Application of intravaginal lactic acid bacteria to lower uterine infections and improve reproductive performance of postpartum dairy cows

by

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Abstract

Transition dairy cows are susceptible to uterine infections due to the compromised immunity around calving and substantial bacterial contamination in the uterus immediately after calving. Cows with uterine infections are at higher odds of developing other periparturient diseases, resulting in lower milk production and impaired fertility. Infertility related to uterine infections has become the main reason for a cow to be culled from the herd. So far, there have been no effective approaches to treat uterine infections. In this study we tested whether intravaginal treatment of transition dairy cows with a mixture of lactic acid bacteria (LAB) can lower the incidence rates of uterine infections, improve the productivity of reproduction and milk yield.

The LAB preparation was composed of *Lactobacillus sakei* and two strains of *Pediococcus acidilactici*, isolated from vaginal mucus of healthy pregnant dairy cows and infused into the vaginal tract of 100 dairy cows with the cell count of 10^8 - 10^9 cfu/dose before or around calving. Results showed that intravaginal infusion of LAB modulated the bacterial composition in the vaginal tract, increased the production of mucosal sIgA, and therefore lowered the incidence rates of metritis and total uterine infections. It also lessened systemic inflammation indicated by the decreased concentration of lipopolysaccharide binding protein and serum amyloid A in the serum. Furthermore, LAB treatment modulated the production of hormones related to reproduction and expedited uterine involution of transitional dairy cows. Cows treated

with LAB before calving had a shorter number of days open; whereas cows treated with LAB around calving accelerated ovarian resumption of cyclicity. In addition, application of LAB intravaginally exerted changes to metabolic status, such as a decrease in the concentration of non-esterified fatty acids (NEFA), and modified milk composition, such as the content of protein and immunoglobulin (Ig) G. More importantly, cows treated with LAB exhibited greater milk production and higher feed efficiency.

In conclusion, application of probiotic supplements intravaginally holds promise to lower the incidence of uterine infections, expedite uterine involution, and improve reproductive and productive performance of postpartum dairy cows.

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. John F. Odhiambo contributed to application of treatments, collection of samples, ultrasound imaging, and evaluation of clinical diseases for part of the experiment. Dr. Umar Farooq assisted with administration of treatments and collection of samples and data for part of the experiment. Tran Lam was involved in sample collection during the whole experimental period. 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#120) entitled ‘Advancement of an intravaginal probiotic treatment into a novel technology for prevention of uterine infections in dairy cows’.

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List of abbreviations

APP	Acute phase proteins
BHBA	β-hydroxy butyric acid
BSC	Body condition score
CD	Cluster differentiation
CL	Corpus luteum
CSA	Cross-sectional areas
CVs	Coefficients of variation
DIM	Days in milk
EHEC	Enterohemorrhagic *E. coli*
EIA	Enzyme immuno-assay
EPEC	Enteropathogenic *E. coli*
G-	Gram negative
G+	Gram positive
GIT	Gastrointestinal tract
GnRH	Gonadotropin releasing hormone
H_2O_2	Hydrogen peroxide
Hp	Haptoglobin
HRP	Horseradish peroxidase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin

LAB	Lactic acid bacteria
LBP	Lipopolysaccharide-binding protein
LH	Luteinizing hormone
LPS	Lipopolysaccharide
MAA	Milk amyloid A
MHC	Major histocompatability complex
MUN	Milk urea nitrogen
NEB	Negative energy balance
NEFA	Non-esterified fatty acid
NF_kB	Nuclear factor kappa B
OD	Optical density
PC	Principle component
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGE_2	Prostaglandin E_2
PMN	Polymorphonuclear neutrophils
r-bST	Recombinant bovine somatotropin
SAA	Serum amyloid A
SCC	Somatic cell count
SCFA	Short chain fatty acid
sIgA	Secretory immunoglobulin A
TAI	Timed artificial insemination
Th	T helper

TMB	3,3', 5,5'-tetramethylbenzidine
TNF	Tumor necrosis factor

Chapter 1 Literature review

1.1 Uterine infections in dairy cows

1.1.1 Importance of uterine infections to dairy industry

Uterine infections affect almost half of the dairy herd (Sheldon et al., 2009a) due to compromised immunity around calving and substantial bacterial contamination in the uterus immediately after calving (Sheldon et al., 2009a; LeBlanc et al., 2011). These infections can easily develop into uterine diseases such as metritis and endometritis, which result in impaired fertility or even infertility by delaying uterine involution and ovulation, or prolonging the luteal phase if ovulation occurs (Huszenicza et al., 1999; Mateus et al., 2002; Sheldon et al., 2009a). Cows with uterine infections have lower conception rate, need more services per conception, and have longer days open, and therefore are culled from the herd earlier (Kasimanickam et al., 2004; Sheldon et al., 2009a). According to recent statistics the culling rate of dairy cows in Canada during 2013 reached 41.7% (CanWest DHI and Valacta, 2013). Among all the culling reasons, reproductive failure was rated as the number one, accounting for 15.4%, which caused a loss of more than 57,600 cows worth $144 million dollars (CanWest DHI and Valacta, 2013). In order to better understand the reasons for the high infertility rates in transition dairy cows we will discuss in more details the physiology and pathology of the uterus.

