reported that retained placentas could be another factor for uterine infections in post-partum buffaloes, providing an ideal environment for bacterial growth. Strains of *E. coli, A. pyogenes, B. fragilis* and *F. necrophorum* species were found in buffalo's bacterial core causing uterine infections. Additionally, *E. coli* strains have been found on healthy and infected uterus in Holstein cows with metritis, even though infected cows presented greater amounts of the pathogenic strains (Wang et al., 2013). Bacterial contamination in the reproductive tract after parturition may be responsible for 90% of the infections in postpartum dairy cows (Herath et al.,2009). Risk factors that are related to the disease are age, parity, calving season, environment, nutritional factor, calving condition and retained placenta which makes the prevention of this pathology difficult to manage (Deng et al., 2014).

According to Sheldon et al., (2006), metritis can be categorized into 3 grades: Grade 1, characterized by having an enlarge uterus and the infection causing the cow to have a purulent uterine discharge 21 day after parturition. Grade 2 also known as puerperal metritis in which signs

of illness are found, fever (>39.5℃), decrease in milk production, and usually characterized by a fetid red-brown uterine discharge. Puerperal metritis is related to problems involving dystocia, retained placentas, twins and abortions which occurs during the first week after parturition; and Grade 3 causing the cow to decrease feed and water intake, which could develop the syndrome of a downer cow (Markusfeld, 1984; Foldi et al., 2006, Konyves et al., 2009).

It has been measured that cows that develop metritis decrease feed consumption 3 weeks before and after parturition, therefore, milk yields and body condition scores (BCS) tend to lower during the first two weeks. Cows with metritis have been known to have a lower BCS than cows with a healthy status. Decrease in feed intake can be an indicator for early detection of metritis, severe metritis is increased when Holstein dairy cows reduced dry matter intake (DMI) of 1 kg per day (Huzzey et al., 2007). An increased risk of developing metritis is greater on cows that need assistance for calving due to the bacterial contamination (Mateus et al., 2002). After parturition cows that developed metritis decrease immunity, therefore, increasing the risk of acquiring other diseases. Reproductive performance is affected negatively delaying the return to a normal estrous cycle which can later become infertile cows and culled from the herd (Sheldon et al. 2002; Deng et al., 2014).

It has been well recorded that regardless of the periparturient period the pH median in the Holstein cows vaginal tract tends to be around 7.50, however, in heifers pH values tend to be slightly higher around 7.75 during the first week after parturition, which may increase the possibility to develop uterine infections due to the cervix's compromised state (Cohen et al., 2012).

### 1.2.2 Endometritis

Endometritis is a localized inflammation of the endometrium whose principal agents are pathogenic bacteria (Boundurant, 1999; Sheldon, 2006). Normally cows that have a detectable

mucopurulent uterine discharge in the vagina after 20 to 33 DIM are defined to have endometritis (LeBlanc et al, 2002; Sheldon et al, 2006). Mucopurulent white or yellowish vaginal discharges (50% pus and 50% mucus) are usually a clinical sign to diagnose endometritis. Several studies have reported prevalence of clinical endometritis (CE) in 18 to 37% of the herds (Etherington et al., 1984; Bartlett et al., 1986; Markusfeld, 1987; Peeler et al., 1994; Drillich et al., 2002; Drillich et al., 2005). While, other studies focusing on subclinical endometritis (SCE) have found prevalence of the infection in 12 to 94% of the herd (Raab, 2004; Kasimanickam et al., 2004; Gilbert et al., 2005; Hammon et al., 2006; Barlund et al., 2008). Subclinical endometritis can be diagnosed and evaluated by the percentage of polymorphonuclear neutrophils (PMN) cells in cytological samples collected from the uterine lumen (Mateus et al., 2002; Gilbert et al., 2005; Sheldon et al., 2006). Cytological samples greater than 18% PMNs after 21 DIM, or greater than 10% PMNs 34 DIM after parturition are considered percentages to define SCE (Sheldon et al., 2006). Pathogens such as: *A. pyogenes, F. necrophorum,* and *Prevotella* have been found in cytology samples of uterine lumens, increasing the risk of developing an endometritis after calving (Ruder et al., 1981; Olson et al., 1984).

Another important method used to detect endometritis in postpartum dairy cows is the use of ultrasonography (Kasimanickam et al., 2004). Ultrasonography measures the clearance of fluids from the uterus and the size of the body of the uterus. The measurements of the uterine horns at the base of the horn can be categorized as follows: small (less than 3.5 cm), medium (3.5-5.0 cm), and large (> 5.0 cm) (Kasimanickam et al., 2004). LeBlanc et al. reported sizes of uterus greater than 8 cm in diameter between 20 and 33 DIM in cows with uterine infections. In the study conducted by Kasimanickam et al., (2004), uterine diameter was measured for 228 cows in two stages: V1(20-33 DIM) and V2(34-47 DIM). The cows in this study had uterine diameters for V1

stage of: 27.6% (<3.5 cm or small), 52.2% (3.5-5.0 cm or medium), and 21.5% (> 5.0 cm). While in V2 stage they could find 68.4% (<3.5 cm or small), 28.1%(3.5-5.0 cm), and 3.5%(> 5.0 cm). These percentages clearly state that more than half of the herd had a normal uterine involution during the first 47 DIM and where diagnosed as healthy cows. Cows with longer duration of uterine infections will result with decrease of estrus detection, conception and pregnancy rates and increased days in first service which could lead to high culling rates in a herd (LeBlanc et al., 2002; Kasimanickam et al.,2004; Raab, 2004; Gilbert et al.,2005).

### 1.2.3 Pyometra

Pyometra is defined as the accumulation of the purulent mucus within the uterine lumen in the presence of a persistent corpus luteum and a closed cervix (Sheldon et al., 2008). Studies have also reported the identification of *F. necrophorum* and *Truepella pyogenes* in microbial composition of cows with pyometra (Sheldon et al., 2008; Knudsen et al., 2015). Pyometra can be diagnosed by transrectal palpation and ultrasonography in the presence of corpus luteum and anestrus (Sheldon et al., 2006).

## 1.3 Immune Response to Uterine Infections

### 1.3.1 Roles of innate immunity in the female reproductive tract

Innate immunity plays an important role in protecting the endometrium from pathogenic bacteria and preventing uterine infections. Cells involved in innate immunity include neutrophils, macrophages, dendritic cells (DC), natural killer cells (NK) and mucosal epithelial cells (Fazeli et al., 2005). Innate immunity is also known as the first line of response system consisting of mechanical, chemical and cellular components (Mackay and Rosen, 2002; Janeway and

Medzhitov, 2002). Chemical reactions include pattern recognition receptor (PRR) like Toll-like receptors (TLR) and nucleotide- binding oligomerization domain (NOD)-like receptors (NLR) (NOD1 and NOD2) (Herath et al, 2009). Additionally, PRR expressed in immune cells detect pathogen-associated molecular patterns (PAMP) and damage-associated molecular patterns (DAMPS) by the TLR, which will recruit immune cells that will secrete cytokines and chemokines that will create an acute inflammatory response (Herath et al, 2006; Farage et al., 2011). A study conducted by Hart et al., (2009) reported the presence of TLR1 through 10 in uterine horns and uterus, TLR1 through 9 in the vagina and cervix which lead to production of IL-8 by epithelial cells. Production of lingual anti-microbial peptides, tracheal antimicrobial peptide and β- defensins are common in the female reproductive tract (FRT) acting as defense bactericides (Gilbert, 2005). Epithelial cells and neutrophils located in the female reproductive tract will produce anti-microbial peptides (AMPs) and neutrophil-activating peptides (NAPSs) which are also components of the chemical immunological barriers (Nasu and Narahara, 2010). Additionally, epithelial cells play an important role in the production of mucus (Ochiel et al., 2008; Capaldo and Nusrat, 2009).

It is known that mucosal epithelial cells in the uterine tract act as protective barrier against unwanted pathogens. The way of action is by inducing pathogens by physiological defense mechanisms which includes apoptosis, necrosis, or phagocytosis (Farage et al., 2011). Mucus protects the host by binding and retaining the pathogenic agent as a physical and immunological barrier, preventing pathogens from entering further into the uterus (Farage et al., 2011; Gilbert, 2005). A study conducted by Carson et al., (1998) reported that mucin glycoproteins Muc-1 found in mucus not only protect the host from infections but also is vital in the reproductive process. Cervical mucus (CM) is produced by goblet cells found in the cervix, some of cervical mucus components are 5AC and 5B gel forming mucins that also form the mucus of the FRT (Gipson,

2001; Andersch-Bjorkman Et al., 2007). Cervical mucus usually mixes with the vaginal fluids also known as cervicovaginal mucus (CVM) (Huggins and Preti, 1981).

Innate immunity in the female reproductive tract (FRT) plays an important role in the defense against Gram negative bacteria, which usually are responsible for uterine infections. Neutrophils and epithelial cells will produce natural NAPs that act as important immune responses against unwanted pathogens (Tjabringa et al., 2005; Wiesner et al., 2010). An example of NAPs in mucus are defensins that play an important role in antibacterial activity, cell proliferation and production of cytokines, chemokines, and modulation of adaptive and innate immune cells which are moderated by estradiol and progesterone (Bowdish et al., 2006; Gilbert, 2005). In the human FRT, the production of elafins and secretory leukocyte protease inhibitor have antimicrobial effects against unwanted pathogens produced by macrophages and epithelial cells (Amjadi et al.,2017). Other components such as lactoferrin and lysozyme produced by neutrophils and epithelial cells also play important roles inhibiting uterine infections and enforcing the innate immune system by producing antibacterial effects (Janeway and Medzhitov, 2002).

It has been known that antimicrobial peptides found in the vaginal mucus destroy the membrane of pathogenic bacteria as a defensive action directly related to B and T cells. Neutrophils interact with pattern recognition receptors and other killing cells against pathogenic bacteria by the production of enzymes, nitric oxide and reactive oxygen species on postpartum cows with endometritis (Sheldon, 2004; Turner et al., 2012). Consequently, the high production of $H_2O_2$ leads to the killing of pathogenic bacteria (Lyer et al., 1961). In addition, PRR's are also directly related to the TLR which manifest as neutrophils, macrophages and DC's and detect pathogenic bacteria associated with immune stimulants such as LPS and peptidoglycan (Alberts et al., 2002; Fazeli et al., 2005).

The negative balance in recruitment of neutrophils and lymphocytes after parturition is known to be associated with uterine infections and post-partum metabolic diseases (Galvao et al., 2011; Gilbert, 2005). Endometrial tissue invaded by pathogenic bacteria goes through an inflammatory response leading to defense mechanisms of white blood cells in forms of macrophages. Normally the incitement of the cytokines by the TLR will attract many more cells to fight the infection of the endometrium (Sheldon, 2004; Turner et al., 2012). These inflammatory responses will increase the presence of cytokines like tumor necrosis factor (TNF), IL-1β, IL-6, IL-8, and IL-12 (Duque et al., 2013).

It has been known the presence of granular lymphocytes and macrophages in the regions of the endometrium (Cobb and Watson, 1995). Interestingly, studies conducted by Galvao et al. (2013) reported that monocytes stimulated by *E. coli* in cows with uterine infections had lower genes expression of key proinflammatory cytokines than healthy cows 14 days after parturition. Additionally, the production of acute phase proteins and the mediators above mentioned help the host to fight inflammation processes but in excess act as endotoxins to the body itself (Beutler et al., 1999). It has been directly related absence of feed intake in cows with low BCS and uterine infections with high serum concentrations of adipokines, TNF-alpha, IL-1 beta, IL-6, and lower concentrations of insulin and insulin-like growth factor-1 (IGF-1) (Kasimanickam et al., 2013). According to Herth et al. (2009), there was a higher ratio of anti-inflammatory mediators such as IL-10, PGE and transforming growth factor beta 1 secreted by endometrial cells and pro-inflammatory mediators such as IL-1 in infertile cows.

### 1.3.2 Roles of Adaptive immunity in the Female Reproductive Tract

Apart from innate immune system, there are cells coming from the bone marrow and the thymus that are able to generate antigen-specific cell surface receptors. B lymphocytes (B cells)

generate B cell receptors (BCR) and this response is also known as humoral immunity and mediated by antibodies while, T lymphocytes (T cells) generate T cell receptors and are known as cell mediated immunity (Brenner and Milstein, 2006).

Adaptive immune cells, multiply by clonal expansion and differentiate into long-lived memory cells. An adaptive response will be triggered when the innate immunity is incapable of controlling an infection; DC will migrate with the captured antigen of the pathogen to the lymph nodes and trigger T cells to activate and differentiate into effector cells (Sallusto et al., 1998). Consequently, naive CD4+ T cells differentiate into Th1 or Th2 cells when antigens are presented by the major histocompatibility complex class II molecules (MHC class II) (Constant and Bottomly, 1997). On the other hand, CD8+ T cells also known as cytotoxic cells are capable of destroying pathogens when presented to MHC class I molecules by DC (Constant and Bottomly, 1997, Shedlock and Shen, 2003, Sun and Bevan, 2003). Activation and differentiation of CD4+ T cells and CD8+ T cells are triggered by secretion of cytokines and chemokines by antigen presenting cells (APC). Additionally, B cells will activate upon binding to (BCRs) B cell receptors and synthesize antibodies specific to different antigens and will differentiate into B1 or B2 cells (Kantor, 1991).

As cited in the innate immunity section CM is composed of antibodies such as secretory immunoglobulin A (sIgA) or inmmunoglobulin G (IgG) which are released by epithelial cells and IgM by B1 cells, bridging the innate and adaptive immunity (Wira et al., 2005, Farage et al., 2011). It has been well studied that sIgA from the reproductive tract acts as polyreactive antibody to antigens of unwanted pathogens that are found in the FRT, in which their way of action is binding and neutralizing unwanted pathogens (Kaushic and Wira, 2007). It is known that IgG is the most abundant immunoglobulin in the FRT, and its way of action is trapping the unwanted pathogens

and then cleared by the host immune cells (Saltzman et al., 1994). Interestingly, Fahrbach et al., 2011 reported interactions between both IgG and sIgA with CM, however only found interactions between IgG with CVM.

B2 cells are distributed in the lymph organs and tissues and are known to produce less Ig. Additionally, B2 cells are able to switch immunoglobulin isotypes and contribute to clonal expansion differentiating into memory B cells (Singh and Lillard, 2008). Innate and adaptive immunity systems are essential for prevention and protection in the host against infections caused by pathogens. As such, it is necessary to develop a prevention technique that will help these systems reduce the possibilities of having an inflammation or infection on the tract after parturition.

## 1.4 Treating uterine infections with probiotics

### 1.4.1 Treating uterine infections in dairy cows the traditional way

Currently there is no effective treatment against uterine infections (Table 1-1). The common methods of treating uterine infection have shown to be lower in effectiveness, these include antibiotics, iodine solutions, and hormone treatments (Lewis et al., 1997b; Azawi et al., 2008). In addition, antibiotics commonly used to combat these infections result in economic and health disadvantages. There are also concerns regarding drug residues in milk and development of microbial resistance to antibacterial drugs (Lewis, 1997a; Ocal et al., 2004). Basically, these methods reduce the bacterial growth and proliferation decreasing the inflammation process and leaving the cells of the endometrium to regenerate by themselves (LeBlanc et al, 2002; Deng, 2014).

**Table 1-1.** Treatment of Postpartum Uterine Infections in Dairy Cows.

| Uterine Infection | Antibiotics | Hormones | Disinfectants |
|---|---|---|---|
| Metritis, endometritis, and pyometra | Penicillin, tetracyclines, aminoglycosides, nitrofurazone and sulfonamides<br>+<br>Distilled water or saline | 1.Prostaglandins and Estrogens<br>2.Oxytocin and ergonovine | Iodine, chlorhexidine or cresol |
| Application | Infusion and injection | Injection | Infusion |
| Disadvantages | -Microbial Resistance<br>-Residues<br>-Production costs up<br>-Endometrial irritation | -Production costs up<br>-Contraction of the uterus regarding the size of the cervix | -Endometrial necrosis and irritation of the epithelium |

(References: Sequin and Morrow, 1974; Gustafsson,1984; Olson and Mortimer, 1985; Olson et al., 1986; Ott, 1986; Kaneene et al., 1986; Pulfer and Riese, 1991)

The postpartum period in dairy cows is known as the most delicate period due to its compromised state to pathogens and reduced immunological status, therefore, prevention and treatments of uterine infections is of major importance. Currently, no preventive system has been designed to decrease uterine infections in commercial dairy farms, therefore, the goal of this project is to validate data collected from previous projects executed by our team and to create a preventive plan to improve uterine health status in dairy cows in several commercial dairy farms in Alberta.

### 1.4.2 Probiotics

Food and Agriculture Organization of the United Nations (FAO) defines the term "probiotics", to live microorganisms, that when administered in adequate amounts, confer a health benefit on the host (Fuller, 1989). Probiotics have a positive effect in the gastrointestinal and genitourinary flora decreasing the growth of pathogens by antimicrobial properties. Probiotics have also been used in human subjects for prevention of diarrheas and respiratory infections (Barrons and Tassone, 2008). Lactobacillus group has been known as one of the most important and studied beneficial microorganisms in treating bacterial vaginosis and urinary tract infections in the FRT (Barrons and Tassone, 2008).

Probiotics constituted with lactobacillus create acidic environments in the vagina and also produced bacteriocins and hydrogen peroxide which play an important role in protecting the FRT against pathogenic bacteria (Aroutcheva et al., 2001). It has been reported that due to the probiotic effect, vaginal pH in women could reach acidic environments with pH values around 4.5. Unlike women, cows vaginal pH is around 7.2, and it is known that the vaginal microbiomes are different. Although pH's and microbiomes are different, uterine infections were decreased in postpartum dairy cows after using beneficial microorganisms as well (Genís et al., 2016).

Some studies have reported lactic acid bacteria (LAB) found on vaginal microflora of healthy pregnant Holstein cows, preventing the colonization of pathogenic microorganisms that could cause uterine infections (; Wang et al., 2013; Ametaj et al., 2014; Genís et al., 2016). It has been found that probiotics constituted with LAB contribute to the production of bacteriocins, which are proteins that have antibacterial effects on pathogenic microorganisms (Lorca et al., 2001; Bach et al., 2003). Recently, Genís et al (2016) confirmed previous findings of Ametaj et al. (2014), and Deng et al. (2014) concluded that probiotics composed of LAB infused in dairy cows, lowered the prevalence of metritis by 62% over dairy cows that were not treated. Differences were

found in reducing the blood concentration of IL-1β, IL-6, IL-8 and tumor necrosis factor alpha (TNF- α), which are parameters for inflammatory mediators, after treating the cows with LAB probiotics provided by immune cells. Probiotics are used as a preventive treatment rather than a curative treatment, colonization of the strains can take days or weeks to multiply the existing flora (Reid et al., 1995; Sanders and Klaenhammer, 2001). Probiotics used to treat yeast vaginitis are usually constituted with skim milk for effectiveness (Bruce and Reid, 1988). Skim milk is a nutritive media composed of lactose and casein that supports growth of lactobacilli (Sneath and Holt, 1986). Probiotics can be used as a preventative treatment for uterine infections, which can decrease the use of antibiotics in Alberta dairy systems.

### 1.4.3 Lactic Acid Bacteria

LAB are Gram-positive, non-respiratory lacking catalase bacteria, which ferment glucose to lactic acid, $CO_2$, and ethanol. LAB grow anaerobically as aerotolerant anaerobes and since they lack catalase, peroxide radicals are detoxified by means of peroxidase enzymes (Mercenier et al., 2003). In the meantime, it is generally accepted that lactic acid bacteria increase microbiological, physiological and immunological defenses on commensal bacteria and the immune system (Mercenier et al., 2003). It has been reported that LAB stimulates the production of sIgA in the vaginal tract of dairy cows (Thomas et al., 2010). It is known that sIgA is the largest immunoglobulin produced in a host. The exact mechanism of how sIgA is stimulated by LAB is not completely understood however there are suggestions that LAB engage epithelial cells and DC (Thomas et al., 2010).

LAB probiotics benefit the host due to their capacity to inhibit or eliminate pathogens through the production of organic acids, bacteriocins, and other antimicrobial compounds (Reid 1999; Espeche et al. 2012). The production of antimicrobial substances, organic acids, and hydrogen

peroxide by LAB maintains the vaginal pH at acidic value creating an inhospitable environment for the growth of pathogenic bacteria (Reid, 2002; Otero et al., 2006a; Rodriguez et al., 2011; Wang et al., 2013).

Genís et al. (2016) evaluated different strands of LAB which include *L. rhamnosus* MOI 25, *P. acidilactici* MOI 25, and *L. reuteri* MOI 2, at preventing *Escherichia coli* infection and maintaining bovine endometrial tissue health. Cells treated with LAB showed healthy epithelium (epithelial cells with normal size and shape and normal aspect of microvilli), whereas in cultures infected with *E. coli*, abundant areas with cell debris and bacilli in epithelial cell surface were observed. Moreover, Deng et al. (2014), reported that intravaginal administration of LAB in dairy cows lowered the incidence rate of uterine infections in the treated animals by 45% compared with the control group. Another important finding in this study is that LAB would also improve metabolic status and milk production and composition of the treated cows. Indeed, results showed that treatment with intravaginal LAB lowered the concentration of non-essential fatty acids (NEFA) in the serum, lowered concentrations of systemic LBP, increased milk IgG, lowered milk pH, and, more importantly, improved milk efficiency. Except for reports done by Otero et al., (2006b); Wang et al., (2013); Ametaj et al., (2014); Deng et al., (2014) and Genís et al., (2016, 2017), there is no literature involving LAB probiotics to treat uterine infections in dairy cows.

### 1.5 Research hypothesis and objectives

It is hypothesized that intravaginal infusion of LAB around and before parturition can prevent and lower the incidence of uterine infections and improve general health status of Holstein and Jersey dairy cows in four commercial farms.

Therefore, the objectives of the study were to evaluate:

1) To ascertain whether administration of LAB intravaginal can improve the overall health status, reproductive performance, expedite uterine involution, and improve milk composition and yields of postpartum dairy cows.

2) To determine whether intravaginal infusion of LAB can improve metabolic status in postpartum dairy cows.

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# Chapter 2 Intravaginal probiotics around parturition lower the incidence rate of uterine infections in transition dairy cows in four dairy farms in Alberta


## Abstract

Metritis is the number one reason for culling of cows in Canada and elsewhere because it is associated with high incidence of infertility. The objectives of this project were to evaluate the effects of infusion of a cocktail of lactic acid bacteria (LAB) in the vaginal tract of dairy cows on uterine health, reproductive performance, clinical diseases, and milk production and composition. Five hundred and twenty-six dairy cows (426 Holstein and 100 Jersey cows) from 4 dairy farms in Alberta (three farms had Holstein dairy cows and one farm had Jersey cows) were assigned into 3 experimental groups as follows: 1) Treatment 1: four doses of 2 mL of carrier solution (saline solution); 2) Treatment 2: four doses of 2 mL of sterile skim milk; and 3) four doses of 2 mL of LAB ($10^8$-$10^9$ cfu/dose). The infusions were conducted on weeks -3 and -2 prior to the expected date of calving and at weeks +3 and +4 after calving. Evaluation of metritis was conducted by rectal ultrasonography and evaluation of vaginal mucus by metricheck. Milk yield and composition for the first 50 DIM, reproductive performance, and health records were collected for each cow. Overall, the results showed that the incidence rate of uterine infections by infusion of probiotics was decreased by 29%. The incidence of uterine infections in Farm A, C, and D (Holstein breed herds) decreased by 19%, 36%, and 21%, respectively. Interestingly, in Farm C (an organically managed Jersey herd) the incidence rate of uterine infections decreased by 50%. There was a difference in the incidence of uterine infections with regards to parity. Multiparous cows in TRT1 and TRT2 had higher odds of developing uterine infections [odds ratio (OR)= 1.25, P = 0.01, and (OR) = 1.61, P = 0.02] compared to TRT3. The effect of treatment interacted with parity with

regards to milk production and feed efficiency. Multiparous cows in the TRT3 group had greater milk production than those in the TRT1 and TRT2 group ($P < 0.01$ and $P < 0.01$, respectively). In conclusion intravaginal infusion of probiotics improved overall health status and milk production in multiparous dairy cows.

## 2.1 Introduction

Infertility accounts for 61% of culling reasons in Albertan dairy farms (Can West DHI, 2016), primarily attributed to uterine infections (LeBlanc, 2002). According to the Government of Canada (2017) roughly 42,251 cows, which is 17.2% of total dairy population in Canada for 2016, were culled due to reproductive issues. Parturition is associated with damage of the uterine epithelial tissue and infection (Sheldon et al., 2008; Källerö,2010). Due to the compromised state of the epithelial layers of the reproductive tract and a state of peripartum immunosuppression, pathogenic bacteria ascending from the lower Female Reproductive Tract (FRT) cause infection of the uterine tissues (Racicot et al., 2013). Bacterial infection of the uterus modulates endometrial prostaglandin secretion, affect ovarian follicle growth and function (Sheldon et al., 2008). In a normal bovine reproductive cycle, successful uterine involution, normal cyclical activity, and good reproductive performance is expected after parturition (Savio et al., 1990; Beam and Butler, 1997; Sheldon et al., 2008). Uterine infections in dairy cows results in decreased estrous detection, lower conception rates, require more services per conception, and increased numbers of days opened (Kasimanickam et al., 2004; Sheldon et al., 2009).

Furthermore, healthy cows will exhibit a better overall performance for production compared to sick cows. High quality standards in production and milk composition is entitled to better prices, therefore, better margins for the farmer. Pricing system is based on components such as fat, protein,

and other solids found in milk. Milk components are also used to measure indicators regarding cows health and nutrition.

A variety of factors influence milk composition after parturition. It is known that during the first week after calving high amounts of protein, fat, constituents of blood serum and immunoglobulins can be found in the first six milkings, also known as colostrum (Senger, 2009). Milk obtained from the first six milkings after parturition is known as transition milk. Previous studies conducted by our team have reported that administration of intravaginal LAB lowers the incidence rate of uterine infections by 45%, improved metabolic status, and increased milk production in transition Holstein dairy cows (Ametaj et al., 2014; Deng et al., 2014).

Traditional methods used to treat uterine infections including antibiotics, iodine preparations, and hormone treatments, but have shown to have low efficiency and be associated with residual effect in meat and milk (Lewis et al., 1997a; Ocal et al., 2004; Azawi et al., 2008). Ametaj et al. (2014) administered 6 doses of intravaginal LAB around calving with a cocktail of 3 LAB (2 doses prepartum and 4 doses postpartum, once per week(wks) dose). Another study from our team administered only two doses of a LAB cocktail prepartum (on -2 and -1 wks) or two doses prepartum and one after calving (at +1 wks) (Deng et al., 2014). Both aforementioned studies reported positive results in lowering the incidence rate of uterine infections, and additionally increasing milk production for treated cows.

Based on previous results reported by our lab, we hypothesized that if intravaginal probiotics would be administered once per week at -3 and -2 wks prepartum as well as at +3 and +4 wks postpartum, similar results could be obtained. At the same time the average labor load for the producers to administer probiotics will be lower. Therefore, the objectives of this study where to test weather administration of intravaginal LAB (once per week) at -3 and -2 wks before the

expected day of parturition and at +3 and +4 wks after parturition (Figure 2-1) will lower the incidence of uterine infections, expedite uterine involution, improve reproductive performance and the overall health status of the cows, and increase milk yield of postpartum dairy cows.

## 2.2 Materials and methods

### *2.2.1 Animals and experimental design*

All experimental procedures were approved by the University of Alberta Animal Care and Use Committee for Livestock (AUP # 00001811) and cows were cared for in accordance with the guidelines of the Canadian Council on Animal Care (1993).

A total of five hundred twenty-six pregnant cows (426 Holstein and 100 Jersey cows) were selected from 4 dairy farms in Alberta (see details of the farms below) and blocked by farm and according to parity, body condition score (BCS), and previous lactation milk yield. Two hundred ninety-nine primiparous cows and 227 multiparous (3.02 ± 1.19 SEM) were assigned randomly to one of the following three treatment groups: Treatment 1: four doses of 2 mL of carrier solution containing sterile 0.9% saline solution; Treatment 2: four doses of 2 mL sterile skim milk; and Treatment 3: four doses of LAB containing $10^8$-$10^9$ cfu/dose. Intravaginal infusions were administered at -3 and -2 wks relative to the expected date of calving, and then after calving at +3 and +4 wks postpartum. Lactic acid bacteria were a lyophilized mixture composed of *Lactobacillus sakei* FUA3089, *Pediococcus acidilactici* FUA3138, and *Pediococcus acidilactici* FUA3140 with a cell count of $10^8$-$10^9$ cfu/dose, as described previously (Wang et al., 2013; Ametaj et al., 2014).

Both LAB, in the form of dry skim milk powder, and skim milk were reconstituted with sterile saline at 0.9% and stored at -86 ºC in vials. Reconstitution of LAB was done using 2 mL of sterile saline at 0.9% before administration. All treatments were infused intravaginally in the

cranial section of the vagina with a 5-ml Luer-Lok Tip syringe (Becton, Dickinson and Company, Franklin Lakes, NJ), attached to an individually wrapped sterile drilled infusion tubes (45.72 cm) (Continental Plastic Corp., Delavan, WI).

### *2.2.2 Dairy farms, and their management systems*

This study was conducted at four different free stall commercial farms (named A, B, C, and D for confidentiality reasons) located in the province of Alberta, Canada. Dairy farms operate under a supply managed production system, but farmers themselves implement their own management system according to their choice to reach the desired production parameters. Farms A, B, and C collect milk under voluntary milking system (VMS**)** which is also known as robotic milking system, in which collection of milk from cows occurs at any time around 24 h periods. Farm D is a parlour dairy system where milk collection occurs 3 times a day approximately at 0400, 1200, and 1800 h by the farm staff. All farms offered a total mixed ration (TMR) on an ad libitum feed and water intake. Each farm formulates their TMR based on their own nutritional requirements following the National Research Council guidelines (2001).

Farm A collects milk under automatic milking system (AMS) (DeLaval VMS Voluntary Milking System, DeLaval International AB), milking around 150 Holstein herd. Farm B is an organic dairy farm and collects milk under a AMS (BouMatic, Madison, WI, USA), milking around 150 Jersey herd. Farm C milks cows with an AMS (Lely Industries N.V., Maassluis, the Netherlands), farm milking around 150 Holstein herd. Farm D uses a parallel parlor milking system to milk the cows (BouMatic, Madison, WI, USA), milking 3 times per day around 400 Holstein dairy cows.

### *2.2.3 Diagnosis of clinical diseases and clinical monitoring*

All cows were monitored for 6 diseases including metritis, mastitis, retained placenta, milk fever, displaced abomasum, and lameness for each farm. Body condition scores (**BCS**) and clinical diseases were monitored starting from -3 wks prior to parturition, and at +3 and +4 wks after parturition. Vaginal examination for detection of metritis was performed by using the Metricheck$^{TM}$ device (Metricheck, Simcro, New Zealand) on +3 and +4 wks. The Metricheck device was disinfected between uses with Nolvasan solution (Zoetis, Kalamazoo, Michigan, USA) contained chlorhexidine diacetate at a concentration of 2% and sanitized using 70% alcohol before each mucus extraction. The vaginal perineal region of the cows was disinfected with 16% iodine solution (Vetoquinol N.-A, Inc., Lavaltrie, Quebec, Canada), and then the metricheck devise was inserted into the reproductive tract obtaining vaginal mucus. Mucus evaluation was done according to Sheldon (2005) where 0 = clear or translucent mucus; 1 = mucus containing flecks of white or off-white puss; 2 = discharge containing < 50% puss or off-white mucopurulent material; 3 = discharge containing > 50% purulent material typically white or yellow or a mixture of mucus and blood color (Sheldon, 2005). We diagnosed for metritis if the mucus score was 3, and whether mucus was accompanied by an odor where 2 scales: 0 = not having smell and 3= odorous smell.

For the purpose of this project, uterine infections where quantified as the sum of metritis and endometritis. Clinical mastitis was diagnosed by each of the farms following somatic cell counts (SCC) in milk > 200,000 cells/ mL (National Mastitis Council, 2001). Retained placenta was diagnosed if the cow failed to expel fetal membranes 24 h after parturition. Milk fever was diagnosed within 72 h after parturition by a veterinary practitioner and farm treatment records. Displaced abomasum was diagnosed by a veterinary practitioner and recorded according to treatment records from the farms. Lameness was detected visually and scored based on locomotion scoring of dairy cattle (Sprecher et al., 1997). For the purpose of this experiment mildly, moderate,

and severely lame was accounted for a lame cow (normal = stands and walks normally with a level back, and lame = stands and walks in an abnormal gait or arched back). Additionally, all the lameness scores were observed in flat surfaces, so the cows could make confident accurate strides making detection accurate. All the diagnosed results were combined with barn and veterinary records to declare any case of the aforementioned diseases.

Body condition scores were measured using the Dairy Cattle Production (342-450A, McGill University and Elanco Animal Health, Indianapolis, Indian, USA). Scores were evaluated at -3 and -2 wks before parturition and at +3 and +4 wks after parturition, based on a scale of 5 with intervals of 0.25 points.

### *2.2.4 Uterine involution*

Evaluation of uterine involution was assessed by transrectal ultrasonography and hand palpation at +2 and +4 wks after parturition on 135 multiparous Holstein cows and 45 multiparous Jersey cows. Diameter of the uterine horns were first measured by rectal palpation, measuring the horns by the evaluator's fingers width. Evaluation of the horns by fingers was done at the base of the uterine horns were the bifurcation could be palpated and divided into the dorsal intercornual ligaments and the ventral intercornual ligament. Evaluation on the uterine retraction was done by palpation of the ventral intercornual ligament due to its larger size and thickness on both gravid and non-gravid horns (Melendez et al., 2004; Deng, et al., 2014).

Additionally, a Sonsonite® (MicroMaxx, Sonsonite, In., Bothell, Washington, USA) ultrasound fitted with a 7.5 MHz probe was used to measure the diameter of uterine horns to completely define involution by both methods. The decrease in size of the bovine uterus after parturition is known to start during the first 9 days after calving, finding diameters of uterine horns between 12 to 14 cm in healthy cows. It is known that at around +2 wks after parturition the uterine

horns can measure between 7 to 8 cm and that at +3 and +4 wks after parturition the uterine horns have diameters ranging between 2 to 4 cm in healthy cows (Leslie,1983; Morrow et al., 1966).

Proper uterine regression was declared if by +2 wks after parturition both uterine horns measured less than 8 cm and if by +4 wks after parturition the diameter was less than 4 cm. Normally, the uterus has involuted enough at +2 wks after parturition to allow rectal palpation. Additionally, uterine fluid collected from the 180 cows (45 cows per each farm in the study) were evaluated and assessed according to Sheldon (2005), as described in the previous section. While, healthy cows will present scores between 2 to 3 the first +2 wks after parturition and then will clear up to score 1 by +4 wks after parturition.

### *2.2.5 Reproductive performance*

Reproductive performance was measured for 135 (45 dairy cows per each of the three Holstein farms) multiparous Holstein cows and 45 multiparous Jersey cows with equally distributed treatments and randomly selected for evaluation. Reproductive performance was measured and based on the first service conception rate and cumulative conception rate. Conception rate is the percentage of animals serviced which become pregnant. For unbiased data collection, evaluation of the inseminator, bull, and management were kept blind. Additionally, pregnancy was declared by farm veterinarian. For the purpose of this study only cows that presented natural heat were evaluated. Days for the first insemination varied in each of the farms included: farm A cows were first inseminated by around 80 ± 10 d, farm B around 70 ± 10 d, farm C around 80 ± 10 d, and farm D by around 60 ± 10 d after parturition.

### *2.2.6 Sampling and Laboratory analyses*

Vaginal mucus was collected from 135 (45 dairy cows for farms A, C, and D) multiparous Holstein cows and 45 multiparous Jersey cows (from farm B) and transferred into sterile polypropylene culture test tube (Fisher Scientific, Toronto, ON, Canada), and stored at -20 °C.

Milk samples were collected once a week from +1 wk to +4 wks after parturition. Daily milk production was measured for the first 50 days in milk (DIM). Samples were collected after three to four streams of milk were removed; 3 mL of milk from the four quarters were collected in a vial containing a preservative pill and stored at 4 °C until analysis of the components. Milk composition was analyzed for fat, crude protein (CP), somatic cell count (SCC), lactose, milk urea nitrogen (MUN), and total solids (TS). Method of analysis was assessed by Central Milk Testing Laboratory located in Edmonton, Alberta, by mid-infrared spectroscopy (MilkoScan 605; A/S Foss Electric, Hillerød, Denmark).

### *2.2.7 Statistical Analyses*

All statistical analyses were performed using SAS software (Release 9.4, SAS Institute, Inc., Cary, NC). In this study cows were blocked by parity and previous milk yields before being assigned to the treatment groups. Data for diseases, uterine involution, mucus scores, palpations, and reproductive performance were analyzed with PROC UNIVARIATE procedure to test assumptions of normality. However, results showed that data followed a non-normal distribution after a Poisson distribution. Because of that, distribution of data by PROC GLIMMIX procedure was used for logistic regression, including farm as random effect and correlated errors as per following equation:

$$Y_{ijk} = \mu + T_i + W_j + (TW)_{ij} + e_{ij}$$

where $\mu$= the overall population mean; $T_i$ = the effect of the treatment; Wj = the effect of the week; $(TW)_{ij}$ = the interaction between treatment and week; and eij = residual error.

PROC FREQ was also used for the binary data to analyze diseases and to test the effect of treatment and analyze frequency. Data are shown as least-squares means (LSM) and the respective standard error of the mean (SEM) as well as odds ratio (OR) and 95% confidence limits (CL). For the odds ratio and 95% confidence intervals (Cl) tables, probability values presented are for each corresponding values. The covariance structure was modeled according to the smallest Akaike information criterion (AIC) and the Bayesian information criterion (BIC) values generated.

To analyze interactions between measurements of the uterine horns, vaginal mucus scores, parity, and weeks after parturition the function of correlation and logistic regression in Medcalc (Medcalc version 16.4.3, 64 bits; Medcalc, Ostend, Belgium) was used. The model followed was:

$$Yijkl = \mu + Ti + Dj + (TD)ij + Pk + eijkl$$

where $\mu$ = the overall population mean; Ti = effect of treatment; Dj = effect of weeks after parturition; (TD)ij = effect of the interaction between treatment and weeks relative to calving; Pk = effect of parity; and eijkl = residual error.

Effect of the treatments on milk yield and composition where tested including previous milk yields and parity, using SAS 9.4 software (SAS Institute Inc., Cary, NC). PROC MIXED procedure was used for modeling effect of treatment on milk yields against parity including farm as random effect following:

$$Y_{ijk} = \mu + T_i + W_j + (TP)_{ij} + e_{ij}$$

where $\mu$= the overall population mean; $T_i$ = the effect of the treatment; Wj = the effect of the week; $(TP)_{ij}$ = the interaction between treatment and parity; eij = residual error. For all the data *P* values of < 0.05 were considered significant, and tendency at $0.05 \leq P \leq 0.10$.

### *2.2.8 Roc Curves*

The accuracy for measuring uterine involution methods were evaluated using a Medcalc's receiver operating characteristic curve (ROC) function for right and left uterine horns. Interpretation of this critical threshold depends on the area under the curve (AUC), such that if the AUC > 0.7 the test is considered accurate or acceptable (Swets, 1988).

## 2.3 Results

### *2.3.1 Effect of intravaginal probiotics on the incidence rates of uterine infection and other periparturient diseases*

The overall results with regards to clinical observations and incidence of periparturient diseases for the 4 dairy farms between +3 and +4 wks postpartum are presented in Table 2-1. Results showed that cows treated intravaginally with probiotic bacteria (TRT3) had lower incidence rates of uterine infections at 26.72% compared with TRT1 and TRT2, with 37.07% and 36.21% incidence rates, respectively.

Results for effect of the treatment on uterine infections are presented in Table 2-2. Furthermore, data showed that TRT3 had lower incidence rates of uterine infections compared to TRT1 and TRT2 with regards to both primaparous cows (15.52% in TRT3 versus 18.97% in TRT2, and 20.60% in TRT1) and multiparous cows (11.21% in TRT3, 17.24% in TRT2, and 16.38% in TRT1). Additionally, the results showed that TRT3 had higher numbers of healthy cows compared to TRT1 and TRT2 (35.57% in TRT3 versus 31.96% in TRT2 and 32.47% in TRT1). Additionally, TRT3 had higher numbers of healthy cows compared to TRT1 and TRT2 in both primaparous (20.36% in TRT3 versus 19.33% in TRT2, and 18.81% in TRT1) and multiparous cows (15.21% in TRT3 versus 12.63% in TRT2, and 13.66% in TRT1).

Interestingly, there was a numerical difference with regards to rates of subclinical mastitis presented on Table 2-3. Results showed that cows treated with probiotics (TRT3) had lower incidence rates of clinical mastitis by 23.08% compared to TRT1 and TRT2 with 35.90% and 41.03% incidence rates, respectively. Furthermore, TRT3 had lower incidence rate of milk fever compared to TRT1 and TRT2 in primaparous (5.13% in TRT3, 25.64% in TRT2, and 15.38% in TRT1) but no similar effect of TRT3 was obtained for multiparous cows (17.95% in TRT3, versus 10.26% in TRT2 and 25.64% in TRT1). Additionally, the results showed that TRT3 had higher numbers of healthy cows with regard to mastitis compared to TRT1 and TRT2 (34.41% for TRT3 versus 32.69% for TRT2 and 32.90% for TRT1). Furthermore, TRT3 had higher numbers of cows free of subclinical mastitis compared to TRT1 and TRT2, in only primaparous cows (20.43% in TRT3 versus 18.71% and 19.57% for TRT2 and TRT1, respectively) but not for multiparous cows (13.98% in TRT3 versus 13.98% in TRT2 and 13.33% in TRT1). Interestingly, TRT2 had numerically lower incidence rates of retained placenta (Table 2-4) compared with TRT3 and TRT1 (25.00% in TRT2 versus 28.57% in TRT3, and 46.43% in TRT1). Milk fever results showed that cows treated with probiotics (TRT3) had lower incidence rates of milk fever by 29.2 % compared to TRT1 and TRT2 with 33.3% and 37.5% incidence rates, respectively. In addition, there was no significant difference among the treatments regarding lameness.

Odds ratio analyses for primiparous and multiparous cows are presented in Table 2-5. Multiparous cows had higher odds of developing uterine infections if infused with TRT1 and TRT2 after calving [odds ratio (OR)= 1.25, $P = 0.01$, and (OR) = 1.61, $P < 0.01$]. Overall multiparous cows had less number of cases of uterine infections after parturition compared with primiparous postpartum dairy cows (Figure 2-2).

There was no numerical difference with regard to rates of retained placenta presented on Table 2-6.

Only 4 cases of displaced abomasum were diagnosed in the 4 farms involved in the study, no significant effect could be measured for this periparturient disease. No significant difference ($P > 0.05$) was found among the treatments regarding BCS at any different time point.

### *2.3.2 Treatment effects under different management systems*

Results of clinical observations for incidence of diseases in cows after parturition for Farms: A, B, C, and D are presented in tables: 2-7, 2-8, 2-9, and 2-10, respectively. Results of this study indicated that different managements or procedures carried out by the farmers affected the results. Uterine infections rates were decreased by 19%, 50%, 36%, and 21% for Farms A, B, C, and D, respectively.

In Farm A, there was a tendency of the probiotic treatment was found with regards to lowering the incidence of metritis ($P = 0.14$), retained placenta ($P = 0.07$), lameness ($P = 0.14$), and mastitis ($P = 0.07$). Since there were only 2 cases for milk fever and displaced abomasum, respectively, no *P*-value is provided.

In Farm B, there was no significant effect of probiotic treatment on the incidence of mastitis ($P = 0.20$), and lameness ($P = 0.25$). Interestingly, there was a significant effect of treatment on metritis ($P = 0.03$) and milk fever ($P = 0.01$). Since there were no cases for retained placenta and displaced abomasum, respectively, no *P*-value is provided.

In farm C, results showed a tendency for retained placenta ($P = 0.18$). However, no significant differences were found for diseases like mastitis ($P = 0.67$) and lameness ($P = 0.56$). However, there was a significant effect of treatment on metritis ($P = 0.05$) and milk fever ($P = 0.005$). Since there were no cases for displaced abomasum, no *P*-value is provided.

Results showed that in Farm D, there was no significant effect of treatment on the incidence of retained placenta ($P = 0.35$). However, there was a significant difference in the cases of metritis ($P = 0.05$), mastitis ($P = 0.04$), and lameness ($P < 0.01$). Tendency was found for milk fever ($P = 0.10$). Since there were no cases for displaced abomasum, no $P$-value is provided.

### *2.3.3 Treatment effects on uterine involution*

There was no effect among treatments regarding involution of uterine horns. Overall the interaction between parity, week, farm and breed presented no effect on the uterine evolution. There was no significant difference ($P > 0.05$) among treatments regarding evaluation of retraction of the uterine right horn ($P = 0.35$) and left horn ($P = 0.22$) for gravid and non-gravid horns at +2 wks postpartum. Furthermore, there was no significant effect among treatments regarding involution of the uterine right horn ($P = 0.23$) and left horn ($P = 0.23$) for gravid and non-gravid horns at +4 wks postpartum.

### *2.3.4 Treatment effects on reproductive performance*

There were no significant effects of treatment ($P = 0.57$) on the first-service conception rate and cumulative pregnancy rate for farms A, B, C, and D. First-service conception rate was 51.0 ± 7.4 % for TRT1, 50.0 ± 7.7 % for TRT2, and 43.3 ± 8.1 % for TRT3. Cumulative pregnancy rate was 84.0 ± 7.7 % in TRT1, 86.0 ± 7.9 % in TRT2, and 79.0 ± 8.4 % in TRT3. No interaction was found between TRT * parity for TRT1 ($P = 0.25$) and TRT2. Interestingly, there was a tendency in the interaction of TRT3* parity for multiparous cows ($P = 0.11$).