1.1.2 Reproductive physiology of transition dairy cows

The fetus grows very fast during the last trimester and reaches the maximum capacity of the uterus. Growth being confined, the fetal hypothalamus start secreting corticotropin releasing hormone (CRH), which stimulates the pituitary gland to secrete adrenal corticotropin hormone (ACTH), and further stimulates corticoid hormone secretion such as cortisol by the adrenal gland. Cortisol can switch endocrine balance from progesterone into estradiol synthesis by inducing the enzyme 17α-hydroxylase and the production of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) due to the increase of oxytocin (Kindahl et al., 2004). While ovary-produced relaxin has ripened the cervix and pelvic ligaments, both estradiol and $PGF_{2\alpha}$ trigger uterine contractions, pushing the fetus towards the lower reproductive tract. When the cervix senses the pressure from the conceptus, oxytocin is released by the posterior pituitary via neuronal reflex, which reinforces the contractions of the uterus (Kindahl et al., 2004). Therefore, the expulsion of the fetus is achieved by increasing estradiol, $PGF_{2\alpha}$, and oxytocin as well as a decrease in progesterone. Hormonal changes during the periparturient period of dairy cows are illustrated in Figure 1-1.

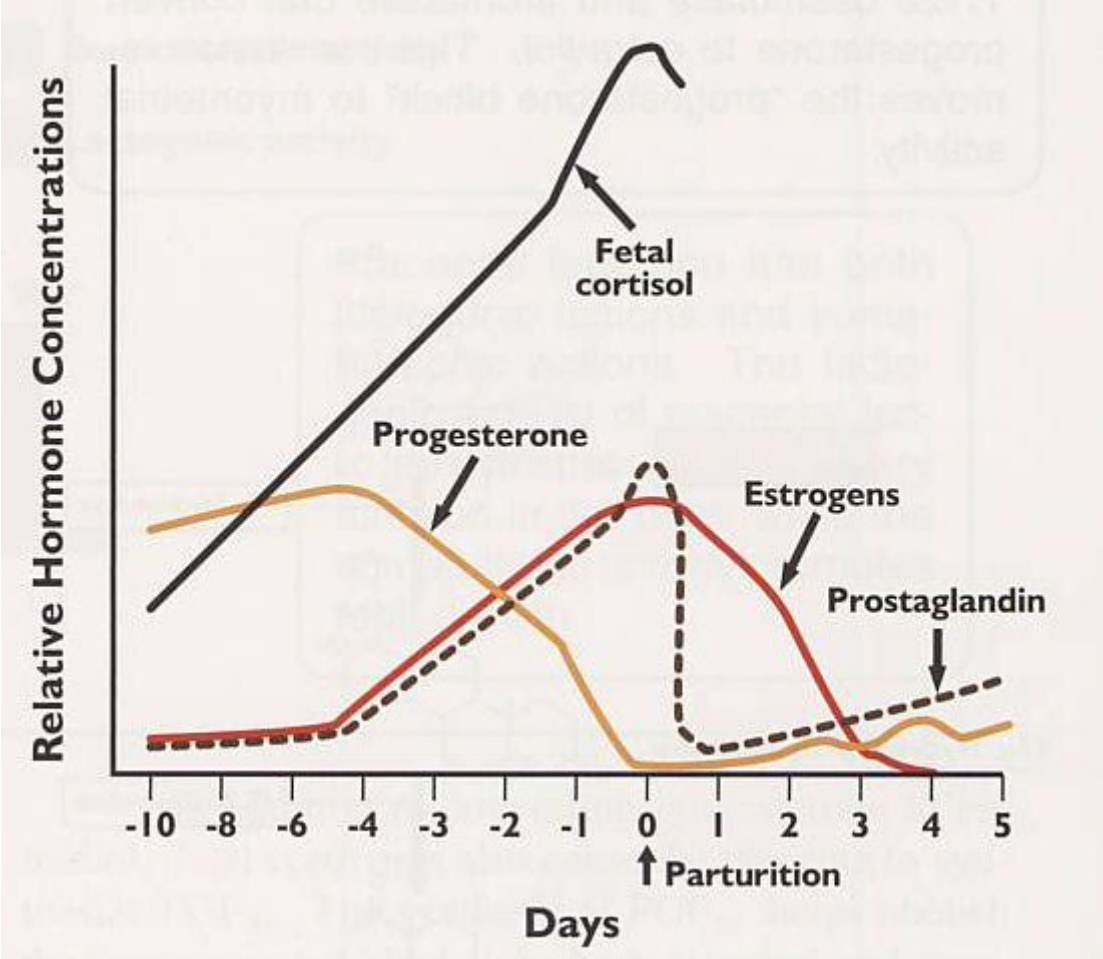

Figure 1-1. Relative hormone profiles in the cow during the periparturient period (Source: Senger, 2005)

After parturition, the uterus undergoes involution, which includes shrink in size, sloughing of the damaged endometrium and endometrial regeneration in order to return to its pre-pregnant state and be able to support the next pregnancy. The main stimulus of uterine involution in postpartum dairy cows is $PGF_{2\alpha}$, which increases sharply in the last week before calving and declines rapidly and returns to the basal level at 2 weeks after calving (Kindahl et al., 2004). $PGF_{2\alpha}$ causes strong myometrial contractions, leading not only to the uterine involution in size but also expulsion of the intrauterine content and lowering the odds of bacterial infections in the uterus. Normally, in dairy cattle, the uterine involution is completed by 3-6 weeks postpartum

(Kindahl et al., 1999; Sheldon, 2004).