Odds ratio analyses for treatments were evaluated for both primiparous and multiparous cows to test the tendency found in the interaction TRT x parity. No significant effect could be found for the odds ratio test even though cows infused with TRT3 (probiotics) had higher odds of

getting pregnant compared to TRT1 and TRT2, respectively [odds ratio (OR) = 1.16, $P = 0.79$, and (OR) = 1.61, $P = 0.38$] (Table 2-10). As an average, primiparous cows required 1.5 insemination per pregnancy compared to multiparous cows that required 1.9 inseminations ($P = 0.01$).

### *2.3.5 Milk production and composition*

Daily milk production was recorded for the first 50 DIM to assess the effect of treatments. Overall there was a significant effect of probiotics on milk production for dairy cows in farms A, B, C, and D ($P < 0.05$). Interestingly, the effect of treatment and parity interaction was found to be ($P = 0.01$). Significant differences were found among treatment groups, parity, and previous 305 DIM for milk productions in Farm A ($P = 0.01$), Farm B ($P = 0.01$), Farm C ($P = 0.01$), and Farm D ($P = 0.03$). Multiparous cows for all treatment groups exhibited higher yields than primiparous cows ($P = 0.01$). Overall, cows treated with probiotics (TRT3) increased 4.6 liters and 3.22 liters per day compared to TRT1 and TRT2, respectively. Odds ratio analyses for treatments were evaluated for all 4 farms to test the effect of probiotics infused intravaginally on milk yields. Interestingly, cows infused with probiotics (TRT3) had higher odds of producing more milk compared with TRT1 and TRT2 after calving [odds ratio (OR) = 1.18, $P < 0.01$, and (OR) = 1.20, $P < 0.01$] (Table 2-12).

Milk composition in all treatment groups is shown in table 2-13. Treatment did not have an effect ($P > 0.05$) on milk composition. There was a difference with respect to milk components such as protein, lactose, somatic cell count, milk urea nitrogen, and total solids at +1 wk and +4 wks postpartum ($P < 0.05$). The aforementioned components were higher during +1 wk compared to +4 wks. There was no significant difference ($P < 0.05$) in the interaction of TRT x Parity for any of the components.

## 2.4 Discussion

We hypothesized that intravaginal infusions of LAB probiotics on weeks -3 and -2 before the expected calving date and at +3 and +4 wks after calving of four doses of LAB (one dose per week) could improve the overall health status, expedite uterine involution, reproductive performance, and improve milk composition and yields of postpartum dairy cows. Indeed, results of this study showed that intravaginal infusion of LAB probiotics, previously isolated from vaginal mucus of healthy cows (Wang et al., 2013; Ametaj et al., 2014), decreased uterine infections in all dairy farms included in this study and improved the overall health status lowering the incidence rates of several other postpartum diseases.

The data reported in this study are in agreement with two previous studies reported by our lab, which also demonstrated lowering of the incidence of uterine infections by intravaginal infusion of the same probiotic cocktail mixture used in our study in multiparous cows (Ametaj et al., 2010; Wang et al., 2013; Deng et al., 2014). This could be attributed to the fact that the probiotics used in this study were isolated from healthy multiparous cows (Wang et al., 2013). Interestingly, we found that primaparous cows had a better reproductive performance compared to multiparous cows. Additionally, our data is in agreement with reports from another lab which also showed that a cocktail mixture of LAB consisting of *L. rhamnosus*, *P. acidilactici*, and *L. reuteri* modulated *E. coli* infection and endometrial inflammation in vitro (Genís et al., 2016). The latter authors have contributed with data regarding the potential mechanism by which intravaginal probiotics can protect vaginal tract from infections from pathogenic bacteria. Additionally, probiotics are known to modify purulent vaginal discharge in dairy cows, lowering concentrations of haptoglobin produced by the liver as an acute phase response (indicative of cows infected with

metritis) during the second week after parturition, a variable used to measure uterine health in dairy cows (Ametaj et al., 2014). Other potential mechanisms of action of LAB include the production of lactic acid, which competes with infectious pathogens by lowering the pH as well as production of hydrogen peroxide and bacteriocins such as AcH/ PA-1 that inhibit the adhesion and growth of pathogenic bacteria in postpartum dairy cows (Aroutcheva et al., 2001; Reid, 200; Wang et al., 2013). Moreover, Otero et al., 2005 reported that *Lactobacillus* strains were able to inhibit *in vitro E. coli* 99/14, by means of acid production.

Another interesting finding of this study was that overall intravaginal probiotics lowered also the incidence rate of mastitis. To the best of our knowledge this is the first report to demonstrate that intravaginal infusion of LAB can lower the number of cases with mastitis in dairy cows. The mechanism by which intravaginal probiotics lower the incidence of mastitis is not clear at present; however, it might be possible that the infected uterus might serve as a source of pathogenic bacteria for infection of the mammary gland. The mechanism by which intrauterine bacteria reach the mammary gland might be through blood circulation, environmental contamination, or by affecting the immune status of the transition dairy cows (Ametaj et al., 2010; Dervishi et al., 2015; Zhang et al., 2015; Eckel and Ametaj, 2016). Indeed, presence of purulent vaginal discharge (PVD) 3 weeks after parturition is present on cows infected with metritis (Sheldon et al., 2002). The purulent vaginal discharge is a source of pathogenic bacteria that causes infection in the female reproductive tract (LeBlanc et al., 2011; Mateus et al., 2002; Dubuc et al., 2010). It is possible that PVD could be responsible for contaminating the mammary gland after parturition due to the contact of the purulent discharge with the cow's bedding. Improving the overall immune response after parturition by decreasing uterine infections infusing probiotics before and after parturition may be responsible for increasing the availability of neutrophil

translocation into the mammary gland and lower the rates of subclinical mastitis. However, further research is warranted to evaluate the effects of intravaginal probiotics on subclinical mastitis, which may have an important impact to the dairy industry.

Intriguingly, results of this study showed that cows treated with intravaginal probiotics had overall lowered the incidence of milk fever. Approximately 5% of a dairy herd are affected by milk fever but can reach up to 25% in some herds (DeGaris and Lean 2008). Milk fever is known as a metabolic disease due to imbalance of Ca around calving, characterized by hypocalcemia (Goff 2008; Reinhardt et al., 2011). Different studies have associated milk fever with periparturient diseases such as mastitis, displaced abomasum, retained placenta, metritis, ketosis, and uterine prolapse (Zwald et al., 2004; DeGaris and Lean 2008; Hossein-Zadeh and Ardalan 2011; Pryce et al., 2016). Our team has identified that periparturient diseases are preceded and associated by chronic inflammation in systemic circulation, contributing to the pathogenesis of multiple diseases simultaneously (Ametaj et al., 2010; Dervishi et al., 2015; Zhang et al., 2015; Eckel and Ametaj, 2016). Endotoxins released by pathogenic bacteria are involved in multiple metabolic diseases (Ametaj et al., 2005). After parturition cows undergo through a negative energy balance and immunity during the periparturient period is impaired, making it a suitable environment for unwanted pathogens to proliferate (Mordak and Anthony, 2015). *Escherichia coli* is among the main types of bacteria found during uterine infections in the female reproductive tract, associated with bacterial endotoxin lipopolysaccharide (LPS) (Sheldon et al., 2002). Uterine infections occur 2-14 days after parturition (Ott, 1986) and endotoxins have been proposed to translocate from mucosal tissues, mammary gland, and uterus into the systemic circulation (Eckel and Ametaj, 2016).

A newly proposed hypothesis suggests how LPS or endotoxins could translocate into the systemic circulation and may be involved in the pathogenesis of milk fever (Ametaj et al., 2010; Zhang et al., 2018). Cows with milk fever have greater concentrations of serum amyloid A (SAA) in the plasma which suggest presence of endotoxins in the blood circulation (Ametaj et al., 2010; Zhang et al., 2018). Probiotics infused intravaginally may be responsible to lower concentrations of unwanted pathogens that produce LPS or endotoxins in the uterus, lowering the incidence rates of milk fever in dairy cows. However, further research is warranted to evaluate the effects of intravaginal probiotics on milk fever.

Analyzing the data for Farm A and D it is noticeable that the *P-values* are not the same as the overall values for rates of uterine infections. Although we saw a numerical improvement in two dairy farms we were no able to reach significance, this might be related to factors such as management, environment, rations, and sample size per farm. Gill (1969) suggested that small experiments are more likely to have experimental errors due to the number of observations. Of note is the overall significant effect of the probiotics infused intravaginally on the incidence rates of uterine infections, mastitis, and milk fever in the 4 dairy farms.

Interesting results were found in Farm B, which is an organic Jersey-based dairy farm. Antibiotics and synthetic hormones are prohibited except when absolutely necessary to improve health, welfare or hygiene of animals, or for safety reasons and administered by a veterinary practitioner (CAN/CGSB-32.310-2006). Dairy animals shall undergo only two treatments per year; if requiring more treatments, the animal should be removed from the herd (Agriculture and Agri-Food Canada, 2018). Organic farms in Canada are distributed in Quebec, Ontario, Alberta, and British Columbia. There are around 222 farms producing 1,110,664 hectoliters of organic milk (Agriculture and Agri-Food Canada, 2016), which cannot administer antibiotic treatment for

uterine infections. Intravaginal probiotics in this organic dairy farm lowered the incidence rates for uterine infections by 50%, which strongly suggest that intravaginal probiotics could be an alternative preventive treatment to decrease uterine infections in organic dairy systems. However, further research is warranted to evaluate the effects of probiotics as a treatment intervention on organic dairy farms.

Techniques for measuring uterine involution include hand rectal palpation and ultrasonography. Accuracy of the palpation methods are important to measure involution of gravid horns in postpartum dairy cows. Even though we could not find a significant difference in the treatment groups on uterine involution, it has been known that postpartum cows infected with metritis reduce the rate of uterine and cervical involution having high scores of vaginal mucus (brownish-fetid odor) (Fonseca et al., 1983; Del Vecchio et al., 1994). Additionally, analysis of vaginal mucus from our study confirms data from our previous studies (Ametaj et al., 2010; Deng et al., 2014) showing that high incidence rates of metritis is related to PVD in postpartum dairy cows (Figure 2-4).

Interestingly, data from our study are also in agreement with previous studies conducted from our team Ametaj et al. (2010) and Deng et al. (2014) which report an increase in milk yields in dairy cows infused with intravaginal probiotics around parturition. The mechanism by which uterine infection lower milk production might involve endotoxins or proinflammatory cytokines that translocate into blood circulation may be involved in milk production as well. Decreasing uterine infections and contributing to improvements in the overall health status of dairy cows, could be one of the potential mechanisms on how probiotics increased milk production. LPS and TNF-α affect expression and release of prolactin which is the hormone that stimulates milk production (Theas et al., 1998). By eliminating infection there is no inhibition of prolactin and

improvement in milk production. However, further research is needed to relate uterine infections and endotoxin translocation into the blood circulation with increased milk yields.

Overall, no significant effect of treatment on milk composition was obtained, which could be accounted for the different management styles adopted by each of the farms. Previous studies from our lab used the same breed, diets, environments and management conditions in comparison to this study. Therefore, we can account that results that were not significant such as milk composition could be attributed to factors mentioned above influencing the outcomes of the study.

## 2.5 Conclusions

The results of this study indicated that LAB probiotics infused intravaginally around parturition lowered the incidence rates of uterine infections, mastitis, and milk fever in treated cows. Additionally, there was an increased overall milk production in the four dairy farms included in the study. Interesting results were found for Farm B, an organic Jersey herd production. At our best knowledge no literature was regarding intravaginal probiotics in an organic Jersey dairy farm. Moreover, cows treated with LAB probiotics increased milk production compared to TRT1 and TRT2. It can be concluded that intravaginal infusions of LAB probiotics lowered the risk of uterine infection, mastitis, and milk fever in transition dairy cows and increased milk production in the 4 farms in the study. However, more research is warranted to better understand the mechanism(s) by which LAB infused intravaginally decreases uterine infection rates and confers overall health status in Holstein and Jersey dairy cows.

## 2.6 Acknowledgements

Dr. Burim N. Ametaj was the principal investigator of this study who contributed to conceptualization and designing of this research work and in organizing and managing the entire study. Dr. Ametaj also supervised all the lab analyses. He contributed in writing, editing as well as in improving the quality of this manuscript with many comments and suggestions. Dr. Andre Luiz Garcia and Ashley Egyedy contributed in sample collection and health evaluation of the cows. Suzanna M. Dunn is acknowledged for her contribution in lab analyses. Likewise, we are grateful to the dairy farmers who provided their cows to this study and for creating a favorable and supportive environment and providing all necessary records to us.

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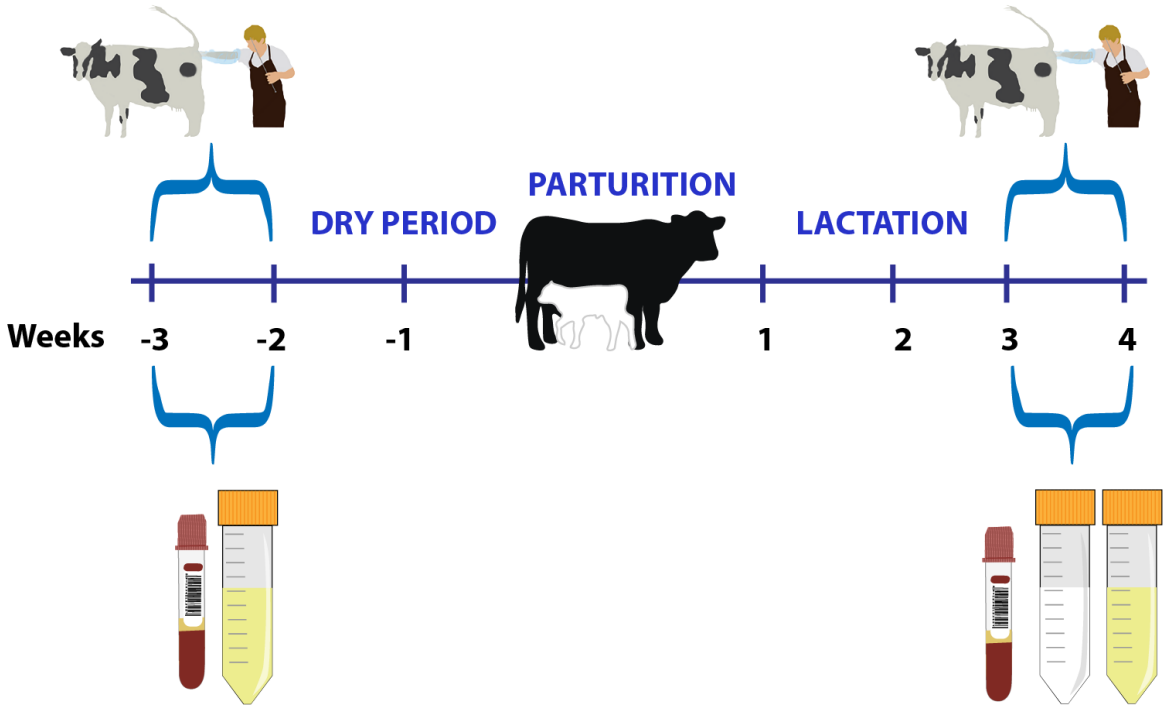


**Figure 2-1.** Timeline of treatments and sample collection. Infusions of LAB probiotics: 1) four doses of 2 mL of carrier solution (sterile 0.9% saline) and sterile skim milk; 2) four doses of 2 mL of carrier solution (sterile 0.9% saline); and 3) four doses of LAB probiotic cocktail (*Lactobacillus sakei* FUA3089, *Pediococcus acidilactici* FUA3138, and *Pediococcus acidilactici* FUA3140 with a cell count of $10^8$-$10^9$ cfu/dose). The infusions of LAB probiotics were divided on -3 wks and -2 wks prior to the expected date of calving (dry period) and at +3 wks and +4 wks after calving. Samples collected at -3 wks: blood and mucus. Samples collected at +3 wks and +4 wk: Blood, milk, and mucus.

**Table 2-1**. Overall effect of intravaginally infused probiotics on 6 periparturient diseases in postpartum dairy cows.

| Variable (%) (case/total) | TRT1 [1] | TRT2 [2] | TRT3 [3] | *P* value |
|---|---|---|---|---|
| Uterine infections | 37.1 (43/116) [a] | 36.1 (44/116) [a] | 26.7 (31/116) [b] | 0.04 |
| Retained placenta | 46.4 (13/28) [a] | 25.0 (7/28) [b] | 28.6 (8/28) [b] | 0.01 |
| Displaced abomasum | 2 | 2 | 0 | N. S. |
| Subclinical Mastitis | 41.0 (16/39) [a] | 36.0 (14/39) [a] | 23.1 (2/39) [b] | 0.01 |
| Milk fever | 33.3 (8/24) [a] | 37.5 (9/24) [a] | 29.2 (7/24) [b] | 0.01 |
| Lameness | 33.3 (8/24) [a] | 29.2 (7/24) [b] | 37.5 (9/24) [a] | 0.01 |

[1]TRT1: Sterile saline 0.9% plus sterile skim milk.
[2] TRT2: Sterile saline solution 0.9%.
[3]TRT3: Probiotic cocktail (*Lactobacillus sakei* FUA3089, *Pediococcus acidilactici* FUA3138, and *Pediococcus acidilactici* FUA3140 with a cell count of $10^8$-$10^9$ cfu/dose) in sterile skim milk and dissolved with sterile saline 0.9%.
[a-b]Numbers within a row with different superscript letters are different at $P < 0.05$.
N.S.: no significance.

**Table 2-2.** Frequencies for uterine infections post-partum among treatments in primiparous and multiparous cows.

| | Uterine infections (%) (case/total) | | | No uterine infections (%) (case/total) | | |
|---|---|---|---|---|---|---|
| TRT[1] | P[2] | M[3] | Total (%) | P[2] | M[3] | Total (%) |
| 1 | 20.69 (24/116) | 16.38 (19/116) | 37.07 | 18.81 (73/388) | 13.66 (53/388) | 32.47 |
| 2 | 18.97 (22/116) | 17.24 (20/116) | 36.21 | 19.33 (75/388) | 12.63 (49/388) | 31.96 |
| 3 | 15.52 (18/116) | 11.21 (13/116) | 26.72 | 20.36 (79/388) | 15.21 (59/388) | 35.57 |
| Total | 55.17 (64/116) | 44.83 (52/116) | 100.00 | 58.51 (227/388) | 41.49 (161/388) | 100.00 |

Note: Total number of observations for +3 wk and +4 wk

[1]TRT: treatment.

TRT1: Sterile saline 0.9% plus sterile skim milk.

TRT2: Sterile saline solution 0.9%.

TRT3: Probiotic cocktail (*Lactobacillus sakei* FUA3089, *Pediococcus acidilactici* FUA3138, and *Pediococcus acidilactici* FUA3140 with a cell count of $10^8$-$10^9$ cfu/dose) in sterile skim milk and dissolved with sterile saline 0.9%.

[2]P: primiparous.

[3]M: multiparous.

**Table 2-3.** Frequencies for subclinical mastitis (> 200 $10^3$ cells/ml) post-partum among treatments in primiparous and multiparous cows.

| | Subclinical mastitis (%) (case/total) | | | No subclinical mastitis (%) (case/total) | | |
|---|---|---|---|---|---|---|
| TRT[1] | P[2] | M[3] | Total (%) | P[2] | M[3] | Total (%) |
| 1 | 15.38 (12/78) | 25.64 (20/78) | 41.03 | 19.57 (182/930) | 13.33(124/930) | 32.90 |
| 2 | 25.64 (20/78) | 10.26 (8/78) | 35.90 | 18.71 (174/930) | 13.98 (130/930) | 32.69 |
| 3 | 5.13 (4/78) | 17.95 (14/78) | 23.08 | 20.43 (190/930) | 13.98 (130/930) | 34.41 |
| Total | 46.15 (128/78) | 53.85 (104/78) | 100.00 | 58.71 (546/930) | 41.29 (384/930) | 100.00 |

Note: Total number of observations for +3 wk and +4 wk
[1]TRT: treatment.
TRT1: Sterile saline 0.9% plus sterile skim milk.
TRT2: Sterile saline solution 0.9%.
TRT3: Probiotic cocktail (*Lactobacillus sakei* FUA3089, *Pediococcus acidilactici* FUA3138, and *Pediococcus acidilactici* FUA3140 with a cell count of $10^8$-$10^9$ cfu/dose) in sterile skim milk and dissolved with sterile saline 0.9%.
[2]P: Primiparous.
[3]M: Multiparous.

**Table 2-4.** Frequencies for milk fever post-partum among treatments in primiparous and multiparous cows.

| | Milk fever (%) (case/total) | | | No milk fever (%) (case/total) | | |
|---|---|---|---|---|---|---|
| TRT[1] | P[2] | M[3] | Total (%) | P[2] | M[3] | Total (%) |
| 1 | 4.17 (2/48) | 29.17 (14/48) | 33.33 | 20(192/960) | 13.54(130/960) | 33.54 |
| 2 | 4.17 (2/48) | 33.33 (16/48) | 37.50 | 20 (192/960) | 12.71(122/960) | 32.71 |
| 3 | 4.17 (2/48) | 25 (12/48) | 29.17 | 20 (192/960) | 13.75 (132/960) | 33.75 |
| Total | 12.50 (6/48) | 87.50 (42/48) | 100.00 | 60 (576/960) | 40 (384/960) | 100.00 |

Note: Total number of observations for +3 wk and +4 wk

[1]TRT: treatment.

TRT1: Sterile saline 0.9% plus sterile skim milk.

TRT2: Sterile saline solution 0.9%.

TRT3: Probiotic cocktail (*Lactobacillus sakei* FUA3089, *Pediococcus acidilactici* FUA3138, and *Pediococcus acidilactici* FUA3140 with a cell count of $10^8$-$10^9$ cfu/dose) in sterile skim milk and dissolved with sterile saline 0.9%.

[2]P: Primiparous

[3]M: Multiparous

**Table 2-5.** Odds ratio (OR) analyses for the incidence rate of uterine infections among treatments (TRT3 vs. TRT1 and TRT2) in primiparous and multiparous cows for the 4 farms.

| | OR[1] | | | OR[1] | | |
|---|---|---|---|---|---|---|
| Variable | TRT1[4] vs. TRT3[6] | 95% CL | *P*-value | TRT2[5] vs. TRT3[6] | 95% CL | *P*-value |
| UI P [2] | 1.34 | 0.72 - 1.57 | 0.15 | 1.46 | 1.07 - 1.99 | 0.27 |
| UI M[3] | 1.25 | 0.83 - 1.89 | 0.01 | 1.61 | 1.18 - 2.19 | 0.02 |

[1]OR = odds ratio.
[2]UI P = uterine infections in primiparous cows.
[3]UI M = uterine infections in multiparous cows.
[4]TRT1: Sterile saline 0.9% plus sterile skim milk.
[5] TRT2: Sterile saline solution 0.9%.
[6]TRT3: Probiotic cocktail (*Lactobacillus sakei* FUA3089, *Pediococcus acidilactici* FUA3138, and *Pediococcus acidilactici* FUA3140 with a cell count of $10^8$-$10^9$ cfu/dose) in sterile skim milk and dissolved with sterile saline 0.9%.

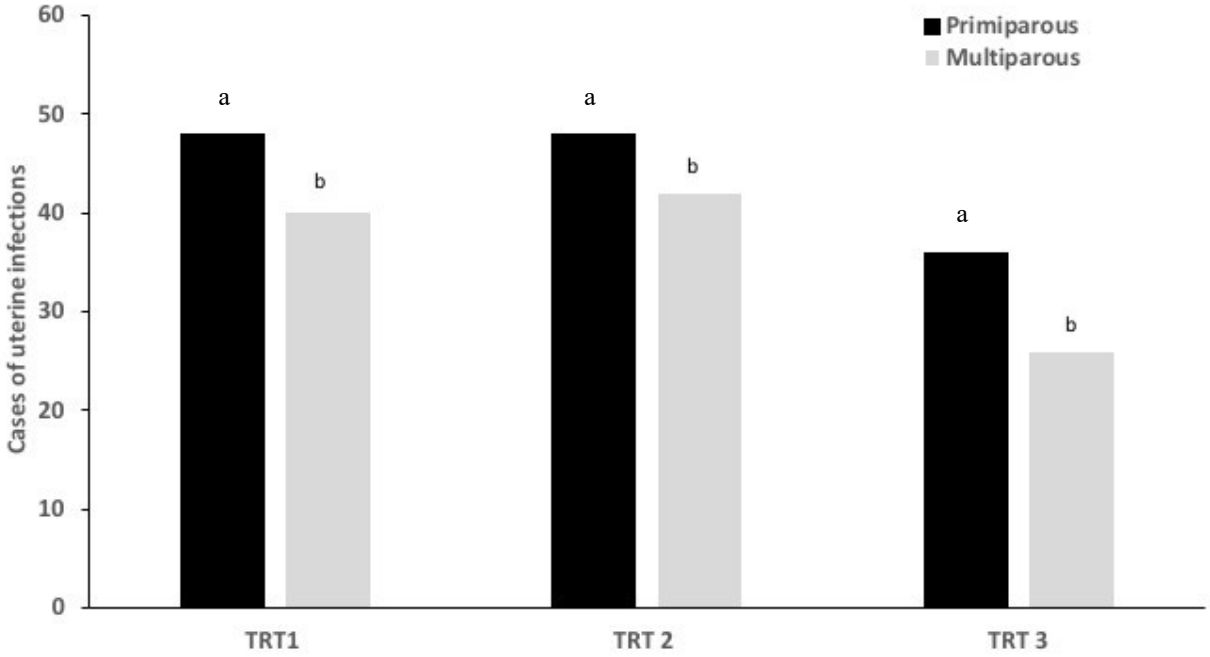


**Figure 2-2.** Number of cases of uterine infections after parturition for multiparous and primiparous postpartum dairy cows (TRT1: sterile saline 0.09% plus sterile skim milk, TRT2: sterile saline solution 0.09%, TRT3: probiotic cocktail (*Lactobacillus sakei* FUA3089, *Pediococcus acidilactici* FUA3138, and *Pediococcus acidilactici* FUA3140 with a cell count of $10^8$-$10^9$ cfu/dose) in sterile skim milk and dissolved with sterile saline 0.9%. [a-b]Numbers within bars with different superscript letters are different at $P < 0.05$.

**Table 2-6.** Frequencies for retained placenta post-partum among treatments in primiparous and multiparous cows.

| | Retained placenta (%) (case/total) | | | No retained placenta (%) (case/total) | | |
|---|---|---|---|---|---|---|
| TRT[1] | P[2] | M[3] | Total (%) | P[2] | M[3] | Total (%) |
| 1 | 28.57 (16/56) | 17.86 (10/56) | 46.43 | 18.7 (178/952) | 14.08 (134/952) | 32.77 |
| 2 | 14.29 (8/56) | 10.71 (6/56) | 25.00 | 19.54 (186/952) | 13.87 (132/952) | 33.40 |
| 3 | 14.29 (8/56) | 14.29 (8/56) | 28.57 | 19.54 (186/952) | 14.29 (136/952) | 33.82 |
| Total | 57.14 (32/56) | 42.86 (24/56) | 100.00 | 57.77 (550/952) | 42.33 (402/952) | 100.00 |

Note: Total number of observations for +3 wk and +4 wk
[1]TRT: treatment.
TRT1: Sterile saline 0.9% plus sterile skim milk.
TRT3: Probiotic cocktail (*Lactobacillus sakei* FUA3089, *Pediococcus acidilactici* FUA3138, and *Pediococcus acidilactici* FUA3140 with a cell count of $10^8$-$10^9$ cfu/dose) in sterile skim milk and dissolved with sterile saline 0.9%.
[2]P: Primiparous.
[3]M: Multiparous.

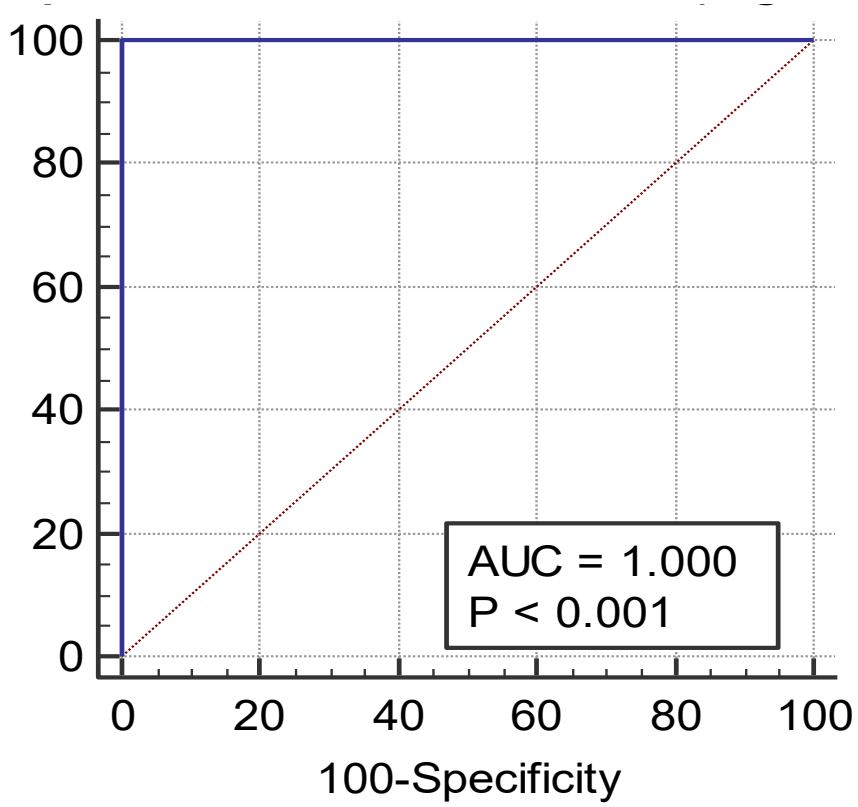


**Figure 2-3.** Receiver- operator characteristic (ROC) curve for palpation method of left and right horn. Interpretation of this critical threshold depends on the area under the curve (AUC), such that if the AUC >0.7 the test is considered accurate or of acceptable performance (Swets, 1988). *P* value =0.001, meaning the method used for palpation, and AUC =1 was accurate way of measuring the horns.

**Table 2-7.** Effect of intravaginal LAB probiotic cocktail on the incidence of peripreturient diseases on postpartum Holstein dairy cows in Farm A.

| Variable (%) (case/total) | TRT1[1] | TRT2[2] | TRT3[3] | *P*-value |
|---|---|---|---|---|
| Metritis | 39.47 (30/76)[a] | 31.58 (24/76)[a] | 28.95 (22/76)[b] | 0.14 |
| Mastitis | 42.86 (12/28)[a] | 35.71 (10/28)[a] | 21.43 (6/ 28)[b] | 0.07 |
| Milk fever | 0 | 100 (2/2) | 0 | NS |
| Retained placenta | 47.83 (22/46)[a] | 26.09 (12/46)[b] | 26.09 (12/46)[b] | 0.07 |
| Displaced abomasum | 0 | 100 (2/2) | 0 | NS |
| Lameness | 36.36 (8/22)[a] | 36.36 (8/22)[a] | 27.27 (6/22)[b] | 0.14 |

[1]TRT1: Sterile saline 0.9% plus sterile skim milk.

[2] TRT2: Sterile saline solution 0.9%.

[3]TRT3: Probiotic cocktail (*Lactobacillus sakei* FUA3089, *Pediococcus acidilactici* FUA3138, and *Pediococcus acidilactici* FUA3140 with a cell count of $10^8$-$10^9$ cfu/dose) in sterile skim milk and dissolved with sterile saline 0.9%.

[a-b]Numbers within a row with different superscript letters are different at $P < 0.05$.

NS.: no significance.

**Table 2-8.** Effect of the treatment on periparturient diseases in postpartum organic Jersey dairy cows in Farm B.

| Variable (%) (case/total) | TRT1[1] | TRT2 [2] | TRT3[3] | *P*-value |
|---|---|---|---|---|
| Metritis | 40.00 (12/30) [a] | 40.00 (12/30) [a] | 20.00 (2/30) [b] | 0.03 |
| Mastitis | 50.00 (8/16) [a] | 25.00(4/16) [b] | 25.00 (4/16) [b] | 0.20 |
| Milk fever | 28.57 (4/14) [a] | 57.14 (8/14) [b] | 14.29 (2/14) [a] | 0.01 |
| Retained placenta | 0 | 0 | 0 | NS |
| Displaced abomasum | 0 | 0 | 0 | NS |
| Lameness | 25.00 (2/8) [a] | 25.00 (2/8) [a] | 50.00 (4/8) [b] | 0.25 |

[1]TRT1: Sterile saline 0.9% plus sterile skim milk.

[2] TRT2: Sterile saline solution 0.9%.

[3]TRT3: Probiotic cocktail (*Lactobacillus sakei* FUA3089, *Pediococcus acidilactici* FUA3138, and *Pediococcus acidilactici* FUA3140 with a cell count of $10^8$-$10^9$ cfu/dose) in sterile skim milk and dissolved with sterile saline 0.9%.

[a-b]Numbers within a row with different superscript letters are different at $P < 0.05$

NS.: no significance.

**Table 2-9.** Effect of the treatment on periparturient diseases in postpartum Holstein dairy cows in Farm C.

| Variable (%) (case/total) | TRT1[1] | TRT2 [2] | TRT3[3] | *P*-value |
|---|---|---|---|---|
| Metritis | 42.42 (28/66)[a] | 33.33 (22/66)[a] | 24.24 (16/66)[b] | 0.07 |
| Mastitis | 41.67 (10/24)[a] | 33.33 (8/24)[a] | 25.00 (6/24)[a] | 0.6 |
| Milk fever | 100 (6/6)[a] | 0[b] | 0[b] | 0.05 |
| Retained placenta | 50.00 (2/4)[a] | 50.00 (2/4)[a] | 0[b] | 0.18 |
| Displaced abomasum | 0 | 0 | 0 | NS |
| Lameness | 28.57 (4/14)[a] | 28.57 (4/14)[a] | 42.86 (6/14) [b] | 0.56 |

[1]TRT1: Sterile saline 0.9% plus sterile skim milk.
[2] TRT2: Sterile saline solution 0.9%.
[3]TRT3: Probiotic cocktail (*Lactobacillus sakei* FUA3089, *Pediococcus acidilactici* FUA3138, and *Pediococcus acidilactici* FUA3140 with a cell count of $10^8$-$10^9$ cfu/dose) in sterile skim milk and dissolved with sterile saline 0.9%.
[a-b]Numbers within a row with different superscript letters are different at $P < 0.05$
NS.: no significance.

**Table 2-10.** Effect of the treatment on periparturient diseases in postpartum Holstein dairy cows in Farm D.

| Variable (%) (case/total) | TRT1[1] | TRT2 [2] | TRT3[3] | *P*-value |
|---|---|---|---|---|
| Metritis | 25.00 (16/64) [a] | 46.88 (30/63) [b] | 28.13 (18/64) [a] | 0.05 |
| Mastitis | 20.00 (2/10) [a] | 60.00 (6/10) [b] | 20.00 (2/10) [a] | 0.04 |
| Milk fever | 23.08 (6/26) [a] | 30.77 (8/26) [b] | 46.15 (12/26) [b] | 0.10 |
| Retained placenta | 25.00 (2/8) [a] | 25.00 (2/8) [a] | 50.00 (4/8) [a] | 0.35 |
| Displaced abomasum | 100 (2/2) | 0 | 0 | NS |
| Lameness | 50.00 (2/4) [a] | 0 | 50.00 (2/4) [a] | 0.01 |

[1]TRT1: Sterile saline 0.9% plus sterile skim milk.

[2] TRT2: Sterile saline solution 0.9%.

[3]TRT3: Probiotic cocktail (*Lactobacillus sakei* FUA3089, *Pediococcus acidilactici* FUA3138, and *Pediococcus acidilactici* FUA3140 with a cell count of $10^8$-$10^9$ cfu/dose) in sterile skim milk and dissolved with sterile saline 0.9%.

[a-b]Numbers within a row with different superscript letters are different at $P < 0.05$

NS.: no significance.

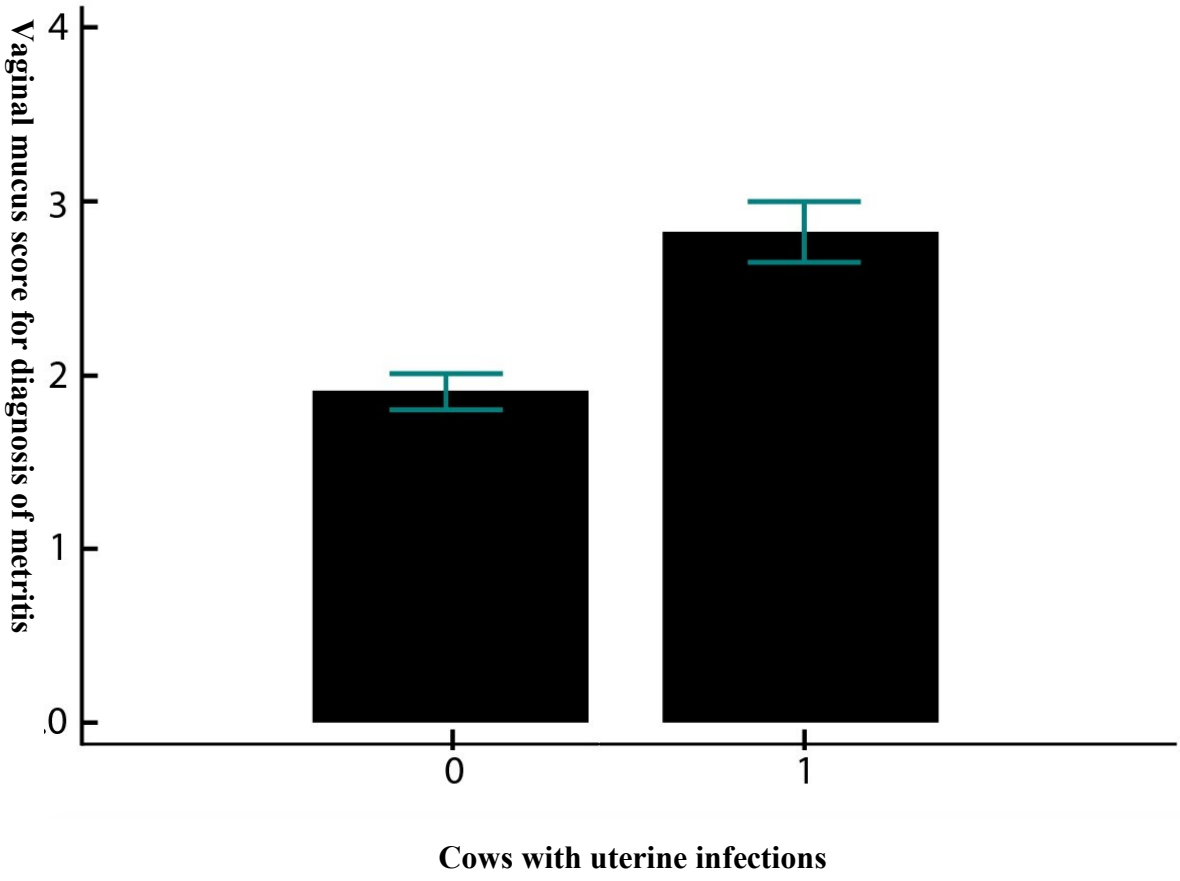


**Figure 2-4.** Relation between purulent vaginal discharge in cows infected with uterine infections vs cows that were infected with uterine infections on postpartum dairy cows (LSM + SEM) (0: not infected with uterine infections, 1: infected with uterine infections).

**Table 2-11.** Odds ratio (OR) analyses for effect of treatments (TRT3 vs. TRT1 and TRT2) on reproductive performance in dairy cows for the 4 farms.

| Class | OR[1] | 95% CL | *P*-value |
|---|---|---|---|
| TRT1[2] vs. TRT3[4] | 0.86 | 0.19-3.88 | 0.84 |
| TRT2[3] vs. TRT3[4] | 1.03 | 0.23-4.62 | 0.96 |

[1]OR = odds ratio.
[2]TRT1: Sterile saline 0.9% plus sterile skim milk.
[3] TRT2: Sterile saline solution 0.9%.
[4]TRT3: Probiotic cocktail (*Lactobacillus sakei* FUA3089, *Pediococcus acidilactici* FUA3138, and *Pediococcus acidilactici* FUA3140 with a cell count of $10^8$-$10^9$ cfu/dose) in sterile skim milk and dissolved with sterile saline 0.9%.

**Table 2-12.** Odds ratio (OR) analyses for effect of treatments (TRT3 vs. TRT1 and TRT2) on milk production in dairy cows for the 4 farms.

| Class | OR[1] | 95% CL | P-value |
|---|---|---|---|
| TRT1[2] vs. TRT3[4] | 1.18 | 1.16-1.20 | 0.01 |
| TRT2[3] vs. TRT3[4] | 1.20 | 1.18-1.22 | 0.01 |

[1]OR = odds ratio.
[2]TRT1: Sterile saline 0.9% plus sterile skim milk.
[3] TRT2: Sterile saline solution 0.9%.
[4]TRT3: Probiotic cocktail (*Lactobacillus sakei* FUA3089, *Pediococcus acidilactici* FUA3138, and *Pediococcus acidilactici* FUA3140 with a cell count of $10^8$-$10^9$ cfu/dose) in sterile skim milk and dissolved with sterile saline 0.9%.

**Table 2-13.** Milk composition of dairy cows from four farms treated with intravaginally mixture of lactic acid bacteria (LAB) around calving (LSM± SEM).

| Variable | Treatments | | | | Wks[4] | TRT*parity |
|---|---|---|---|---|---|---|
| | TRT1[1] | TRT2 [2] | TRT3 [3] | *P*-value [a] | *P*-value [b] | *P*-value [c] |
| Fat (%) | 4.95 ± 0.15 | 4.93 ± 0.13 | 4.97 ± 0.14 | 0.35 | 0.75 | 0.71 |
| Protein (%) | 3.69 ± 0.02 | 3.68 ± 0.03 | 3.69 ± 0.03 | 0.11 | <0.01 | 0.22 |
| Lactose (%) | 4.23 ± 0.01 | 4.25 ± 0.01 | 4.25 ± 0.01 | 0..92 | <0.01 | 0.61 |
| [5]TS (%) | 10.78 ± 0.24 | 10.76 ± 0.28 | 10.78 ± 0.27 | 0.37 | 0.01 | 0.37 |
| [6]MUN (mg/dL) | 17.26 ± 0.04 | 17.23 ± 0.04 | 17.32 ± 0.03 | 0.65 | 0.03 | 0.55 |
| [7]SCC ($10^3$/mL) | 841 ± 0.23 | 836 ± 0.23 | 849 ± 0.22 | 0.97 | 0.23 | 0.31 |

[1]TRT1: Sterile saline 0.9% plus sterile skim milk.
[2] TRT2: Sterile saline solution 0.9%.
[3]TRT3: Probiotic cocktail (*Lactobacillus sakei* FUA3089, *Pediococcus acidilactici* FUA3138, and *Pediococcus acidilactici* FUA3140 with a cell count of $10^8$-$10^9$ cfu/dose) in sterile skim milk and dissolved with sterile saline 0.9%.
[5] TS: total solids
[6] MUN: milk urea nitrogen
[7] SCC: somatic cell count
[a] Comparisons between TRT1, TRT2 and TRT3 in all cows
[b] Comparison between TRT1, 2, and 3 and +1 wk and +4 wks
[c] Interaction between all treatments and parity

## Chapter 3 Effects of administration of intravaginal probiotics on metabolic status and vaginal mucus microbiota in transition dairy cows


### Abstract

The objective of this study was to evaluate the effects of peripartal intravaginal infusion of probiotics on metabolic status of dairy cows around parturition. A total of five hundred twenty-six pregnant cows were assigned randomly to one of the following three treatment groups: 1) TRT1: four doses of 2 mL of carrier solution (sterile 0.9% saline) plus sterile skim milk; 2) TRT2: four doses of carrier solution (sterile 0.9% saline); and 3) TRT3 four doses of LAB at $10^8$-$10^9$ cfu/dose. The infusions of a lyophilized mixture of lactic acid bacteria (LAB) isolated from healthy Holsteins dairy cows were infused around calving at -3 and -2 wks prior to the expected day of parturition and at +3 and +4 wks postpartum. The probiotic cocktail is composed of *Lactobacillus sakei* FUA3089, *Pediococcus acidilactici* FUA3138, and *Pediococcus acidilactici* FUA3140 with a cell count of $10^8$-$10^9$ cfu/dose. Blood was sampled -3, +1 and at +4 wks for 180 dairy cows. Results showed that probiotics infused intravaginally lowered concentrations of glucose and cholesterol in the serum of both primiparous and multiparous cows diagnosed with uterine infections compared to TRT1 and TRT2 cows at +1 wk and +4 wks after parturition. No significant differences among the three groups were observed for lactate, non-esterified fatty acids (NEFA), and β-Hydroxybutyric acid (BHBA) for both primiparous and multiparous cows diagnosed with uterine infections and infused with TRT1, TRT2, and TRT3. Additionally, probiotics (TRT3) did not alter the quantity of total bacteria and lactobacilli strains in transition dairy cows. Furthermore, the most dominant phyla populating the vaginal mucus were unclassified *Ruminococcacceae*, *Ureaplasma*, *Fusobacterium*, *unclassified Pasteurellaceae*, *unclassified Clostridiales*, and *Porphyromonas*. In conclusion, intravaginal infusion of probiotics modulated serum glucose and

cholesterol after parturition in both primiparous and multiparous dairy cows affected by uterine infections but had no effect on serum lactate, NEFA, and BHBA or vaginal mucus microbiota.

### 3.1 Introduction

Periparturient diseases are a consequence of many factors that may include feed quality, ease of calving, and lactation which can induce negative energy balance (NEB) in postpartum dairy cows. Metritis, mastitis, retained placenta, ketosis, and milk fever are common periparturient disease that have been reported to be related to NEB and low dry matter intake during the transition period (Drackley, 1999; Urton et al., 2005; Huzzey et al., 2007).