Besides uterine involution, the reproductive axis needs to resume the normal cyclicity before the cow regains the pregnancy capability. Anterior pituitary becomes responsive to hypothalamus at 3-5 days postpartum and then starts releasing follicle stimulating hormone (FSH), which initiates the first new follicular wave around 7-10 days postpartum (Crowe, 2008). Although the average lifespan of follicles is 7-10 days (Crowe, 2008), the ovulation can not occur during the first 10-15 days postpartum due to the dominance of $PGF_{2\alpha}$ (Kindahl, 1999). The occurrence of the first ovulation postpartum depends on when the luteinizing hormone (LH) pulse frequency returns to one pulse every one hour from one pulse every four hours in the gestation period (Crowe, 2008). It has been reported that most dairy cows have a silent first ovulation after calving, which means an ovulation accompanied by no anestrous signs. In addition, the first ovulation is followed mostly by a short cycle around 9-11 days due to a shorter luteal phase in 70% of dairy cows instead of 18-24 days for the normal estrous duration (Kindahl, 1999; Crowe, 2008). The short cycle is not fertile but it is believed to prime the hypothalamus to return to a pulsatile state after a long period of suppression by high progesterone levels in blood.

The first 3 weeks after parturition are impossible for the cow to remain pregnant due to the uterine damage from calving and dominance of $PGF_{2\alpha}$ in circulation (Kiracofe, 1980; Kindahl, 2004). For the second 3 weeks after parturition, pregnancy is possible but not optimal due to the incomplete uterine involution and presence of negative energy balance (NEB) (Kiracofe, 1980). In postpartum dairy cows, the LH

pulse frequency is greatly influenced by the energy status, an interactive result of body condition score (BCS) loss, feed intake and milk production; the return of normal LH pulse frequency is impeded by NEB (Crowe, 2008). Therefore, the dairy producers should wait until 9-10 weeks postpartum to breed the cow when the energy balance turns positive, a period known as voluntary waiting period. Nowadays, more than 90% of dairy producers use artificial insemination (AI) to breed their cows. The fertilization rate usually reaches 90-100% (Diskin and Morris, 2008). Taking the average conception rate as 30-40%, if submission rate (heat detection rate) is 40-50%, the pregnancy rate is between 12-20%. In order to improve the reproductive outcome, synchronization of ovulation is employed to obtain 100% submission rate, hence increasing the pregnancy rate.

One of the prevalent breeding programs for reproductive management is 'presynch + ovysynch' combined with the timed artificial insemination (TAI, Figure 1-2). In this protocol, two injections of $PGF_{2\alpha}$ are given at 14 days apart, which is called 'presynch', with the 1st $PGF_{2\alpha}$ given on 35 days postpartum. The 1st $PGF_{2\alpha}$ injection is given to trigger luteolysis of the corpus luteum (CL) if there is one; the 2nd $PGF_{2\alpha}$ injection is given to cause luteolysis of the old CL that did not respond to administration of the 1st $PGF_{2\alpha}$, or the newly formed CL in the cows that either had a responsive CL to the 1st $PGF_{2\alpha}$ or did not have a CL present when the 1st $PGF_{2\alpha}$ was given. The 'ovysynch' involves two injections of gonadotropin releasing hormone (GnRH) and one injection of $PGF_{2\alpha}$. The 1st GnRH given 12 days after the 2nd $PGF_{2\alpha}$ induces the dominant follicle to ovulate, to form a new CL, and initiate a new

follicular cohort. The 3rd $PGF_{2\alpha}$ is given 7 days later to cause this newly formed CL to regress. The follicles continue growing and become mature. Two days later a 2nd GnRH is administered to synchronize ovulation. Then, 16-18 hours later, the herd can receive a timed AI without estrus detection (Diskin et al., 2002). Normally, a cow will ovulate 24-32 hours after experiencing her heat, and the ovum can remain viable in the oviduct for 6-12 hours. The sperm remains viable in the female reproductive tract for 24-30 hours; they need 9-10 hours to acquire capacitation before they acquire the ability to fertilize the ovum. Therefore, conducting AI at 16-18 hours after the timed ovulation can ensure optimal fertilization result.