Common indicators used to evaluate the state of NEB in dairy cows are concentrations of non-esterified fatty acid (NEFA) and β-hydroxy butyric acid (BHBA) in the serum (Duffield et al., 2009). High prepartum concentrations of NEFA and BHBA before parturition have been associated with periparturient diseases such as mastitis, metritis, and ketosis in transition dairy cows, causing impairments in milk production and reproduction performances (Dubuc et al., 2010; LeBlanc, 2011; Dervishi et al., 2015; Zhang et al., 2015). Changes in the concentrations of plasma NEFA has been directly related to the function of immune cells by changing the composition of the cellular membrane of those cells (Sordillo et al 2009). Additionally, high concentrations of NEFA in the plasma are known to be associated with pro-inflammatory cytokines such as IL-1β (LeBlanc, 2011).

Mobilization of energy in cows has been shown to be associated with periparturient diseases after parturition (Cai et al., 1994; Hammon et al., 2006). In addition, availability of glucose in dairy cows decreases during NEB, impairing immune cell functions and causing immunosuppression (Roth and Kaeberle, 1982). High concentrations of blood glucose are

associated with increased cortisol levels further exacerbating hyperglycemia and triggering insulin resistance after parturition, which is associated with immunosuppression (Galvao et al 2010). Innate immune cells need glucose as a source of energy for processes such as phagocytosis and microbial elimination (Borregaard and Herlin, 1982; Weisdorf et al., 1982).

Additionally, high concentrations of lactate after calving have been reported, whereas concentrations of cholesterol decrease weeks before calving and increase after parturition (Gross et al., 2015). It has been reported that serum lactate could be used as a predictive and diagnostic biomarker metabolite to identify cows that might develop lameness (Demir et al., 2012; Zhang et al., 2015). High concentrations of the metabolites mentioned above have been also associated with impairment of neutrophil functions and increase periparturient diseases risk around parturition (Dosogne et al., 2001). *Lactobacillis sakei* and isolates *Pediococcus acidilactici* have been known to decrease uterine infections and lower concentrations of plasma haptoglobin, an acute phase protein produced by the liver as part of the acute phase response to bacterial infection in the reproductive tract (Huzzey et al., 2009; Ametaj et al., 2010).

Uterine infections in transition dairy cows involve a broad spectrum of bacteria during the first 4 wks after parturition (Wang et al., 2013). The compromised state of the reproductive tract after parturition allow microorganisms to ascend to the reproductive tract (Sheldon et al., 2017). Pathogenic bacteria such as *Escherichia coli*, *Arcanobacterium pyogenes*, *Fusobacterium necrophorum*, *Prevotella melaninogenica* and *Proteus* species have been associated with endometrial inflammation and uterine infections (Williams et al., 2007). Additionally, it has been reported that presence of *Lactobacilli* strains inhibits growth of pathogenic microorganisms in healthy female reproductive tract (Redondo-Lopez et al., 1990). The mechanism by which non-harmful bacteria like *Lactobacillus* spp. involve include competitive exclusion for nutrients,

stimulation of immune system, production of acetic and lactic acid and hydrogen peroxide as well as antimicrobial peptides (Eschenbach et al.,1989). However, detailed alterations in vaginal mucus microbiota of dairy cows in dairy farms treated with intravaginal probiotics in transition dairy cows remain poorly documented.

Recent studies infusing probiotics intravaginally in cows around parturition have shown decreased uterine infections and alterations in blood metabolites as well as innate immunity variables (Wang et al., 2013; Deng et al., 2014, Ametaj et al., 2014). In this study we hypothesized that infusing four doses of LAB probiotics intravaginally at -3 wks, -2 wks, +3 wks, and +4 wks around calving (Figure 2-1) may influence the metabolic status of transition dairy cows. Additionally, the aim of this study was to characterize vaginal microbiota in transition dairy cows by next-generation DNA sequencing of the bacterial 16S rRNA gene. Therefore, the objectives of this study where to test weather administration of probiotics intravaginally at -3 and -2 wks before the expected day of calving as well as at +3 and +4 wks after calving at $10^8$ to $10^9$ cfu/mL could influence indirectly metabolic status of transition dairy cows.

## 3.2 Material and methods

### *3.2.1 Animals and treatments*

A total of five hundred twenty-six pregnant dairy cows were blocked by farm and according to parity, body condition score (BCS), and previous lactation milk yield. Cows were assigned randomly to one of the following three treatment groups: 1) TRT1: four doses of 2 mL of carrier solution (sterile 0.9% saline) plus sterile skim milk; 2) TRT2: four doses of carrier solution (sterile 0.9% saline); and 3) TRT3 four doses of LAB at $10^8$-$10^9$ cfu/dose. Cows were infused at -3 and -2 wks prior to the expected date of calving and at +3 and +4 wks after calving.

Probiotics used in this study were a lyophilized mixture in sterile skim milk composed of *Lactobacillus sakei* FUA3089, *Pediococcus acidilactici* FUA3138, and *Pediococcus acidilactici* FUA3140 with a cell count of $10^8$-$10^9$ cfu/dose. All experimental procedures were approved by the University of Alberta Animal Care and Use Committee for Livestock and cows were cared for in accordance with the guidelines of the Canadian Council on Animal Care (1993).

Both LAB and skim milk were stored at -86 ºC in vials, and each vial of LAB was reconstituted with 2 mL of sterile 0.9% saline (Vetoquinol, Lavaltrie, QC) before administration. The three different treatments where infused intravaginally with a 5-mL Luer-Lok Tip syringe (Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA) attached to an individually wrapped sterile drilled infusion tube (45.72 cm) (Continental Plastic Corp., Delavan, WI). This experiment was conducted at four different free stall commercial farms (A, B, C, and D) located in the province of Alberta, Canada. Each farm was operated under different management systems as already explained in Chapter 2.

### *3.2.2 Sample collection and clinical monitoring*

Blood samples were collected from the coccygeal vein, once per week, from each cow (i.e., 180 cows). Samples were collected at -3 wks prior to the expected day of parturition and at +1 and +4 wks after calving using 10-mL vacutainer tubes without anticoagulant (BD Vacutainer Systems, Plymouth, UK). Samples were stored in ice and transported to the laboratory within 4 h until separation of the serum. Blood samples were centrifuged at 2,090 x g at 4 °C for 20 min (Beckerman Coulter, Pasadena, California, USA). Serum was stored into 10-mL plastic tubes (Fisher Scientific, Toronto, ON, Canada) at -20 °C until analysis.

Vaginal mucus was collected from 135 (45 dairy cows per each of the three Holstein farms) multiparous Holstein cows and 45 multiparous Jersey and transferred into sterile polypropylene

culture test tube (Fisher Scientific, Toronto, ON, Canada), and stored at -20 °C until analysis. The Metricheck device was disinfected between uses with Nolvasan solution (Zoetis, Kalamazoo, Michigan, USA) contained chlorhexidine diacetate at a concentration of 2% and sanitized using 70% alcohol before each mucus extraction. The vaginal perineal region of the cows was disinfected with 16% iodine solution (Vetoquinol N.-A, Inc., Lavaltrie, Quebec, Canada), and then inserted into the reproductive tract obtaining vaginal mucus.

Body condition scores (BCS) were measured using the BCS in Dairy Cattle Production (342-450A, McGill University and Elanco Animal Health, Indianapolis, Indiana, USA). BCSs were evaluated at -3 and -2 wks before parturition and +3 and +4 wks after parturition, based on a scale of 5 with intervals of 0.25 points.

### *3.2.3 Laboratory analyses*

Serum samples from 120 cows (10 per treatment/per farm/3-time points) (Figure 2-1) collected at -3, +1, and +4 wks around calving were randomly analyzed to evaluate concentrations of glucose, cholesterol, lactate, NEFA, and BHBA.

Concentrations of glucose in the serum were quantified by an enzymatic method using hexokinase procedure with a commercial kit in accordance with the manufacturer's instructions (Glucose-SL Assay) (Sekisui Diagnostics, Liphook Way, Allington, Maidstone, Kent, UK). Standards were provided by the kit and diluted to a set detection range of 19 to 152 mg/dL. The test principle involves glucose phosphorylated by hexokinase into glucose-6-phosphate and adenosine diphosphate, which when oxidized leads to production of NADH. The serum glucose concentration was determined by reading the optical densities on a microplate spectrophotometer at 340 nm (Spectramax 190, Molecular Devices Corp, Sunnyvale, CA, USA). The intra-assay variations were at ≤ 10%.

Concentration of cholesterol in the serum was measured using an enzymatic colorimetric method with a commercial kit in accordance with the manufacturer's instructions (Cholesterol-SL Assay) (Sekisui Diagnostics, Kent, UK). The test principle for cholesterol concentration involves hydrolysis of cholesterol esters into free cholesterol by cholesterol esterase (CE). Oxidation of free cholesterol by cholesterol oxidase yields cholest-4-ene-3-one and hydrogen peroxide. Hydrogen peroxide couples with 4-aminoantitipyrine and p-hydroxbenzoate in the presence of peroxidase to yield a chromogen. Detection range of 20.5 to 164 mg/dL were used for the dilution of standards, which were provided by the kit. Concentration of cholesterol in the serum was measured by reading the plate on a microplate spectrophotometer at 505 nm (Spectramax 190, Molecular Devices Corp, Sunnyvale, CA, USA). The intra-assay variations were at ≤ 10%.

Concentrations of NEFA were measured in duplicate by a bovine kit using a NEFA standard assay as instructed by the manufacturer (Randox Laboratories Ltd., Antrim, UK). The basic principle of the test involves acylation of coenzyme A by fatty acids and acyl-CoA synthetase and production of hydrogen peroxide in the presence of Acyl-CoA oxidase. Hydrogen peroxide, in the presence of peroxidase, permits the oxidative condensation of N-Ethyl-N-(2hydroxy-3-sulphopropyl)-m-toluidine with 4-aminoantipyrine to form a purple adduct, which is proportional to NEFA concentration within the sample. The standards for NEFA, provided by the kit, were diluted to set a detection range of 0.25 to 2 mM. Concentrations of NEFA in the serum were measured by reading the optical densities at 550 nm on a microplate spectrophotometer (Spectramax 190, Molecular Devices Corp, Sunnyvale, CA, USA). The intra-assay variations were at ≤ 10%.

Concentrations of lactate were measured in duplicate using a L-Lactate Assay Kit (Cat# A-108L) (Biomedical Research Service Center, University at Buffalo, State University of NY). The

test's principle for lactate assay involves a reduction of the tetrazolium salt INT in a NADH-coupled enzymatic reaction to formazan. Measurements by the assay is the concentration of intracellular and extracellular L-lactate. Standards for lactate were set to a detection range of 125 to 1,000 μM. All samples were tested in duplicate and diluted 10-fold with distilled ice $H_2O$. All results for lactate were multiplied by dilution factor of 10x. The serum lactate was determined by reading the optical densities at 492 nm on a microplate spectrophotometer (Spectramax 190, Molecular Devices Corp, Sunnyvale, CA, USA). The intra-assay variations were at ≤ 10%.

The amounts of β-Hydroxybutyric acid (BHBA) in the serum were measured using a Cayman Chemical kit (Ann Arbor, Michigan, USA). The test basic principle for BHBA involves the oxidation of D-3-hydroxybutyrate to acetoacetate under the influence of 3-hydroxybutyrate dehydrogenase. As a result, the reaction produces cofactor $NAD^+$ which is reduced to NADH. NADH then reacts with the colorimetric detector WST-1 in the presence of diaphorase yielding a formazan dye. Standards for BHBA were diluted to set a detection range of 0.5 to 0.0652 μM. The serum BHBA was measured by reading optical densities at 445-455 nm on a microplate spectrophotometer (Spectramax 190, Molecular Devices Corp, Sunnyvale, CA, USA). The intra-assay variations were at ≤ 10%.

### *3.2.4 DNA Extraction of vaginal mucus microbiota*

Vaginal mucus samples from 35 multiparous cows, from farms A and C, were selected to determine whether they contained the probiotic strains after parturition that could be detected using qPCR. Total bacterial DNA was extracted from samples using the QIAamp Fast DNA Stool Mini Kit (Qiagen Inc., Mississauga, ON, Canada). Evaluation of bacterial contaminants was tested by setting 6 nuclease-free water samples as negative controls and performed the same process of DNA extraction using the same kit. Quality and quantity of DNA were checked on a NanoDrop

spectrophotometer system ND-1000, software version 3.3.0 (Thermo Fisher Scientific Inc., Wilmington, Delaware, USA). Samples were stored at -20 ℃ for qPCR analyses later.

### *3.2.5 PCR Amplifications*

The PCR amplifications of L. *sakei* FUA 3089, P. *acidilactici* FUA 3140, and P. *acidilactici* FUA 3138 with four types of primer pairs in Table 3-1 were performed in GeneAmpW PCR System 9700 (Applied Biosystems, Foster City, CA, USA). The reaction mixture contained 5 μL of 10x Ex Taq buffer, 4 μL of 10 mM dNTP mix (2.5 mM each), 2.5 μL of 10 mM primer forward, 2.5 μL of 10 mM primer reverse, 0.5 μl of TakaRa Ex Taq (5 units/uL), 34.5 μL of nuclease-free water, and 1 μL of probiotic DNA or nuclease-free water as the negative control. The PCR programs included an initial denaturation at 94 °C for 5 min. Then, 35 cycles of denaturation, annealing and extension were performed at 94 °C for 30 sec, at the listed annealing temperatures in Table 3-1 for 30 sec, at 72 °C for 1 min, followed by a final extension step at 72 °C for 7 min. The electrophoresis was performed in a 1% DNA gel stained agarose gel with Tris/Borate/EDTA (TBE) buffer at 130 V for 40 min. The gel was photographed in UV light to confirm that each PCR product had a single clear band at the target amplicon size and negative controls did not get contaminated. The PCR products were purified by the QIAquick PCR purification kit (Qiagen, Hilden, Germany) and then stored at -20 ℃.

### *3.2.6 Quantitative PCR Analyses*

The DNA concentrations of purified PCR products were measured by the NanoDrop spectrophotometer system and were used to calculate copy numbers for standard curves. To create standard curves, ten-fold serially dilution of purified PCR products (10-3 to 10-11) was prepared. Quantitative PCR was used as a sensitive tool to quantify vaginal bacteria at -3 wks, +1 wk, and

+4 wks around parturition. Primers were the same as PCR (Table 3-1). The qPCR reaction mixture contained 0.5 μL of 10 mM primer forward, 0.5 μL of 10 mM primer reverse, 12.5 μL of SYBP green, 10.5 μL of nuclease-free water, and 1 μL of diluted PCR products (triplicates), vaginal mucus samples (duplicates) or nuclease-free water as negative controls (triplicates). The reaction mixture obtained was loaded in MicroAmp Fast Optical 96-well reaction plates, sealed with MicroAmp Optical Adhesive Film (Applied Biosystems, Foster City, CA, USA), and spun down at 5000 rpm for 1 min using the S5700 Swinging Bucket Rotor (Beckman Coulter). Quantitative PCR was performed in a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with similar programs to PCR, except for 40 cycles instead of 35 in the cycling stage and a default melt curve stage. Six nuclease-free water samples extracted by the stool kit were also performed qPCR in triplicates to evaluate whether the clean stool kit contained any bacteria that might show false positive results.

### *3.2.7 Statistical Analyses*

All statistical analyses were performed using SAS software (Version 9.4, SAS Institute, Inc., Cary, NC). In this study cows were blocked by parity and treatment (TRT1, TRT2, TRT3) before being assigned to the treatment groups. For analysis of serum variables, 10 healthy cows were selected from each treatment group that were not diagnosed with post-partum uterine infections, and 10 sick cows from each treatment group that were diagnosed with postpartum uterine infection (30 multiparous and 30 primiparous).

Data for blood variables were analyzed with PROC UNIVARIATE procedure to test assumptions of normality. However, results showed that data followed a non-normal distribution after a Poisson distribution. Because of that, distribution of data by PROC GLIMMIX procedure

was used for logistic regression, including farm as random effect and correlated errors as per following equation:

$$Y_{ijkl} = \mu + TRT_i + eijkl$$

where $\mu$ = the overall population mean; $TRT_i$ = the fixed effect of treatment i (i = skim milk, saline solution 0.9%, LAB probiotic cocktail), and $e_{ijkl}$ = the residual error.

Data are shown as least-squares means (LSM) and the respective standard error of the mean (SEM). Effect of farm and cow were considered a random effect in the model statement. The effect of sampling week was considered a repeated measure. The covariance structure was modeled according to the smallest Akaike information criterion (AIC) and the Bayesian information criterion (BIC) values generated.

Additional model fixed effects for week and parity were investigated along with their corresponding interactions for each serum parameter (Parameter = trt hs week trt*hs, week*trt, trt*parity, week*hs, week*hs*parity). A backwards elimination from a saturated model was performed if the effect was not significant on the response variable. Since there was no significance in the 3-way and 4-way interactions for glucose, NEFA, and lactate, those were removed from the statistical model. There was a significant effect of the two-way interaction between treatment and parity for glucose and therefore it was kept in the statistical model. Moreover, there was a significant effect of the 3-way interaction between week, health status, and parity for cholesterol as well as the 2-way interactions between treatment and health status, week and treatment, treatment and parity, and week and health status. These were therefore kept in the statistical model for cholesterol. Significance was declared at $P < 0.05$ and tendency at $0.05 \leq P \leq 0.10$.

Copy numbers in PCR products were calculated as (X ng * 6.0221 * 1023 molecules / mole) / (N bp * 660 g / mole) * 1* 109 ng / g), in which X was the amplicon amount measured by the NanoDrop spectrophotometer system, and N was the amplicon length (Table 3-1). Quantitative PCR generated a linear regression of Ct means as the independent variable and log10 (copies for 10-fold serially dilution) as the dependent variable. Original copy numbers in animal samples per gram mucus were calculated using the standard curve and the Ct mean of duplicates, and corrected by dilution factors, elution volume and sample weight (Microsoft Excel, 2014). A one-way Analysis of Variance (ANOVA) test was conducted to analyze whether gene copies were significantly different among three treatments and between prepartum and postpartum periods using SigmaPlot version 13 from Systat Software, Inc., San Jose California USA. Significance was declared at $P < 0.05$.

## 3.3 Results

### *3.3.1 Overall effect of intravaginal probiotics on the metabolic status*

It should be pointed out that cows were selected randomly for measurements of blood variables and that concentration of glucose and cholesterol in the serum of probiotic treated cows was lower at -3 wks (prior to treating cows with probiotics) for reasons that are not understood. Concentration of glucose in the serum differed among treatments at -3 wks, +1 wk, and +4 wks ($P < 0.01$) (Table 3-2). Overall, after infusion at +1 wk and +4 wks, glucose concentrations were lower for probiotic group (TRT3) compared to TRT1 ($P = 0.01$) and TRT2 ($P = 0.03$). Concentrations of glucose in the serum of cows infused with probiotics (TRT3) were lower after parturition at +1 wk compared to cows from TRT1 ($P < 0.07$) and TRT2 ($P < 0.04$). Furthermore, concentrations of glucose in cows infused with probiotics (TRT3) were lower at +4 wks compared

to TRT1 ($P < 0.01$), but had no significant difference compared to TRT2 ($P = 0.29$). Serum glucose decreased gradually in cows infused with probiotics (TRT3) from -3 to +4 wks ($P < 0.01$), whereas in TRT2 which increased at +1 wk and then decreased at +4 wks ($P < 0.01$). Interestingly, serum glucose decreased gradually in cows infused with TRT1 from -3 to + 4 wks ($P < 0.01$).

Concentration of cholesterol in the serum differed among treatments for -3 wks, +1 wk, and +4 wks ($P = 0.01$) (Table 3-2). Overall, after infusion at +1 wk and +4 wks, cholesterol concentrations were lower for probiotic group (TRT3) compared to TRT1 ($P = 0.01$) and TRT2 ($P = 0.01$). Concentrations of cholesterol in the serum of cows infused with probiotics (TRT3) were lower after parturition at +1 wk compared to cows from TRT1 ($P = 0.03$) and TRT2 ($P = 0.04$). Furthermore, concentrations for cholesterol in cows infused with probiotics (TRT3) were lower at +4 wks compared to TRT1 ($P = 0.01$) and TRT2 ($P = 0.01$). Serum cholesterol increased gradually in cows infused with probiotics (TRT3) from -3 wks to + 4 wks ($P < 0.01$), whereas in TRT1 and TRT2 which decreased at +1 wk, and then increased at + 4 wks ($P < 0.01$).

No difference was observed among treatment groups in terms of concentration of lactate ($P = 0.33$), NEFA ($P = 0.39$), and BHBA ($P = 0.54$). Concentrations of lactate, NEFA, and BHBA in the serum for treatment groups on -3 wks, +1 wk, and +4 wks are presented in Tables 3-4, 3-5. No interaction between health status and parity and TRT was found with regard to the above-mentioned variables.

### *3.3.2 Effect of intravaginal probiotics on the metabolic status of multiparous and primiparous cows*

Probiotics infused intravaginally lowered glucose concentrations in blood serum of dairy cows infused with probiotics (TRT3) compared to TRT1 and TRT2 ($P < 0.01$, Figure 3-1). Multiparous and primiparous cows diagnosed with uterine infections and infused intravaginally with TRT3 had

lower ($P < 0.01$) concentrations of glucose in the serum compared to cows infused with TRT1 and TRT2 at +1 wk and +4 wks after parturition. Additionally, multiparous cows that were not diagnosed with uterine infections and infused with TRT2 had lower ($P < 0.01$) concentrations of glucose in the serum compared to TRT1 and TRT3 at +1 wk and +4 wks. Interestingly, primiparous cows that were infused with probiotics and free of uterine infections had lower ($P < 0.01$) concentrations of glucose compared to TRT1 and TRT2 at +1 wk and +4 wks. There was an interaction between TRT and parity, however, no interactions were obtained between TRT and week.

Probiotics infused intravaginally (TRT3) in multiparous cows lowered cholesterol in the serum compared to TRT1 and TRT2 ($P < 0.01$, Figure 3-2). Multiparous and primiparous cows that were not diagnosed with uterine infections and infused intravaginally with TRT3 had lower ($P < 0.01$) concentrations of cholesterol in blood compared to cows infused with TRT1 and TRT2 in +1 wk and +4 wks postpartum. Additionally, multiparous cows that were diagnosed with uterine infections and infused with TRT3 increased cholesterol concentrations at +1 wk, gradually decreasing by +4 wks ($P = 0.01$) compared to TRT1 and TRT2. Interestingly, primiparous cows that were infused with TRT3 and were diagnosed with uterine infections had lower ($P = 0.01$) concentrations of cholesterol compared to TRT1 and TRT2 at +1 wk and +4 wks. There was an interaction between TRT and parity, moreover, no interactions were found for TRT and week.

No significant difference was found for probiotics infused intravaginally (TRT3) regarding concentrations of lactate (Figure 3-3), NEFA (Figure 3-4), and BHBA (Figure 3-5) in the serum of multiparous and primiparous dairy cows compared to TRT1 and TRT2 ($P > 0.05$). Serum NEFA varied over time in primiparous and multiparous cows that were diagnosed with uterine infections, increasing significantly at +1 wk ($P = 0.01$). Additionally, multiparous cows that were not

diagnosed with uterine infections and were intravaginally infused with TRT3 had a steady increase of serum concentrations of BHBA from -3 to + 4 wks ($P < 0.01$), however, no difference was obtained between TRT3 and TRT1 and TRT2. Moreover, there was an interaction between TRT and parity, but no interactions between TRT and week.

### *3.3.3 Characterization and dynamics of bacterial microbiota after probiotic infusions on vaginal mucus of transition dairy cows*

The phyla identified in the vaginal mucus samples are presented in Table 3-7. The most dominant phyla identified in the vaginal mucus of dairy cows in the study were *Firmicutes* (44.30% ±1.39), *Bacteroidetes* (19.75% ±0.93), *Proteobacteria* (10.54% ±1.33), *Tenericutes* (9.41% ±1.22), *Fusobacteria* (7.60% ±1.13), and *Actinobacteria* (3.79% ±0.42). Furthermore, microbiota composition of the vaginal mucus at genus level is presented in Table 3-8. The most dominant genera of bacteria were unclassified *Ruminococcacceae* (17.02% ±0.82), *Ureaplasma* (8.02% ±1.22), *Fusobacterium* (5.56% ±0.79), unclassified *Pasteurellaceae* (4.86% ±1.05), unclassified *Clostridiales* (4.31% ±0.21), and *Porphyromonas* (4.18% ±0.74).

Microbiota was compared between the probiotic (TRT3) group and the two control groups around parturition for Farms A (Figure 3-6) and C (Figure 3-7). There was no significant effect of probiotics (TRT3) on the number of *L. sakei* FUA 3089, *P. acidilacti* FUA 3140, and *P. acidilacti* FUA 3138 at three different time points (-3 wks, +1 wk and + 4wks around calving). Within probiotics-treated samples, the number of *Lactobacilli* from farms A and C showed a significant decrease from wk -3 to wk +1, and a significant restoration of *Lactobacill*i from +1 wk to +4 wks ($P < 0.05$). Of note, sequences for *P. acidilacti* FUA 3140 and *P. acidilacti* FUA 3138 were hard to distinguish. Moreover, for Farms A and C no significant difference was found in the total

number of *L. sakei* FUA 3089, *P. acidilacti* FUA 3140, and *P. acidilacti* FUA 3138 at different time points studied.

### 3.4 Discussion

In this study we hypothesized that infusing four doses of LAB probiotics intravaginally at around calving may indirectly influence the metabolic status of transition dairy cows. Indeed, results of this study showed that intravaginal infusion of LAB probiotics, previously isolated from vaginal mucus of healthy cows (Wang et al., 2013; Ametaj et al., 2014), affected several blood variables related to carbohydrate and lipid metabolism.

Results of our study demonstrated that cows infused intravaginally with probiotics (TRT3) around calving had lower concentrations of glucose in the plasma compared to control cows, treated with sterile saline or skim milk dissolved in sterile saline (TRT1 and TRT2) respectively. Data indicated that concentrations of glucose in the serum decreased postpartum at +1 and +4 wks after parturition. Glucose concentrations were lower in multiparous and primiparous cows diagnosed with uterine infections and infused with probiotics (TRT3) compared to TRT1 and TRT2. It is not clear whether high glucose concentrations after calving are responsible for increased risk of uterine infections, but it has been reported that females with chronic hyperglycemia are susceptible to bacterial infections related to reduced neutrophil functions (Saito at al., 2013). Moreover, Bicalho et al. (2017) and Credille et al. (2014), reported higher glucose concentrations in cows diagnosed with metritis, endometritis, and retained placenta. Although the mechanism of how probiotics lower serum glucose is not known, it is possible that this effect might be related to prevention of uterine bacterial infections.

It has been reported that cows experience a negative energy balance (NEB) around parturition followed by a decrease in nutrients for milk production and body maintenance (Galvao et al.,2010). Glucose is known as a critical nutrient for milk production and reproduction purposes (Lucy et al., 2014). It has been shown that concentrations of glucose in the serum decrease and body fatty acids mobilized to provide the necessary energy to transition dairy cows (Roth and Kaeberle, 1982; Galvao et al., 2010; Bicalho et al., 2017). Alterations in blood metabolites immediately after parturition in cows experiencing NEB predispose them to metabolic and microbial diseases (Esposito et al., 2014). Roth and Kaeberle, 1982 indicated that higher blood glucose around calving may be responsible for impairment of polymorphonuclear neutrophil functions. Additionally, high levels of blood glucose are responsible for increased cortisol levels causing hyperglycemia after parturition and immunosuppression (Roth and Kaeberle, 1982). It has also been reported that high blood glucose induces secretion of tumor necrosis factor (TNF)-α, which is known to induce insulin resistance (Gonzalez et al., 2012). Insulin resistance is associated with pathological conditions including infections (Borst, 2014). During uterine infections macrophages release proinflammatory cytokines such as TNF-α which is translocated into the systemic circulation in response to bacterial lipopolysaccharide (LPS) (Dervishi et al., 2015; Eckel and Ametaj, 2014), triggering insulin resistance (Cheung et al., 1998; Lang et al., 1992). Insulin response in dairy cows has been associated with cell proliferation and differentiation; glucose uptake by skeletal and adipose tissue; lipogenesis and lipolysis in adipose tissue; and glucogenesis in the liver (De Koster and Opsomer, 2013). Galvao et al., (2010) showed that concentrations of glycogen in PMN were lower 3 wks after parturition in cows diagnosed with uterine infections. Moreover, Van den Oever et al., (2010) associated increased glucose concentrations with impaired endothelial functions in humans.

Uterine diseases are associated with *Escherichia coli, A. pyogenes,* and *Fusobacterium necrophorum* stimulating the immune system and triggering an inflammatory response including release of acute phase proteins, proinflammatory cytokines, and expression of complement system which require large amounts of energy (Sheldon et al., 2017). Occasionally, *Streptococci*, *Staphylococci*, *Proteus* or *Clostridium* spp. are also associated with metritis (Dohmen et al., 1995).

As already discussed previously (Chapter 2), cows treated with intravaginal probiotics (TRT3) produced 4.6 L/d and 3.22 L/d more milk compared to TRT1 and TRT2, respectively. This might be attributed to the flow of glucose to the mammary gland for synthesis of lactose in the mammary epithelial cells (Lucy et al., 2014; Eger et al., 2015). Lactose is a disaccharide made of glucose and galactose and the main osmotic determinant of milk volume (Mohammad et al., 2012).

Another important finding of this study was that cows infused intravaginally with probiotics (TRT3) had lower concentrations of cholesterol in the serum compared to TRT1 and TRT2 cows. It should be noted that, although cholesterol concentrations were lower in TRT3 there was a gradual increase over time from -3 to +4 wks around calving. It should also be pointed out that although cows were randomly selected for measurement of blood variables concentration of cholesterol in the serum of probiotic-treated cows was lower starting from -3 wks (prior to treating cows with probiotics) prepartum. The reason for this is not understood at present. However, it has been reported that oral administration of LAB probiotics in human subjects lowers blood cholesterol (Kumar et al., 2012; Tsai et al., 2014).

In dairy cows, high blood cholesterol has been associated with fatty liver around calving (Grummer, 1993; Maxfield and Tabas, 2005). Additionally, it has been reported that high blood cholesterol can affect both B- and T-cell functions by disrupting antigen presentation and affecting their regulatory mechanisms (Widenmaier and Hotamışlıgil, 2016). It should be indicated that

there are conflicting results on the role of cholesterol on the incidence of uterine infections and other periparturient diseases in postpartum dairy cows. For example, Yvan-Charvet et al. (2008) stated that although production of monocytes and neutrophils in the bone marrow is stimulated by accumulation of cholesterol, high cholesterol levels can worsen metabolic diseases associated with inflammation. Sarkar et al. (2016) also demonstrated lower cholesterol in cows diagnosed with uterine infections.

Concentrations of lactate, NEFA, and BHBA in the serum of probiotic-treated cows were not found to be different from those of the two control groups ($P > 0.05$) and will not be discussed further.

After parturition vagina and uterus of postpartum dairy cows contain a combination of both Gram-negative and Gram-positive bacteria with unknown origin (Sheldon et al., 2017). Some of those bacteria are responsible for uterine infections (Bekana et al., 1996; Sheldon et al 2017). In most cases metritis is resolved by the host during the first 3 wks after parturition; however, some pathogenic bacteria persevere and are difficult to be treated (Wang et al., 2013). The most abundant phyla of the vaginal mucus of dairy cows in this study were *Firmicutes* and *Bacteriodetes* at 44% and 19%, respectively. The other less dominant phyla of the vaginal mucus were *Proteobacteria*, *Tenericutes*, and *Fusobacteria*. Furthermore, the most dominant genera populating the vaginal mucus were unclassified *Ruminococca*cceae (i.e., family), *Ureaplasma*, *Fusobacterium*, *unclassified Pasteurellaceae* (i.e., family), *unclassified Clostridiales* (i.e., family), and *Porphyromonas*.

Quantitative PCR of vaginal mucus samples at Farm C showed a significant increase in the total bacterial load in the same cows after parturition. Enhancement of bacterial load might be

related to the compromised state of the immune status and increased numbers of pathogenic bacteria in the reproductive tract (Wang et al., 2013; Deng et al., 2014).

Intriguingly, cows infused with sterile saline (TRT1) or sterile skim milk (TRT2) showed no significant alterations in the number of *Lactobacilli* spp. However, within the probiotics group, there was a significant decrease from wk -3 to wk +1, and a significant restoration from wk +1 to wk +4. However, the difference was not significant ($P > 0.05$) (approximately 1 log). This could be attributed to the fact that the probiotic might have been flushed out by discharges of lochia from the uterus. Furthermore, probiotics administered might have been attached to the epithelial cells of the vagina and were not free in the vaginal mucus. Additionally, probiotics colonization relied on their competitive abilities against *Escherichia coli* and *Staphylococcus aureus* for adhesion sites in the vaginal epithelium (Charlier et al., 2009). Of note is the finding that isolates of *P. acidilactici* were below the detection limits. It is important to recall that in farm A, cows that were diagnosed with uterine purulent discharges were immediately lavaged with a solution containing penicillin. This antibiotic provides bactericidal activity against Gram-positive and Gram-negative bacteria and could be the reason for the observed decrease in the total bacteria load. Moreover, the increase of *Lactobacilli* spp. after +1 wk could be attributed to the fact that health status in cows infused with probiotics (TRT3) was improved (Ametaj et al., 2014; Deng et al., 2014).

Although administration of probiotics was not found to modulate vaginal mucus microbiota in the treated cows in this study, our lab has reported previosuly that administration of LAB around calving increased abundance of *L. sakei* in the vaginal mucus and lowered the number of pathogenic bacteria after parturition (Deng et al., 2014). Additionally, Genis et al., (2016) reported lower expression of B-defensins and MUC1 in the endometrium of cows treated intravaginally with similar probiotic *Lactobacilli*, two indicators of uterine involution. *Lactobacilli*

adhere to vaginal epithelial cells and displace well known vaginal pathogens (Boris et al., 1996). Microbiota in this study was measured on vaginal mucus and not on *Lactobacilli* adhered to epithelial cells. This might be one of the reasons for not detecting changes in vaginal mucus Lactobacilli numbers. However, the effects of probiotics on bovine vaginal microbiota warrants further investigation.

## 3.5 Conclusions

Overall, treatment with intravaginal probiotics was associated with alterations in some of the metabolites measured including glucose and cholesterol. More specifically probiotics lowered concentrations of both glucose and cholesterol in the serum of the treated cows. High blood glucose has been associated with high incidence of uterine infection. Therefore, lowering of blood glucose can be seen as a beneficial effect of intravaginal probiotics. Additionally, high cholesterol has been associated with negative effects on immune cells. Therefore, lowered cholesterol in the probiotic-treated cows could be a positive effect of probiotics. Treatment did not have an effect on several other blood variables measured including NEFA, lactate, and BHBA and did not alter the quantity of total bacteria load and Lactobacilli strains in the treated cows. This means that probiotics are safe and do not cause harmful alterations in the vaginal tract.

## 3.6 Acknowledgements

Dr. Burim N. Ametaj was the principal investigator of this study who contributed to conceptualization and designing of this research work and in organizing and managing the entire study. Dr. Ametaj also supervised all the lab analyses. He contributed in writing, editing as well as in improving the quality of this manuscript with many comments and suggestions. Dr. Andre

Luiz Garcia and Ashley Egyedy contributed in sample collection and health evaluation of the cows. Dr. Michael G. Gänzle and Dr. Yuanyao Chen contributed on DNA extraction from vaginal mucus microbiota, in designing strain-specific primers for intravaginal mucus samples and processing of data. Suzanna M. Dunn is acknowledged for her contribution in lab analyses. Likewise, we are grateful to the dairy farmers who provided their cows to this study and for creating a favorable and supportive environment and providing all necessary records to us.

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**Table 3-1.** Primers and PCR conditions.

| Primers | Target Sequence (5' - 3') | Amplicon Length (bp) | $T_m$ (℃) | Reference |
|---|---|---|---|---|
| Total bacteria primer | 16S rRNA<br>F: CGGYCCAGACTCCTACGGG<br>R: TTACCGCGGCTGCTGGCAC | 200 | 65 | Lee et al., 1996 |
| Lactobacilli primer | 16S rRNA<br>F: AGCAGTAGGGAATCTTCCA<br>R: CACCGCTACACATGGAG | 341 | 55 | Walter et al., 2001; Heiling et al., 2002 |
| *Pediococcus acidilactici* FUA 3140 specific primer 2* | Hypothetical protein<br>F: CAT CCA AAA TGG AAT TTA CAA AAC TCG ATT ATT<br>R: AGT CAG GTG ATC GGG TAA CAA GAA GAA A | 332 | 57 | This study, 2018 |
| *Lactobacillus sakei* FUA 3089 specific primer 4* | Hypothetical protein<br>F: ACG ATA TGT GTA GGA AGG GAA GTG GTT<br>R: TTG GTT TGA ATT AAA GAG GCT ATC AAC AAG | 320 | 57 | This study, 2018 |

Tm, melting temperature; F, forward; R, reverse. Strain-specific primers indicated by * were designed by Dr. Yuanyao Chen.

**Table 3-2.** Overall concentration of glucose (mg/dL) in the serum of dairy cows affected by uterine infections around parturition in 4 farms.

| | Weeks relative to parturition | | | | | |
|---|---|---|---|---|---|---|
| TRT | -3 weeks | *P* | +1 weeks | *P* | +4 weeks | *P* |
| 1[1] | 76.48 ± 5.75 | | 64.21 ± 4.50 | | 60.74 ± 3.08 | |
| 2[2] | 61.35 ± 5.74 | 0.01 | 64.98 ± 4.48 | 0.01 | 52.43 ± 3.06 | 0.01 |
| 3[3] | 60.72 ± 5.82 | | 51.30 ± 4.56 | | 48.63 ± 3.12 | |

[1]TRT1: Sterile saline 0.9% plus sterile skim milk.

[2] TRT2: Sterile saline solution 0.9%.

[3]TRT3: Probiotic cocktail (*Lactobacillus sakei* FUA3089, *Pediococcus acidilactici* FUA3138, and *Pediococcus acidilactici* FUA3140 with a cell count of $10^8$-$10^9$ cfu/dose) in sterile skim milk and dissolved with sterile saline 0.9%.

**Table 3-3.** Overall concentration of cholesterol (mg/dL) in the serum of dairy cows affected by uterine infections around parturition in 4 farms.

| | Weeks relative to parturition | | | | | |
|---|---|---|---|---|---|---|
| TRT | -3 weeks | *P* | +1 weeks | *P* | +4 weeks | *P* |
| 1[1] | 115.31 ± 7.74 | | 104.76 ± 6.67 | | 68.24 ± 6.62 | |
| 2[2] | 102.02 ± 7.71 | 0.01 | 96.14 ± 6.60 | 0.01 | 68.24 ± 6.62 | 0.01 |
| 3[3] | 64.96 ± 7.85 | | 68.24 ± 6.62 | | 68.24 ± 6.62 | |

[1]TRT1: Sterile saline 0.9% plus sterile skim milk.

[2] TRT2: Sterile saline solution 0.9%.

[3]TRT3: Probiotic cocktail (*Lactobacillus sakei* FUA3089, *Pediococcus acidilactici* FUA3138, and *Pediococcus acidilactici* FUA3140 with a cell count of $10^8$-$10^9$ cfu/dose) in sterile skim milk and dissolved with sterile saline 0.9%.

**Table 3-4.** Overall concentration of NEFA (μM) in the serum of dairy cows affected by uterine infections around parturition in 4 farms.

| | Weeks relative to parturition | | | | | |
|---|---|---|---|---|---|---|
| TRT | -3 weeks | *P* | +1 weeks | *P* | +4 weeks | *P* |
| 1[1] | 654.31 ± 182.99 | | 1048.74 ± 182.7 | | 852.64 ± 160.34 | |
| 2[2] | 757.58 ± 185.3 | 0.01 | 1225.38 ± 188.02 | 0.01 | 750.93 ± 165.35 | 0.01 |
| 3[3] | 757.58 ± 185.3 | | 1373.03 ± 210.58 | | 750.93 ± 165.35 | |

[1]TRT1: Sterile saline 0.9% plus sterile skim milk.
[2] TRT2: Sterile saline solution 0.9%.
[3]TRT3: Probiotic cocktail (*Lactobacillus sakei* FUA3089, *Pediococcus acidilactici* FUA3138, and *Pediococcus acidilactici* FUA3140 with a cell count of $10^8$-$10^9$ cfu/dose) in sterile skim milk and dissolved with sterile saline 0.9%.

**Table 3-5.** Overall concentration of lactate (μM) in the serum of dairy cows affected by uterine infections around parturition in 4 farms.

| | Weeks relative to parturition | | | | | |
|---|---|---|---|---|---|---|
| TRT | -3 weeks | *P* | +1 weeks | *P* | +4 weeks | *P* |
| 1[1] | 4931.3 ± 992.44 | | 4733.29 ± 428.27 | | 4284.76 ± 710.04 | |
| 2[2] | 7336.97 ± 1017.43 | 0.01 | 3983.15 ± 455.14 | 0.01 | 4730.03 ± 736.7 | 0.01 |
| 3[3] | 5551.04 ± 1125.45 | | 3840.24 ± 545.7 | | 5285.8 ± 844.56 | |

[1]TRT1: Sterile saline 0.9% plus sterile skim milk.

[2] TRT2: Sterile saline solution 0.9%.

[3]TRT3: Probiotic cocktail (*Lactobacillus sakei* FUA3089, *Pediococcus acidilactici* FUA3138, and *Pediococcus acidilactici* FUA3140 with a cell count of $10^8$-$10^9$ cfu/dose) in sterile skim milk and dissolved with sterile saline 0.9%.

**Table 3-6.** Overall concentration of BHBA (mM) in the serum of dairy cows affected by uterine infections around parturition in 4 farms.

| | Weeks relative to parturition | | | | | |
|---|---|---|---|---|---|---|
| TRT | -3 weeks | *P* | +1 weeks | *P* | +4 weeks | *P* |
| 1[1] | 0.68 ± 0.07 | | 0.86 ± 0.17 | | 0.82 ± 0.09 | |
| 2[2] | 0.73 ± 0.07 | 0.01 | 0.95 ± 0.17 | 0.01 | 0.83 ± 0.09 | 0.01 |
| 3[3] | 0.64 ± 0.08 | | 1.08 ± 0.20 | | 1.04 ± 0.11 | |

[1]TRT1: Sterile saline 0.9% plus sterile skim milk.

[2] TRT2: Sterile saline solution 0.9%.

[3]TRT3: Probiotic cocktail (*Lactobaillus sakei* FUA3089, *Pediococcus acidilactici* FUA3138, and *Pediococcus acidilactici* FUA3140 with a cell count of $10^8$-$10^9$ cfu/dose) in sterile skim milk and dissolved with sterile saline 0.9%.

**Table 3-7.** Overall vaginal mucus microbiota at phylum level.

| Phylum | Mean (%) | SEM |
|---|---|---|
| *Firmicutes* | 44.30 | 1.39 |
| *Bacteroidetes* | 19.75 | 0.93 |
| *Proteobacteria* | 10.54 | 1.33 |
| *Tenericutes* | 9.41 | 1.22 |
| *Fusobacteria* | 7.60 | 1.13 |
| *Actinobacteria* | 3.79 | 0.42 |
| *Verrucomicrobia* | 1.29 | 0.07 |
| *TM7* | 0.36 | 0.03 |
| *Lentisphaerae* | 0.34 | 0.02 |
| *Spirochaetes* | 0.34 | 0.02 |
| *Cyanobacteria* | 0.19 | 0.01 |
| *Planctomycetes* | 0.10 | 0.01 |
| *Chloroflexi* | 0.05 | 0.01 |
| *Chlamydiae* | 0.03 | 0.02 |
| *Acidobacteria* | 0.01 | 0.01 |
| *Unclassified* | 1.57 | 0.23 |
| *Euryarchaeota (Archaea)* | 0.21 | 0.06 |

* Data belongs to Farms A, B, and C.

**Table 3-8.** Overall vaginal mucus microbiota at genus level.

| Genus | Mean (%) | SEM |
|---|---|---|
| Unclassified_*Ruminococcaceae* | 17.02 | 0.82 |
| *Ureaplasma* | 8.02 | 1.22 |
| *Fusobacterium* | 5.56 | 0.79 |
| Unclassified_*Pasteurellaceae* | 4.86 | 1.05 |
| Unclassied_*Clostridiales* | 4.31 | 0.21 |
| *Porphyromonas* | 4.18 | 0.74 |
| Unclassified_*Bacteroidales* | 3.65 | 0.19 |
| *Bacteroides* | 3.37 | 0.57 |
| *Streptococcus* | 2.98 | 0.80 |
| Unclassified_*Lachnospiraceae* | 2.48 | 0.13 |
| *Helcococcus* | 1.91 | 0.30 |
| Unclassified_*Leptotrichiaceae* | 1.66 | 0.56 |
| *5-7N15* | 1.63 | 0.09 |
| *Corynebacterium* | 1.47 | 0.16 |
| Unclassified_*Peptostreptococcaceae* | 1.45 | 0.12 |
| *Clostridium* | 1.44 | 0.42 |
| Unclassified_*Rikenellaceae* | 1.36 | 0.07 |
| Unclassified_*RF16* | 1.20 | 0.07 |
| *Trueperella* | 1.14 | 0.37 |
| *Phascolarctobacterium* | 0.98 | 0.06 |

* Data belongs to Farms A, B, and C.