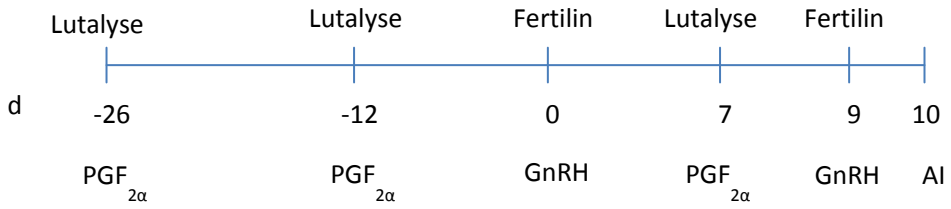

Figure 1-2. Presynch + Ovsynch protocol to time the ovulation in dairy cows

After the TAI, pregnancy is checked via ultrasound per rectum 30-32 days later. If the cow is open, the ovysynch protocol is repeated. If not, at 60 days after the TAI the cow is check again for pregnancy. If the pregnancy fails, ovysynch protocol is repeated to breed the cow, otherwise, the pregnancy is declared.

Usually, in a dairy herd there is a 30% loss of pregnancy; sometimes in high producing herds this number can reach even 40% of the herd. Up to 65% of the pregnancy loss (accounting for 20% pregnancy loss in a herd) in cows is attributed to embryonic loss, which means that the loss occurs within 42 days of gestation before the fertilized egg completes differentiation and implantation (Vassilev et al., 2005).

Embryonic loss mostly occurs in the early embryonic phase, namely within 27 days of gestation, before the pregnancy can be identified via ultrasound or other indicators. Out of this 20%, about 10-16% is lost between 8-16th day of gestation, during which the embryo is floating in the uterus and its survival completely depends on the uterine environment (the embryo starts differentiation on day 8 and hatches out from zona pellucida on day 9-10, and starts implantation on day 19-21 by caruncle-cotyledon attachment) (Vassilev et al., 2005; Diskin and Morris, 2008). The pregnancy loss occurred between day 42-260 of gestation is usually called abortion, and that after 260 days called stillborn.

1.1.3 Immune characteristics of the reproductive tract of transition dairy cows

Normal uterine lumen is a sterile environment ensured by several defense mechanisms. The first one is called mechanical or anatomical defense, mainly the vulva and the cervix. Vulva prevents fecal contamination of the tubular genitalia (Azawi, 2008). The cervix is capable of stopping most of the materials flowing ahead to the uterus by contracting its circular musculature. The large amounts of mucus in the vagina and cervix can be regarded as the second mechanical barrier. The function of the cervical-vaginal mucus is to serve as a physical barrier for holding pathogenic microorganisms back from ascending the reproductive tract. The sticky and collagenous mucus accumulated at cervix forms another excellent barrier against micro-organisms and is known as mucus plug during gestation (Azawi, 2008).

Two important members of the uterine cellular immunity are granular

lymphocytes present in the epithelial and subepithelial regions of the bovine endometrium and macrophages present in the stromal regions of endometrium (Cobb and Watson, 1995). Neutrophils are the earliest phagocytic cells to be recruited from the peripheral circulation to the uterine lumen by chemotactic factors, such as interleukin (IL)-8, in case of presence of inflammation in dairy cows (Sheldon and Dobson, 2004; Földi et al., 2006). Once migrated, they can not re-enter the circulation any longer, but to perform their bactericidal functions and die by apoptosis in the tissue, contributing to the formation of pus (Burton et al., 2005). They kill invading bacteria by producing multiple compounds, such as enzymes, nitric oxide, and reactive oxygen species (Sheldon, 2004). However, it is believed the infected lochia depresses the reactive oxygen species generation capacity of neutrophils dramatically (Zerbe et al., 2002). Consequently, the phagocytic ability of uterine neutrophils is inclined to be reduced compared to those circulating in the blood (Hussain, 1989). Even blood PMN obtained from cows with endometritis possess significantly less competent phagocytosis capability than those from the healthy cows (Kim et al., 2005).

Macrophages are also active in recognizing and responding to bacterial challenge and migrate to the uterus to help with bacterial elimination (Sheldon, 2004). Activated macrophages are the most important source of pro-inflammatory cytokines such as IL-1β, IL-6, and tumor necrosis factor (TNF) in case of local inflammation, which stimulate hepatocytes to secrete acute phase proteins (APP) and further trigger the general immune response (Sheldon, 2004).

Apart from neutrophils and macrophages, there are many placenta- and endometrium-derived cells involved in immune defense such as epithelial cells and trophoblastic cells. They are active in secreting both gestation-supporting hormones and immunity-related molecules. The pure endometrial epithelial cells have been reported to be able to express tracheal antimicrobial peptide (TAP) (Davies et al., 2008), lingual antimicrobial peptide (LAP), and bovine neutrophil β-defensins (BNBD4), and their expression is up-regulated when treated with lipopolysaccharide (LPS) (Sheldon et al., 2009a). Both the epithelial and stromal cells can detect and respond to bacteria by releasing interleukins and increasing the production of prostaglandins (Herath et al., 2006). Uterine epithelial cells also serve as antigen-presenting cells which can be enhanced by estrogen in females (Wirth, 2007). The polymeric Ig receptor (pIgR) expressed on uterine epithelial cells can be increased by estradiol in the presence of IL-4 and interferon (IFN)-γ to facilitate transportation of IgA to the uterine lumen (Wirth, 2007).