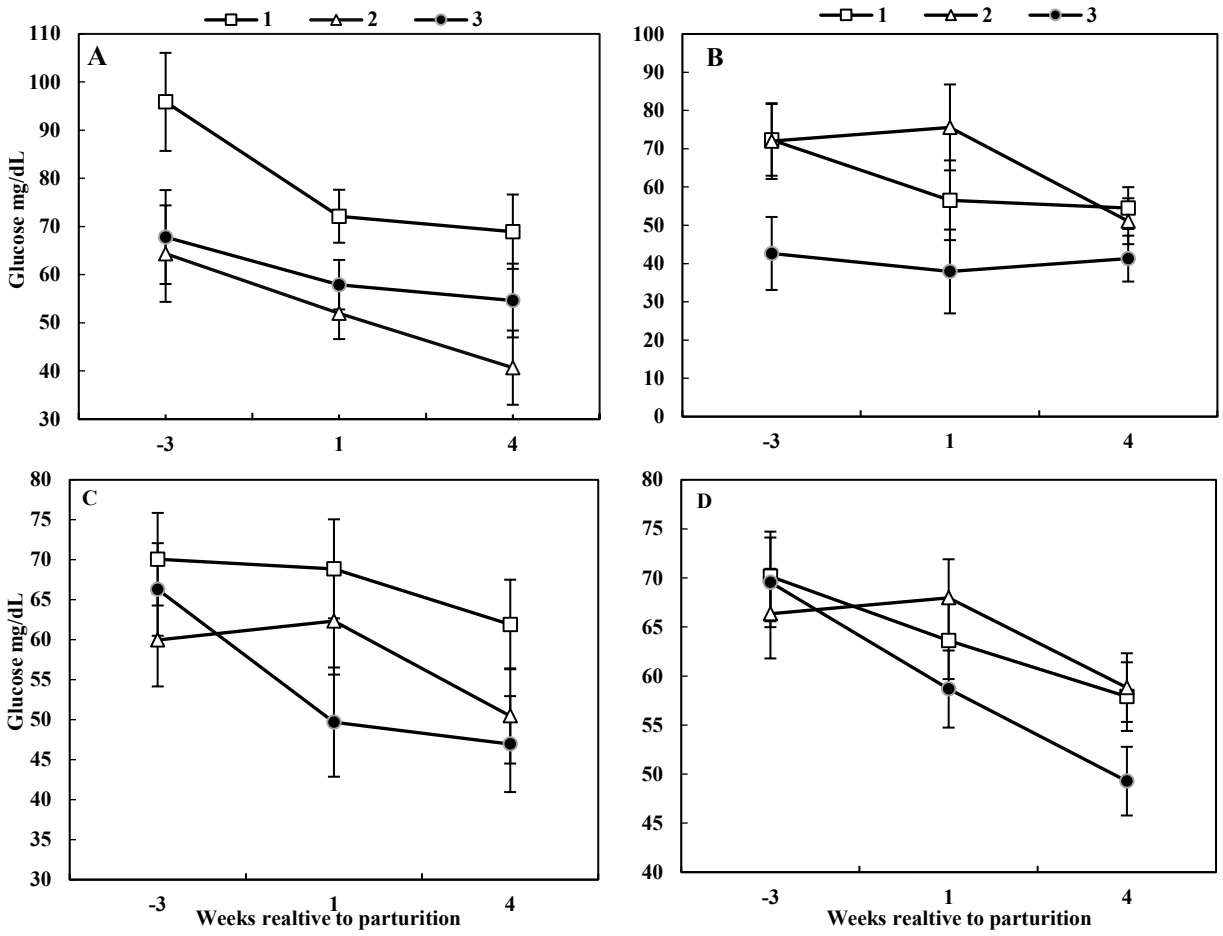


**Figure 3-1.** Least squares means and standard error of the mean (SEM) of lactate (mmol/L) concentrations in plasma blood for (A) multiparous and (B) primiparous cows that weren't diagnosed with uterine infections (C) multiparous and (D) primiparous cows that were diagnosed with uterine infections. Treatment 1 = sterile skim milk; Treatment 2 = sterile 0.9% saline solution; Treatment 3 = *Lactobacillus sakei* FUA3089, *Pediococcus acidilactici* FUA3138, and *Pediococcus acidilactici* FUA3140 dissolved in sterile skim milk and sterile 0.9% saline with cell count of $10^8$-$10^9$ cfu/dose).

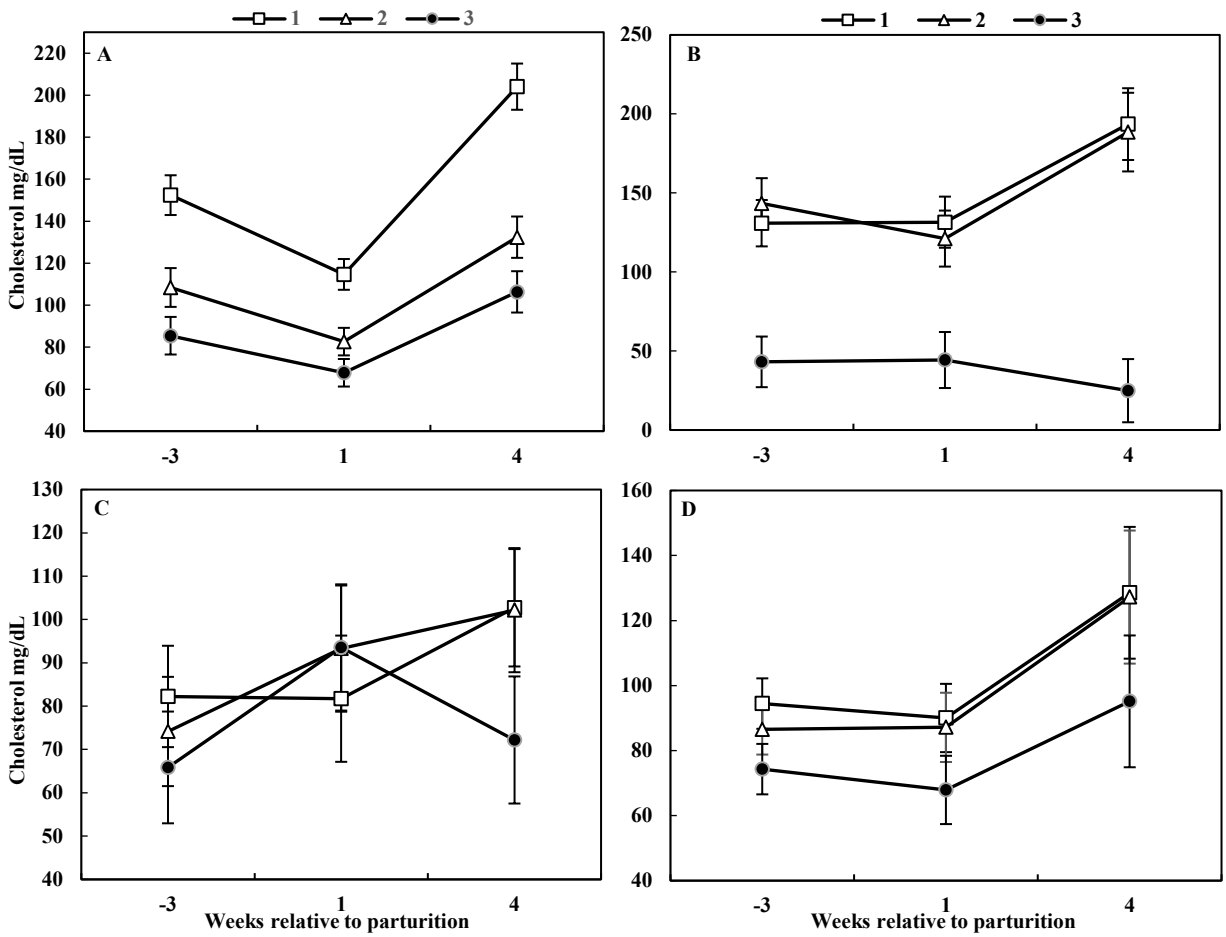


**Figure 3-2.** Least squares means and standard error of the mean (SEM) of lactate (mmol/L) concentrations in plasma blood for (A) multiparous and (B) primiparous cows that weren't diagnosed with uterine infections (C) multiparous and (D) primiparous cows that were diagnosed with uterine infections. Treatment 1 = sterile skim milk; Treatment 2 = sterile 0.9% saline solution; Treatment 3 = *Lactobacillus sakei* FUA3089, *Pediococcus acidilactici* FUA3138, and *Pediococcus acidilactici* FUA3140 dissolved in sterile skim milk and sterile 0.9% saline with cell count of $10^8$-$10^9$ cfu/dose).

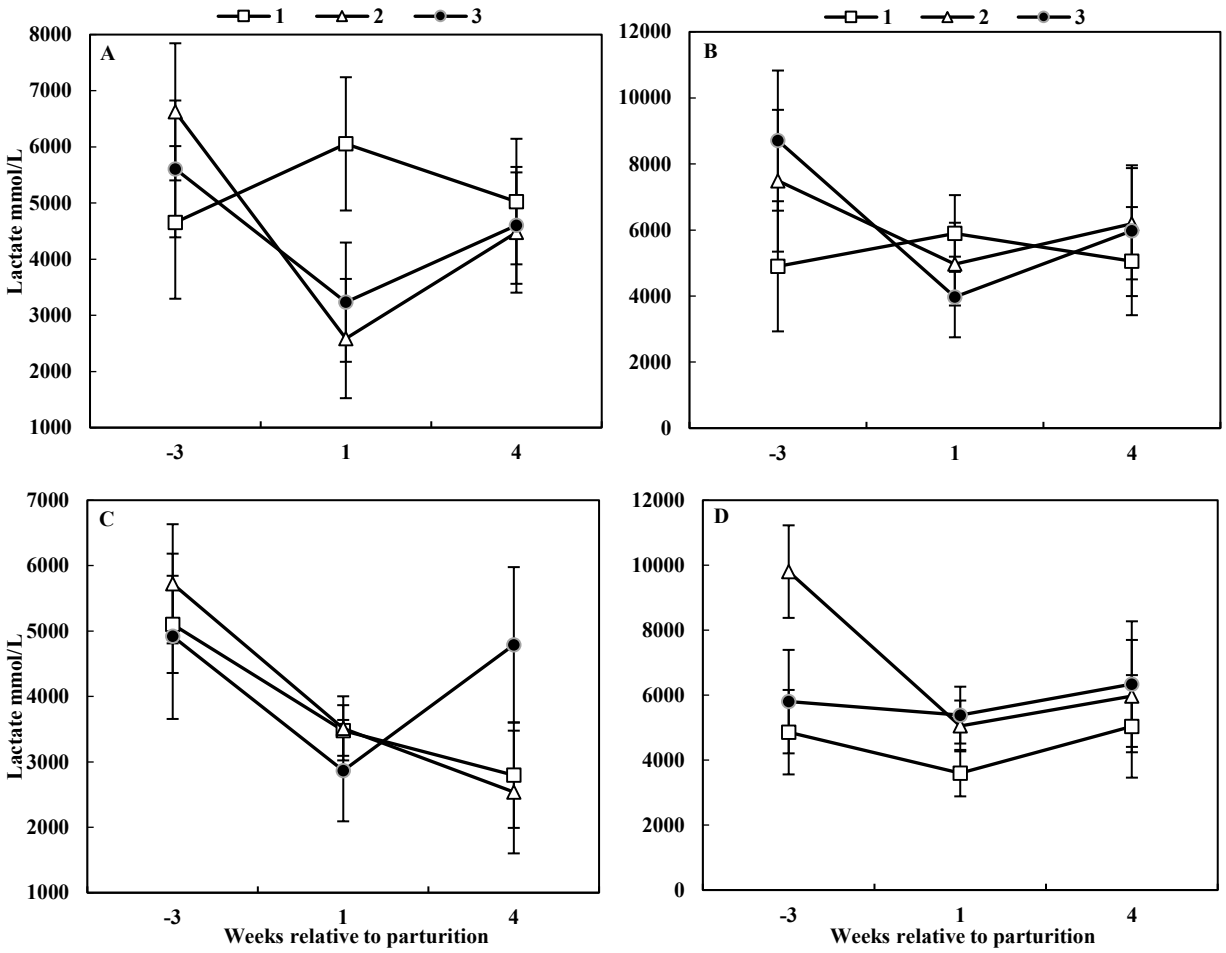


**Figure 3-3.** Least squares means and standard error of the mean (SEM) of lactate (mmol/L) concentrations in plasma blood for (A) multiparous and (B) primiparous cows that weren't diagnosed with uterine infections (C) multiparous and (D) primiparous cows that were diagnosed with uterine infections. Treatment 1 = sterile skim milk; Treatment 2 = sterile 0.9% saline solution; Treatment 3 = *Lactobacillus sakei* FUA3089, *Pediococcus acidilactici* FUA3138, and *Pediococcus acidilactici* FUA3140 dissolved in sterile skim milk and sterile 0.9% saline with cell count of $10^8$-$10^9$ cfu/dose).

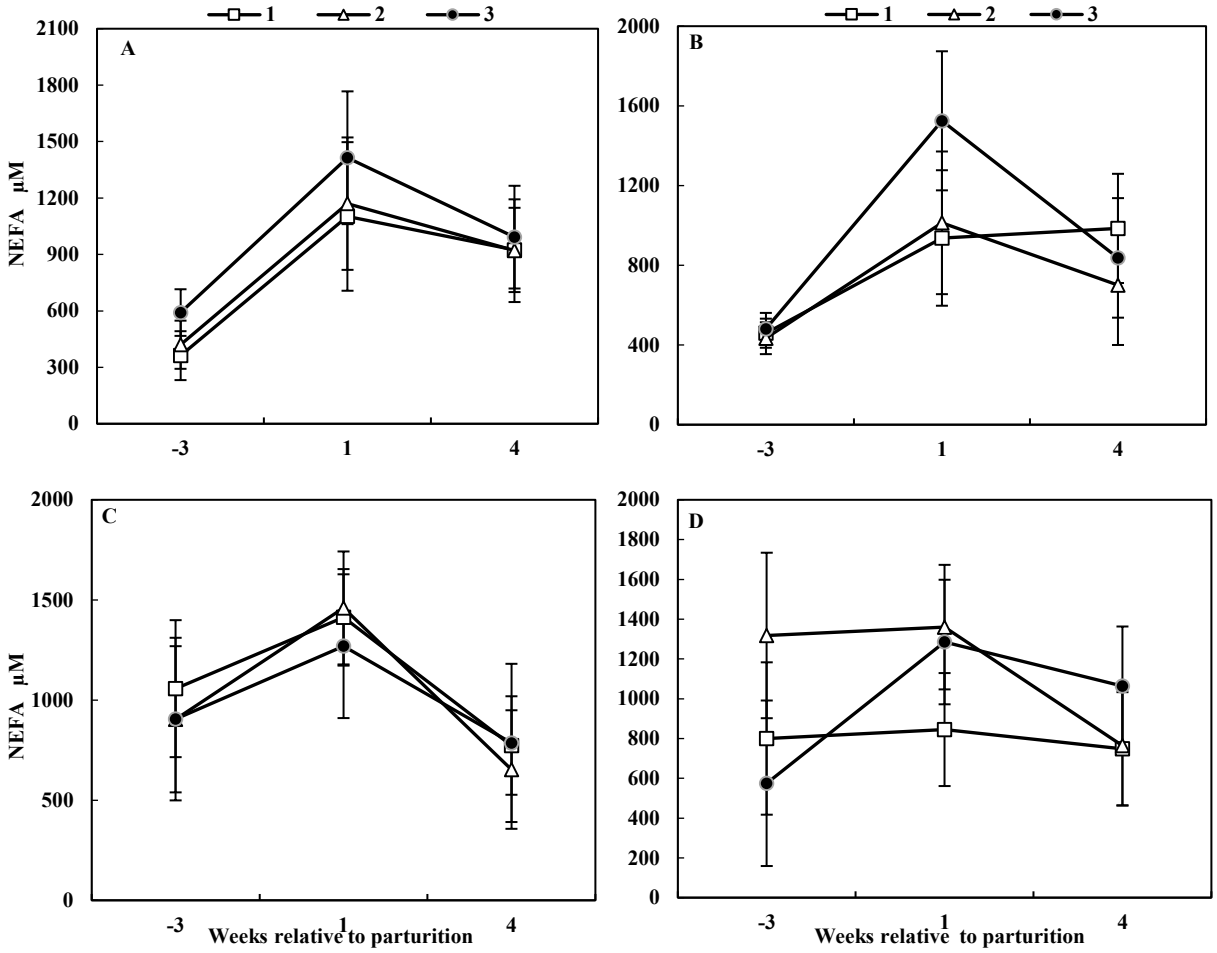


**Figure 3-4.** Least squares means and standard error of the mean (SEM) of lactate (mmol/L) concentrations in plasma blood for (A) multiparous and (B) primiparous cows that weren't diagnosed with uterine infections (C) multiparous and (D) primiparous cows that were diagnosed with uterine infections. Treatment 1 = sterile skim milk; Treatment 2 = sterile 0.9% saline solution; Treatment 3 = *Lactobacillus sakei* FUA3089, *Pediococcus acidilactici* FUA3138, and *Pediococcus acidilactici* FUA3140 dissolved in sterile skim milk and sterile 0.9% saline with cell count of $10^8$-$10^9$ cfu/dose).

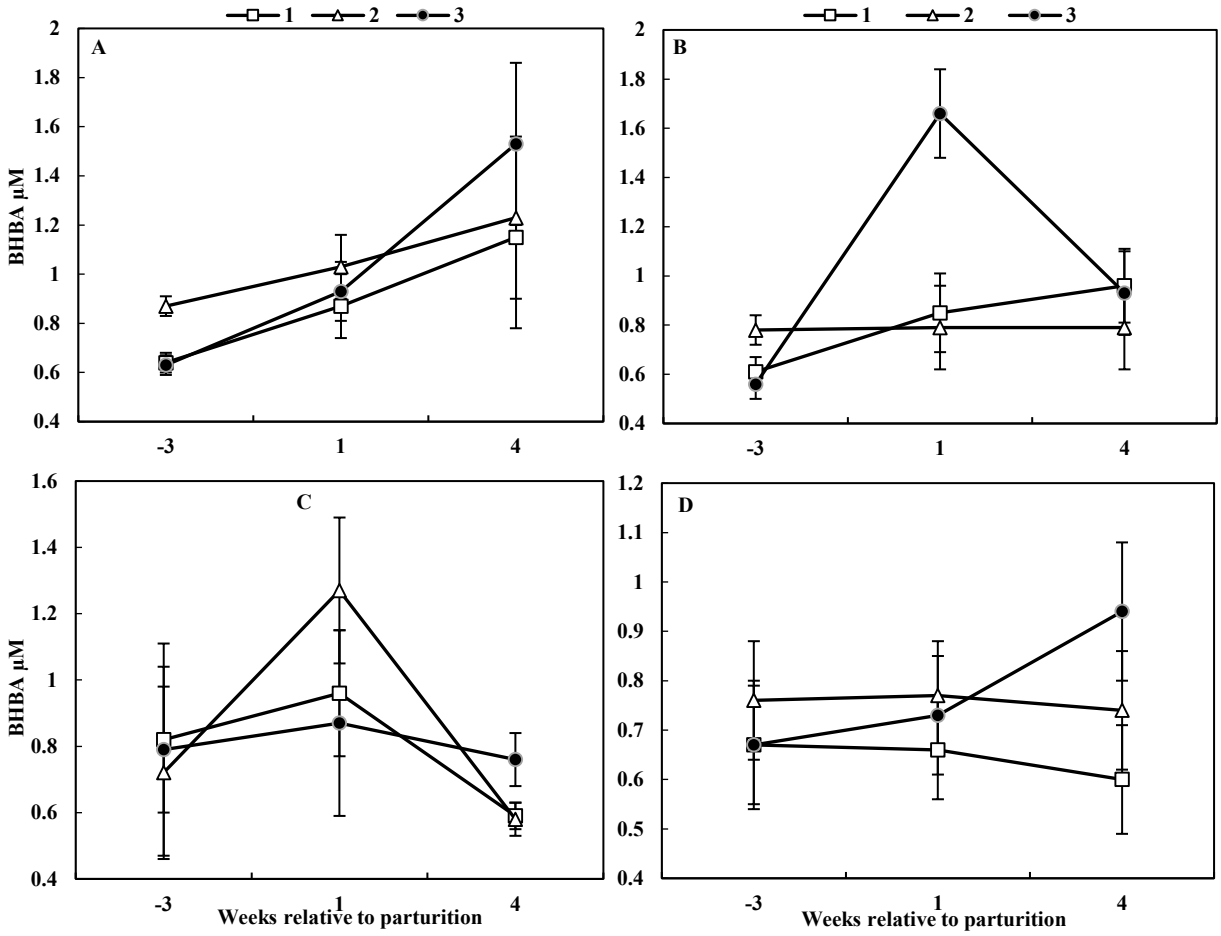


**Figure 3-5.** Least squares means and standard error of the mean (SEM) of lactate (mmol/L) concentrations in plasma blood for (A) multiparous and (B) primiparous cows that weren't diagnosed with uterine infections (C) multiparous and (D) primiparous cows that were diagnosed with uterine infections. Treatment 1 = sterile skim milk; Treatment 2 = sterile 0.9% saline solution; Treatment 3 = *Lactobacillus sakei* FUA3089, *Pediococcus acidilactici* FUA3138, and *Pediococcus acidilactici* FUA3140 dissolved in sterile skim milk and sterile 0.9% saline with cell count of $10^8$-$10^9$ cfu/dose).

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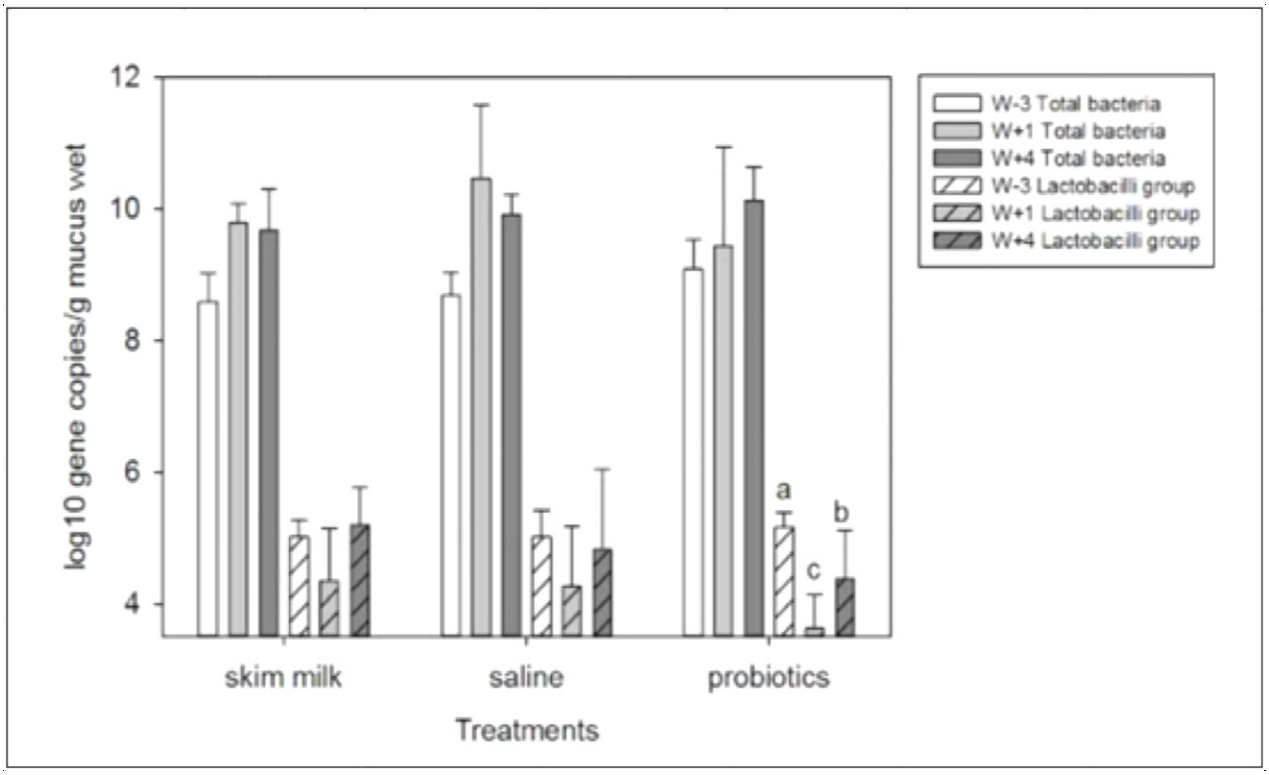


**Figure 3-6.** Differences in log gene copies of periparturient vaginal bacteria with 3 treatments at Farm A. W-3, 3 wks prepartum; W+1, 1 wks postpartum; W+4, 4 wks postpartum. Vaginal mucus was sampled from 18 dairy cows infused with skim milk (TRT1) (N = 6), saline solution 0.9% (TRT2) (N = 5), and probiotics (TRT3) (N = 7) at -3 wks, +1 wks, and +4wks. Total bacteria, lactobacilli, *Pediococcus acidilactici* and *Lactobacillus sakei* strains were quantified by qPCR in duplicates. Numbers of *P. acidilactici* and *L. sakei* were consistently undetermined regardless of probiotics treatments (data not shown). Statistical significance ($P < 0.05$) was indicated by letters above bars. Among 3 treatments, no significance was observed at each time point. In the probiotic group, lactobacilli showed significant decrease from wk -3 to wk +1 and restoration from wk +1 to wk +4.