Normally, uterine immune responses are mainly cell-mediated and humoral immunity is less involved. This is supported by the wide distribution of T lymphocytes but rare distribution of B lymphocytes (Cobb and Watson, 1995). Although cell-mediated immunity plays the leading role in normal immune defense, when the uterus is subject to infection, all the three immunoglobulins (Ig) M, IgA, and IgG appear in cervical and vaginal regions, with IgG as the major immunoglobulin to defend against infectious agents (Cobb and Watson, 1995; Dhaliwal et al., 2001). These immunoglobulins are popular in different sites due to the regional distribution

of B cells. Uterine lumen-dominated IgG demonstrates a gradually lessened concentration gradient from blood to uterine lumen (Wirth, 2007). Therefore, it's believed that IgG is partly synthesized in the endometrium and the remaining is derived from peripheral circulation (Singh et al., 2008). IgA is synthesized locally at the uterine mucosal surface and dominates in the vagina (Wirth, 2007).

Lymphocyte distribution is site specific in the uterus of pregnant cows. In the placentomes, lymphocytes are completely absent from the syncytial layer and dramatically lower in the connective tissue of the caruncular endometrium to avoid immunological fetal rejection during early and mid-pregnancy (Low et al., 1990; Meeusen et al., 2001; Singh et al., 2008). Substantial lymphocytes and macrophages are found in the epithelium and connective tissues in the inter-caruncular areas (Low et al., 1990; Meeusen et al., 2001; Singh et al., 2008). During the early pregnancy, the stroma contains more T helper (Th) lymphocytes, B lymphocytes and macrophages than other regions of endometrium and myometrium (Leung et al., 2000). T lymphocytes account for 10-20% of the leukocytes in the uterine mucosa and increase with gestational age (around 45% in mid-pregnancy), but decline at parturition (around 20%) (Wirth, 2007; Singh et al., 2008). Cluster differentiation (CD)4+ T lymphocytes are restricted to the subepithelial stroma while CD8+ T lymphocytes almost occupy all the location of glandular and luminal epithelium and stroma, close to epithelium, making up the main body of T lymphocytes in the uterus (Cobb and Watson, 1995; Meeusen et al., 2001). The low ratio of CD4+/CD8+ is probably related to the high expression of major histocompatability complex (MHC) class I

protein and low expression of MHC class II protein. It is generally accepted that CD8+ cells act as suppressor T lymphocytes and CD4+ cells as helper T lymphocytes, so the reduced CD4+/CD8+ ratio underpins the immune suppression of pregnant cows.

Besides preventing uterine contractility, progesterone inhibits cervical mucus production (Rodriguez-Martinez et al., 1987) and lowers immunoprotective responses of the reproductive tract (Wira and Rossoll, 1995). Progesterone has also demonstrated an inhibitory effect on bovine cellular response (Lewis, 2003). It induces Th0 cell conversion to Th2 cell, promoting IL-4 and IL-6 secretion and therefore predisposes Th2-type immune response in pregnant cows (Ishikawa et al., 2004). In pregnant women progesterone seems to contribute to the high Th2 cytokines by stimulating lymphocytes to produce progesterone-induced blocking factor (PIBF), which can potentially intensify the production of Th2 cytokines (IL-3, IL-4, and IL-10) while blocking IL-12 secretion (Wirth, 2007). Besides its direct inhibitory effect on blood lymphocytes and decreasing the activity of pro-inflammatory molecules, progesterone can induce other immune response-suppressive substances, such as uterine milk protein (UTMP) (Hansen, 1995), as well as inhibit uterine eicosanoid synthesis such as leukotriene and prostaglandin, which are very important in uterine immune defense (Lewis, 2004). Consequently, pregnancy tends to shift immune response from Th1-type to Th2-type in order to provide immune tolerance for the fetus, and Th1-type immune response was found to be associated with abortion in humans (Hill et al., 1995) and in mice (Krishnan et al., 1996). This, on the other hand,

predisposes the uterus to infections. After parturition, the immune system returns to Th1-type from Th2-type in order to protect the uterus from birth canal-ascending bacterial infections (Ishikawa et al., 2004).

Periparturition is a dynamic time period for host immunity with usually disrupted immunological profile. Before parturition, the uterus is a sterile environment, harboring fewer antigens than peripheral blood, so the main defense force lies in blood, characterized by leukocytosis (Mateus et al., 2002; Kim et al., 2005; Singh et al., 2008). Because of the abrogation of progesterone at labor, production of TNF, IL-1β, IL-6, and IL-8 increases in the uterine membrane as well as the cervix to induce cervical ripening and labor (Peltier, 2003), whereas IFN-γ declines at calving compared to prepartum (Karcher et al., 2008). The surge of glucocorticosteroids around parturition is always concomitant with neutrophilia due to the abundant glucocorticosteroid receptors on neutrophils (Burton et al., 2005), but with a major negative impact on the polymorphonuclear neutrophils (PMN) oxidative burst capacity (Vangroenweghe et al., 2005). A large number of leukocytes surge in the cervix, preceded by increased numbers of neutrophils and macrophages but not T or B cells (Peltier, 2003).

After parturition, the uterus is exposed to a variety of bacteria ascending from the lower part of birth canal. PMN phagocytic activity remains high throughout the periparturient period (Zerbe et al., 1996; Mateus et al., 2002), but their bactericidal capacity is impaired, especially after parturition (Zerbe et al., 1996; Mateus et al., 2002; Hammon et al., 2006), which is substituted by macrophages (Sheldon, 2004).

During the first week postpartum, before deep invasion of pathogens, the local immune system in the uterus plays the leading role in resolving acute bacterial contamination. This is supported by the findings that phago-PMN (percentage of phagocytizing PMN) and phagocytic index (mean number of phagocyted bacteria per phagocytizing PMN) of intrauterine PMN are high while both the count and oxidative burst of blood PMN declines compared to prepartum levels (Mateus et al., 2002; Kim et al., 2005; Singh et al., 2008). A significant negative correlation has been reported between uterine fluid phago-PMN and the blood PMN oxidative burst activity, so it seems that there is a complementary effect between the intrauterine and peripheral PMN capability during periparturition (Mateus et al., 2002). The number of bacteria phagocytized by each neutrophil and the percentage of neutrophils in the uterine fluid reaches maximum levels within two weeks postpartum, and then declines gradually until the 3rd week postpartum (Hussain and Daniel, 1992). As pathogens invade further, systemic immune responses gradually take over from local immunity to clear the pathogens and improve recovery from reproductive system damages or infections. During the following several weeks, phago-PMN and phagocytic index of intrauterine PMN declines, coincident with an enhancement of blood PMN oxidative burst activity (Mateus et al., 2002; Kim et al., 2005).

During the periparturient period, the percentage of T lymphocytes in the peripheral circulation drops from 45% in mid-lactation to 20%, accompanied by a decrease in the proportion of CD4+/CD8+ T lymphocytes, hence suppressive immune status (Singh et al., 2008). In fact, no matter whether the cows are infected or not,

they will undergo a decrease in CD3+, CD4+, and CD8+ T lymphocytes around calving (Ohtsuka et al., 2004). IL-6 is a pro-inflammatory cytokine secreted by many immune cells, mainly by macrophages and Th2 cells. The plasma level of IL-6 was observed to be greater before parturition and declines significantly after parturition (Ishikawa et al., 2004). The high plasma IL-6 is beneficial for the contraction of myometrium to expulse the fetus and debris by enhancing plasma calcium levels as well as prostaglandin synthesis (Davidson et al., 1995; Singh et al., 2008).

Concentrations of IgG and IgM in the serum of dairy cows decrease dramatically from 8 week prepartum until 4 week postpartum (Hussain, 1989; Herr et al., 2011), and both have a nadir at calving (Herr et al., 2011). However, concentrations of IgG recover by 4 week postpartum, while IgM remains at a low level (Herr et al., 2011). Low γ-globulin in the uterine secretion (below 0.40 gm% on the first day postpartum) demonstrates a decreased bactericidal activity and predisposes the development of postpartum uterine infections (Hussain, 1989).

1.1.4 Microbial characteristics in the reproductive tract of dairy cows

Based on bacterial presence, the reproductive tract of dairy cows can be divided into the upper and lower part. The upper part, which consists of fallopian tubes, uterus, and endocervix, is normally bacteria-free, whereas the lower part, which consists of ectocervix and vagina, is populated by bacteria. The vaginal tract of dairy cows harbors various bacteria including aerobic, facultatively anaerobic, and obligately anaerobic ones (Wang et al., 2013). Plate culture analysis has shown that

Enterococcus and *Staphylococcus* are the predominant bacteria of the vaginal tract of healthy heifers, followed by *Enterobacteriaceae* and *Lactobacilli* (Otero et al., 2000).

During gestation, the cervix is closed with a mucus plug, isolating the bacteria harboring vagina and the sterile uterine body. After parturition, the cervix is open to allow the drainage of intrauterine fluid, which also provides a passage for bacteria to ascend into the uterine body via the vaginal tract. The early postpartum uterus is colonized by a wide range of microorganisms, derived from feces, skin, and environment. Furthermore, bacterial contamination undergoes a dynamic fluctuation in the first few weeks postpartum (Sheldon and Dobson, 2004). In a recent article we reported that *Bacillus* and lactic acid bacteria (LAB) such as *Enterococcus*, *Lactobacillus*, and *Pediococcus* as well as *Enterobacteriaceae* and *E. coli* were present in both healthy cows and those with uterine infection, with *E. coli* being the most abundant in infected cows (Wang et al., 2013).