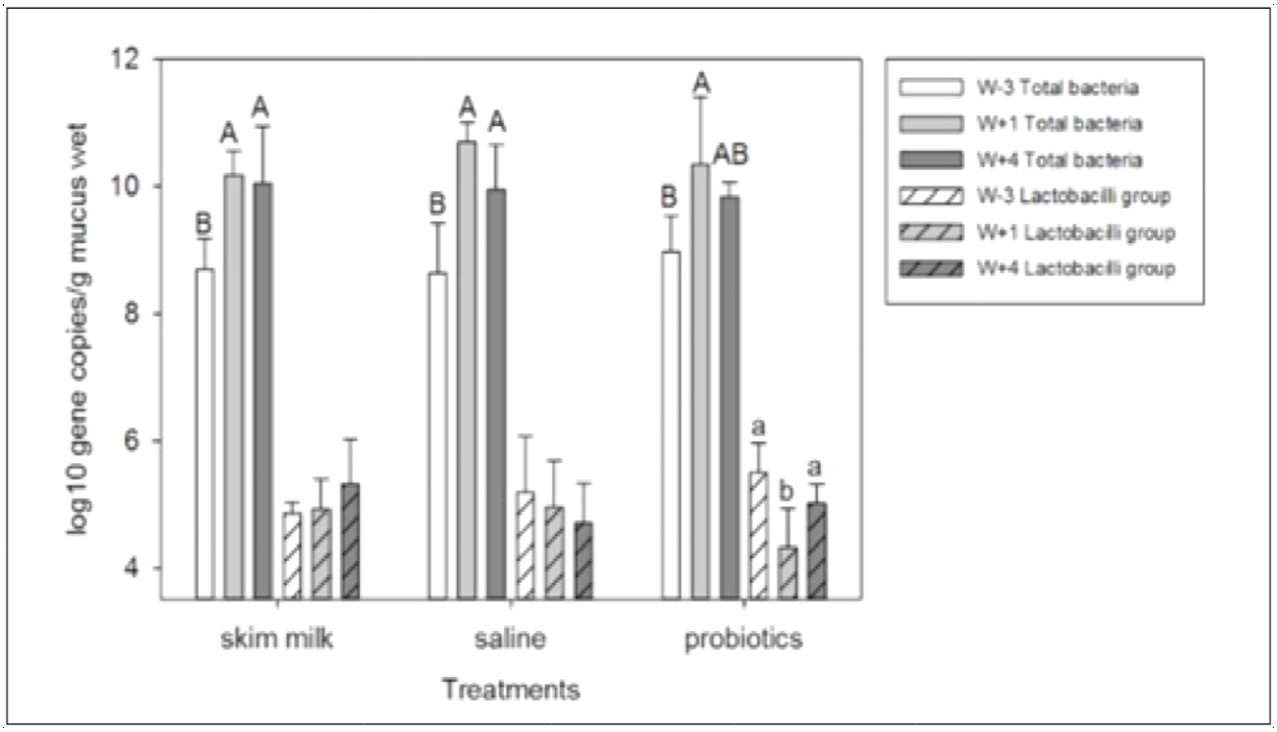


**Figure 3-7.** Differences in log gene copies of periparturient vaginal bacteria with 3 treatments at Farm C. W-3, 3 wk prepartum; W+1, 1 wk postpartum; W+4, 4 wk postpartum. Vaginal mucus was sampled from 17 dairy cows infused with sterile skim milk and sterile 0.9% saline (TRT1) (N = 6), sterile 0.9% saline solution (TRT2) (N = 6), and probiotics (TRT3) dissolved in sterile skim milk and sterile 0.9% saline (n = 5) at -3 wks, +1 wk, and +4wks. Total bacteria, lactobacilli, *Pediococcus acidilactici* and *Lactobacillus sakei* strains were quantified by qPCR in duplicates. Numbers of *P. acidilactici* and *L. sakei* were consistently undetermined regardless of probiotics treatments (data not shown). Statistical significance ($P < 0.05$) was indicated by letters above bars. Among 3 treatments, no significance was observed at each time point. In the probiotic group, lactobacilli showed significant decrease from wk -3 to wk +1 and restoration from wk +1 to wk +4.

# Chapter 4 Overall discussion.

## 4.1 Intravaginal administration of probiotics around parturition had beneficial effects on productive performance and overall health status of transition dairy cows in four dairy farms in Alberta

### *4.1.1 Intravaginal probiotics and health status of the treated cows*

The main hypothesis of this study was that intravaginal infusion of four doses of LAB probiotics (once per week) around parturition (on weeks -3 and -2 before the expected day of calving as well as at +3 and +4 wks after calving) could improve reproductive health and the overall health status of dairy cows, expedite uterine involution, and improve milk composition and yields of postpartum dairy cows. Indeed, results of this study showed that intravaginal infusion of LAB probiotics, previously isolated from the vaginal mucus of healthy cows (Wang et al., 2013; Ametaj et al., 2014), decreased uterine infections in all dairy farms included in this study. Contrary to previous results from our lab, intravaginal LAB did not have an effect on uterine involution or milk composition. Previously Deng et al. (2014) showed that intravaginal probiotics at 2 and 3 doses prior to or around calving expedited uterine involution of the treated cows. Additionally, the same authors demonstrated that intravaginal probiotics increased the overall concentration of milk IgG and lowered milk serum amyloid A. The reason for this discrepancy is not understood at present. However, the present results confirm that probiotics isolated from vaginal mucus of healthy dairy cows are capable of helping the host to lower the incidence of uterine infections in susceptible cows.

Another main objective of the present study was to test whether the same probiotic strains used under strict University of Alberta research dairy farm would work under both conventional

and organic dairy farming. Data showed that this was the case. Intravaginal probiotics lowered the incidence of uterine infections in both Holsten conventional farms and one organically managed Jersey farm included in the study. These results confirmed our hypothesis that although LAB isolated from vaginal mucus of Holstein dairy cows they conferred health benefits also to a Jersey herd suggesting a global effect of probiotics independently of the host's genotype.

Another interesting finding was that intravaginal probiotics in this study conferred health benefits with regards to lowered incidence of subclinical mastitis (SCM) and milk fever (See Chapter 2). Mastitis is one of the three most important reasons, together with uterine infections and lameness, for culling of dairy cows from dairy herds. Lowering the incidence of SCM was an unexpected health benefit of intravaginal probiotics. The mechanistic effects of this benefit need to be studied further. However, our data suggest that by improving the overall health and reducing the overall inflammation and potential endotoxin translocation in the host, might have improved immune responses. Additionally, it suggests the importance of uterine health on the health of the mammary gland. Another reason for lower incidence of SCM in cows treated with probiotics is that those cows had less purulent vaginal discharges. This has lowered the contact of the udder to bacteria present in purulent vaginal discharges of cow's bedding indirectly decreasing the rates of uterine infections in postpartum dairy cows.

Lowering the incidence of milk fever also was unexpected. Milk fever has been considered as a decrease in blood ionized calcium. Recent publications indicate that hypocalcemia might not be a calcium deficiency but simply a host response to endotoxemia where lowering of calcium in the blood might help the host to quickly eliminate endotoxins from systemic circulation (Eckel and Ametaj, 2016). Uterine infections might contribute substantial amounts of translocated endotoxin and lowering the incidence of uterine infections by probiotics might have eased host

responses related to endotoxemia and indirectly has established normal calcemia and prevented milk fever.

### *4.1.2 Intravaginal probiotics as an alternative to treat uterine infections in organic dairy farms*

The most intriguing finding was that the effect of intravaginal probiotics was more pronounced in the organically managed Jersey herd versus the three Holstein herds in the study. Indeed, the decrease in uterine infections in this herd reached 50% compared to 19% (Farm A), 36% (Farm C), and 21% (Farm D). We are not sure whether this effect is random, or it has to do with the management system. There are no reports on the microbiota composition of the vaginal mucus of Jersey cows. This warrant further research in the future. However, reports on microbiota composition in Holstein cows conducted by our lab showed that the abundance of *Lactobacillus* spp. in the vaginal mucus of those cows are at a very low level (Wang et al., 2013). Therefore, increasing the numbers of *Lactobacillus* spp. in the vaginal tract by infusion around calving might help the host in preventing pathogenic bacteria to establish their niches in the vagina and cause infection of the uterus postpartum.

Organic dairy farms are not allowed to treat dairy cows with antimicrobial agents. Treating cows with antimicrobials results in elimination of those cows from the organic chain and withdrawal of milk during the remaining milking cycle. Because of this, most cows in organic farms either are left to heal themselves, which affects their welfare and wellbeing, or simply are treated systemically with anti-inflammatory compounds. Given that intravaginal probiotics can lower the incidence of uterine infections drastically (i.e., 50%), this novel product can be used by organic dairy farms in the near future as a preventive treatment against uterine infections. To our best knowledge, this is first time that intravaginally infused probiotics have been tested in an

organically managed dairy farm in Canada and beyond. Our data suggests that intravaginal probiotics may be a preferred alternative to decrease the incidence rates of uterine infections in organic dairy management systems.

### *4.1.3 Milk yield and metabolic status*

Overall, cows treated with probiotics (TRT3) increased 4.6 liters and 3.2 liters per day compared to TRT1 and TRT2, respectively. Milk yields were recorded for the first 50 DIM for all cows, a parameter used to test models when predicting productive performance in dairy cows (Olori et al., 1999). Significant differences were tested using odds ratio analysis, comparing TRT3 vs TRT1 and vs TRT2 for all 4 farms to compare milk yields. Interestingly, cows infused with probiotics (TRT3) had higher odds of producing more milk than if infused with TRT1 (OR=1.18) and TRT2 (OR= 1.20) after calving. It should be noted that results of this study are in agreement with the two other previous studies conducted by our lab that showed intravaginal probiotics were associated with 2-3 L/d higher milk yields in the treated cows during the first 56 d after parturition (Ametaj et al., 2014; Deng et al., 2014). One of the potential mechanisms of how probiotics increased milk production in this and previous experiments conducted by our lab (Ametaj et al., 2010, Wang et al., 2013; Deng et al., 2014) could be related to a decrease of uterine infections and as a consequence prevention of translocation of endotoxins into the blood circulation (Ametaj et al., 2010; Eckel and Ametaj, 2016), which has been shown to inhibit the release of prolactin, a hormone that stimulates milk production (Smith and Wagner, 1984). Additionally, higher milk production could be associated to greater utilization of glucose (See Chapter 3), by the udder, for lactose synthesis. Concentration of glucose in the serum of probiotic-treated cows was lower

compared to the other control groups suggesting that the udder could have extracted more glucose for lactose synthesis (Lucy et al., 2014; Eger et al., 2015).

If the advantage that the probiotic group (TRT3) had over TRT1 and TRT2 with regards to milk yield (3.9 liters per day) is extrapolated to a dairy farm of 100 cows there would be an increase of 390 L/d, 11,700 L/mo, or 118,950 L per 305 DIM. A cow treated intravaginally with probiotics could produce around 1,189 L more per lactation (305 DIM). However, this warrants further investigation in more dairy farms due to the variety of management systems.

Another important finding of this study was that cows infused with probiotics (TRT3) exhibited lower concentrations of cholesterol in the serum compared to TRT1 and TRT2. It has been reported that elevated amounts of cholesterol in the serum can be accumulated in immune cells provoking immune dysfunction, associated with promoting inflammatory response including increased Toll-like receptor activity augmenting production of cytokines and chemokines (Tall and Yvan-Charvet, 2015). However, there are conflicting results on the role of cholesterol levels in the incidence of uterine infections and other periparturient diseases in postpartum dairy cows. This warrants further investigation on how probiotics infused in transition dairy cows affects cholesterol levels in serum.

### 4.2 Future implications

The idea of administrating probiotics to decrease uterine infection rates in dairy farms sound promising. Administration of probiotics by one or two dairy farm personnel is totally doable, a suggestion based by our experience of infusing 526 cows in 4 dairy farms. Accounting different management systems in various dairy farms and different styles of barn structures, we calculated that 1-2 persons are able to administer probiotics to 10 cows in 20 minutes. In fact, it takes more

time to treat a cow with antibiotics by means of a uterine lavage. Additionally, treating uterine infections the traditional way with antimicrobials (Discussed in Chapter 1) have shown to be an economic and health disadvantage (Lewis et al., 1997b; Azawi et al., 2008).

An issue needed to be improved for this technology might be the consistency of the probiotic mixture. Under very cold winter conditions the probiotic solution tends to freeze due to the fact that it is reconstituted in saline solution (0.9%), especially in farms that keep their close-up cows outside. Suggesting that the administration of the probiotic must be done inside a barn where temperatures don't reach freezing points or that the probiotic could be mixed in a gel-like material with its own infusing device making infusions even faster.

It would be of interest to isolate and identify vaginal microbiota composition of Jersey breeds and compare those to microbiota from Holstein breeds. Additionally, it would be of importance to economically identify the exact cost of using probiotics and compare it with the traditional techniques of treating uterine infections in postpartum dairy cows.

### 4.3 Overall conclusions

Results from this study demonstrated that probiotics infused intravaginally around calving improved the overall health status decreasing the incidence rates of uterine infections, mastitis, and milk fever in postpartum dairy cows. Intravaginal probiotics were associated with alterations in glucose and cholesterol metabolism which indirectly pinpoints the metabolic benefits of using intravaginal probiotics as a preventive treatment against metritis. Administration of probiotics around calving was associated with increased milk yields. Moreover, probiotics isolated in Holstein dairy cows lowered the incidence of uterine infection in Jersey cows. Finally, the incidence rate of uterine infection in a Jersey dairy farm was lowered by 50% by infusing

intravaginal probiotics. With small adjustments this technology could be used efficiently by dairy producers as a preventive treatment to lower the incidence of uterine infections in periparturient dairy cows.

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# Appendices

## Appendix A: Other serum parameter data

**Table A-1.** Concentration of glucose (mg/dL) in the serum in dairy cows affected by uterine infections around parturition in 4 farms.

| | -3 weeks | | | +1 weeks | | | +4 weeks | | |
|---|---|---|---|---|---|---|---|---|---|
| TRT | No UI[4] | UI[4] | *P* | No UI[4] | UI[4] | *P* | No UI[4] | UI[4] | *P* |
| 1[1] | 58.6 ± 5.45 | 61.9 ± 5.40 | | 58.1 ± 4.96 | 61.9 ± 4.92 | | 58.0 ± 3.64 | 62.1 ± 3.60 | |
| 2[2] | 64.8 ± 5.40 | 62.1 ± 5.40 | 0.01 | 64.5 ± 4.92 | 62.4 ± 4.92 | 0.02 | 64.0 ± 3.60 | 62.4 ± 3.60 | <0.01 |
| 3[3] | 51.9 ± 5.40 | 61.7 ± 5.40 | | 49.7± 4.92 | 61.8 ± 4.92 | | 48.6 ± 3.60 | 61.8 ± 3.60 | |

[1]TRT1: Sterile saline 0.9% plus sterile skim milk.
[2] TRT2: Sterile saline solution 0.9%.
[3]TRT3: Probiotic cocktail (*Lactobacillus sakei* FUA3089, *Pediococcus acidilactici* FUA3138, and *Pediococcus acidilactici* FUA3140 with a cell count of $10^8$-$10^9$ cfu/dose) in sterile skim milk and dissolved with sterile saline 0.9%.
[4]UI: uterine infections

**Table A-2**. Concentration of cholesterol (mg/dL) in the serum in dairy cows affected by uterine infections around parturition in 4 farms.

| | -3 weeks | | | +1 weeks | | | +4 weeks | | |
|---|---|---|---|---|---|---|---|---|---|
| TRT | No UI[4] | UI[4] | *P* | No UI[4] | UI[4] | *P* | No UI[4] | UI[4] | *P* |
| 1[1] | 118.9 ± 8.23 | 91.2 ± 8.12 | | 117.8 ± 9.36 | 90.7 ± 9.23 | | 116.6 ± 12.54 | 89.8 ± 12.37 | |
| 2[2] | 148.4 ± 8.12 | 92.9 ± 8.12 | 0.01 | 146.6± 9.23 | 92.5 ± 9.23 | <0.01 | 145.3 ± 12.37 | 92.7 ± 12.37 | <0.01 |
| 3[3] | 69.7± 8.12 | 89.3 ± 8.12 | | 67.1± 9.23 | 88.8 ± 9.23 | | 68.3± 12.37 | 88.8 ± 12.37 | |

[1]TRT1: Sterile saline 0.9% plus sterile skim milk.

[2] TRT2: Sterile saline solution 0.9%.

[3]TRT3: Probiotic cocktail (*Lactobacillus sakei* FUA3089, *Pediococcus acidilactici* FUA3138, and *Pediococcus acidilactici* FUA3140 with a cell count of $10^8$-$10^9$ cfu/dose) in sterile skim milk and dissolved with sterile saline 0.9%.

[4]UI: uterine infections

**Table A-3.** Concentration of NEFA (μM) in the serum in dairy cows affected by uterine infections around parturition in 4 farms.

| | -3 weeks | | | +1 weeks | | | +4 weeks | | |
|---|---|---|---|---|---|---|---|---|---|
| TRT | No UI[4] | UI[4] | *P* | No UI[4] | UI[4] | *P* | No UI[4] | UI[4] | *P* |
| 1[1] | 871 ± 163.21 | 942 ± 137.94 | | 870 ± 198.77 | 947 ± 67.99 | | 856 ± 174.94 | 951 ± 147.85 | |
| 2[2] | 764 ± 163.21 | 892 ± 163.21 | 0.45 | 771 ± 198.77 | 923 ± 198.77 | 0.15 | 766 ± 174.94 | 923 ± 174.94 | 0.32 |
| 3[3] | 989 ± 163.21 | 1039 ± 210.71 | | 990 ± 198.77 | 1094 ± 256.61 | | 1026 ± 174.94 | 1045 ± 225.85 | |

[1]TRT1: Sterile saline 0.9% plus sterile skim milk.

[2] TRT2: Sterile saline solution 0.9%.

[3]TRT3: Probiotic cocktail (*Lactobacillus sakei* FUA3089, *Pediococcus acidilactici* FUA3138, and *Pediococcus acidilactici* FUA3140 with a cell count of $10^8$-$10^9$ cfu/dose) in sterile skim milk and dissolved with sterile saline 0.9%.

[4]UI: uterine infections

**Table A-4.** Concentration of lactate (μM) in the serum in dairy cows affected by uterine infections around parturition in 4 farms.

| | -3 weeks | | | +1 weeks | | | +4 weeks | | |
|---|---|---|---|---|---|---|---|---|---|
| TRT | No UI[4] | UI[4] | *P* | No UI[4] | UI[4] | *P* | No UI[4] | UI[4] | *P* |
| 1[1] | 5235 ± 1045 | 4793 ± 882 | | 5230 ± 636 | 4820 ± 537 | | 5196 ± 856 | 4821 ± 723 | |
| 2[2] | 5373 ± 1041 | 4777 ± 1041 | 0.05 | 5294 ± 634 | 4729 ± 634 | 0.56 | 5384 ± 853 | 4726 ± 853 | 0.37 |
| 3[3] | 5215 ± 1041 | 4638 ± 1352 | | 4996 ± 634 | 4688 ± 823 | | 5250 ± 853 | 4856 ± 1108 | |

[1]TRT1: Sterile saline 0.9% plus sterile skim milk.
[2] TRT2: Sterile saline solution 0.9%.
[3]TRT3: Probiotic cocktail (*Lactobacillus sakei* FUA3089, *Pediococcus acidilactici* FUA3138, and *Pediococcus acidilactici* FUA3140 with a cell count of $10^8$-$10^9$ cfu/dose) in sterile skim milk and dissolved with sterile saline 0.9%.
[4]UI: uterine infections

**Table A-5.** Concentration of BHBA (mM) in the serum in dairy cows affected by uterine infections around parturition in 4 farms.

| | -3 weeks | | | +1 weeks | | | +4 weeks | | |
|---|---|---|---|---|---|---|---|---|---|
| TRT | No UI[4] | UI[4] | *P* | No UI[4] | UI[4] | *P* | No UI[4] | UI[4] | *P* |
| 1[1] | 0.63± 0.04 | 0.73± 0.14 | | 0.89 ± 0.29 | 0.81 ± 0.13 | | 1.10 ± 0.19 | 0.59 ± 0.05 | |
| 2[2] | 0.82 ± 0.03 | 0.67 ± 0.15 | 0.42 | 0.98 ± 0.29 | 0.95 ± 0.13 | 0.46 | 1.01 ± 0.18 | 0.66 ± 0.06 | 0.71 |
| 3[3] | 0.60 ± 0.03 | 0.67 ± 0.18 | | 1.30± 0.29 | 0.78 ± 0.16 | | 1.20 ± 0.18 | 0.85 ± 0.09 | |

[1]TRT1: Sterile saline 0.9% plus sterile skim milk.
[2] TRT2: Sterile saline solution 0.9%.
[3]TRT3: Probiotic cocktail (*Lactobacillus sakei* FUA3089, *Pediococcus acidilactici* FUA3138, and *Pediococcus acidilactici* FUA3140 with a cell count of $10^8$-$10^9$ cfu/dose) in sterile skim milk and dissolved with sterile saline 0.9%.
[4]UI: uterine infections