The microbiota in the uterus of postpartum dairy cows is dominated by *A. pyogenes* and *E. coli* combined with certain Gram negative (G-) anaerobic bacteria such as *Fusobacterium necrophorum*, *Bacteroides* spp., and *Prevotella* spp. during the first 10-14 days postpartum even if there is no sign of evident disease (Del Vecchio et al., 1994; Földi et al., 2006). As stated above, in the first week postpartum, the local immune system plays the leading role in resolving bacterial contamination. Bacterial infection is confined in the uterus. The local release of inflammatory products, such as TNF, leukotriens, and also other eicosanoids, and/or their absorption from the uterus might be limited (Földi et al., 2006). Indeed, puerperal metritis caused by bacterial

complications often occurs during this period (Sheldon et al., 2006). The most frequently reported bacteria associated with uterine disease in cattle are *E. coli*, *Arcanobacterium pyogenes*, *Prevotella melaninogenicus* and *F. necrophorum* (Williams et al., 2005; Singh et al., 2008).

Although *E. coli* has been found in both healthy and infected uterus of postpartum dairy cows they were found in a much greater numbers in infected cows (Wang et al., 2013). Also, except for the enteroinvasive *E. coli* (EIEC) and enteroaggregative *E. coli* (EAEC) all of the enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC), and necrotoxic *E. coli* (NTEC) have been reported in the uterus of various animals (DebRoy and Maddox, 2001). However, Sheldon et al. (2010) found that the endometrial pathogenic *E. coli* (EnPEC) isolated from the uterus of infected cows lacked some of the genes commonly associated with adhesion and invasion by enteric or extraintestinal pathogenic *E. coli*, but they were more adherent and invasive for endometrial epithelial and stromal cells compared to those isolated from the uterus of healthy cows.

Arcanobacterium pyogenes is a Gram-positive (G+) facultative anaerobe. It possesses many virulence genes encoding attaching factors and toxins. *Arcanobacterium pyogenes* expresses fimbriae and extracellular matrix binding proteins, such as collagen-binding protein, which acts to strengthen the adherence and colonization of the bacterium to host collagen-rich tissue after the host surface tissue is damaged (Jost and Billington, 2005). The major virulent factor of *A. pyogenes* is

cholesterol-dependent cytotoxin pyolysin (PLO), which binds to the cholesterol-rich domain of the cell membranes to form a pore, resulting in cytolysis (Jost and Billington, 2005). In addition, *A. pyogenes* secretes several enzymes such as DNase, neuraminidases, and protease (Jost and Billington, 2005). DNase is used to degrade nucleic acid and utilize it as a nutrient source. Neuraminidase can reduce mucous viscosity, exposing host attaching site and making IgA susceptible for protease. Protease can degrade proteinaceous component of the host and make it a nutrient source for *A. pyogenes*. Furthermore, *A. pyogenes* is said to be able to invade epithelial cells and survive in macrophages when engulfed (Jost and Billington, 2005). The biofilm-forming ability makes *A. pyogenes* a chronic infectious agent.

Fusobacterium necrophorum is a G- anaerobe which converts lactic acid to propionic acid (Shinjo, 1983). The most important virulent factor of *F. necrophorum* is leucotoxin, which is a heat stable large protein specific to bovine and human PMN (Nagaraja et al., 2005). *Fusobacterium necrophorum* expresses adhesins, hemagglutinin, dermonecrotic toxin, and extracellular proteases, which all contribute to its adherence to the epithelial cells. It induces apoptosis of PMN when in a low concentration but causes cellular lysis when in a high concentrations. As a G- bacterium, presence of endotoxin in the outer wall membrane is also detrimental to the host. Moreover, it produces hemolysins, which cause hemolysis and provide iron access to this intruder. In addition, it has a platelet aggregation factor, which is believed to help create an anaerobic environment for the bacteria (Nagaraja et al., 2005).

Prevotella melaninogenicus is a strictly anaerobic G- bacterium, which is sensitive to bile acids and usually forms biofilms. *Prevotella melaninogenicus* produces hemolysin under iron-limited conditions to make iron accessible to itself and other bacteria (Allison et al., 1997). It also produces neuraminidase and collagenase which facilitate the process of adherence. In addition, it produces some proteases specific to IgG and IgA as well as phospholipase A (Bulkacz et al., 1981; Kilian, 1981).

Pathogenic bacteria aggravate uterine infection via synergistic actions. For instance, *E. coli* is mostly isolated from the uterus of cows in the first 2 weeks postpartum and it is believed to increase the susceptibility to *A. pyogenes,* whereas *A. pyogenes* is found to be more associated with chronic infections of the uterus (Sheldon et al., 2008). *Arcanobacterium pyogenes* provides a catalase to hydrolyze H_2O_2 (Singh et al., 2008; Sheldon et al., 2009a). It produces lactic acid which can be utilized by *F. necrohphorum* as a fermentation substrate. *Fusobacterium necrohphorum* produces leucotoxin to inhibit phagocytosis, protecting other bacteria from neutrophils and macrophage.

The species and numbers of bacteria in the uterine lumen are supposed to decrease gradually as the uterus involutes. Usually cows are able to self-resolve bacterial infections within 3-4 weeks postpartum by discharge of uterine content, rapid involution of the uterus and cervix as well as mobilization of immune responses (Sheldon et al., 2006; Azawi, 2008). The presence of bacteria is sporadic from 4-5 wk postpartum and the uterus is supposed to be sterile thereafter (Földi et al., 2006).

1.1.5 Uterine infections of postpartum dairy cows

The development of uterine infections or disease depends on the balance of the host immune function and bacterial invasion. As indicated above, dairy cows undergo a period of immunosuppression during the transition period. Meanwhile, unavoidable bacterial contamination of the uterus after parturition casts a great challenge to the immune system; the disrupted surface epithelium together with fluids and tissue debris provides a nutritious environment for bacterial growth and proliferation (Azawi, 2008). Normally the cervix plays an important role in preventing bacterial entrance as a gatekeeper. The dilation of the cervix during and after parturition is responsible for 90% of the infections of cows within 21 days post-partum (Singh et al., 2008). Risk factors that predispose a cow to uterine infections are listed in Table 1-1.

Table 1-1. Risk factors associated with uterine infections of dairy cows

Risk factors	Increased risk	Sources
Age and parity	Older cows	Erb and Martin, 1980; Onyango et al., 2014
Calving season	Warmer season	Erb and Martin, 1980; Benzaquen et al., 2007; Onyango et al., 2014
Environment	Dirty, congested stall	Lewis, 1997
Nutritional factor	NEB; BCS <3 or >4 on 1-5 scale; Inadequate calcium, selenium, vitamin A or E	LeBlanc et al., 2011; Galvão, 2013

Calving condition	dystocia, twines, abortion, assisted calving	Sheldon et al., 2008; Potter et al., 2010; Salasel et al., 2010; Onyango et al., 2014
Retained placenta	--	Sheldon et al., 2008; Potter et al., 2010; Salasel et al., 2010

Uterine infections refer to infections of the uterus that cause inflammation and histological lesions to various anatomic layers (endometrium, submucosa, muscularis, and serosa) of the uterus. This pathology involves adherence of pathogenic organisms to the mucosa, colonization and penetration of the epithelium as well as release of bacterial toxins that cause histological lesions (Sheldon et al., 2006). Several pathologies are closely related to uterine inflammation and are named according to their inflammatory extension. Metritis is an inflammation involving all layers of the uterine wall, i.e. mucosa, submucosa, muscularis, and serosa. Endometritis is termed for superficial inflammation limited to the endometrium (mucosa and submucosa), while perimetritis is limited to serosa, and parametritis is limited to the suspensory ligaments (Sheldon et al., 2006). Metritis occurs within the first 21 days postpartum, whereas infections occurring within the first 10 days postpartum are called puerperal metritis (Sheldon et al., 2006). Metritis is characterized by an abnormally enlarged uterus and a watery red-brown uterine discharge with a fetid odor, accompanied by fever and systemic illness, such as decreased feed intake and milk production

(Sheldon et al., 2006). Clinical metritis is diagnosed by the presence of purulent uterine discharge in the vagina and an abnormally enlarged uterus, but no systemic symptoms of illness (Sheldon et al., 2006). Endometritis occurs after 21 days postpartum and can last till service. Clinical endometritis is defined in cattle with purulent uterine discharge detectable in the vagina after 21 days or more postpartum or mucopurulent discharge detectable in the vagina after 26 days postpartum (Sheldon et al., 2006). Subclinical endometritis is diagnosed if neutrophils exceed 18% of total cells in uterine cytology samples between d 21-33 postpartum, or 10% between d 34-47, or 3% thereafter, in the absence of clinical endometritis (Sheldon et al., 2006). In addition, the accumulation of purulent materials in the uterine lumen in the presence of a persistent CL is defined as pyometra, which usually occurs after 6 wk postpartum (Sheldon et al., 2006).

It has been estimated that up to 90% of the cows are contaminated by bacteria within the first week after parturition (Herath et al., 2009; Sheldon et al., 2009a). Approximately 36-50% of the cows are affected by clinical metritis, and 20% by metritis, during the first 3 weeks after calving (Sheldon et al., 2006). Normally, during the uterine involution period, the immune system is activated to fight the invading pathogens, and most dairy cows can self-resolve the infections within 3 weeks (Bekana et al., 1996; Bondurant, 1999; Sheldon et al., 2006). However, some pathogenic bacteria are not cleared efficiently and persist thereafter. It is estimated that even 3 weeks after calving there are still 15-20% of the cows having clinical endometritis and 30% having subclinical endometritis (Sheldon et al., 2009a).

Furthermore, around 8% of the cows still suffer severe endometritis even 6 or 7 weeks after calving (Lewis, 1997).

Contractions of the myometrium provide physical propulsion of uterine content as well as trapped bacteria (Azawi, 2008; Singh et al., 2008). Uterine contractility is subject to hormonal regulation, mainly by prostaglandins. Prostaglandin $F_{2\alpha}$ is very effective in stimulating myometrial contraction and aiding in the expulsion of uterine contents (Paisley et al., 1986). The production of $PGF_{2\alpha}$ by epithelial and stromal cells is increased following infectious challenge (Herath et al., 2006), and its low concentration is associated with the development of uterine infections (Seals et al., 2002). Cows with retained placenta have a longer release of $PGF_{2\alpha}$ than those without and the duration of high levels of $PGF_{2\alpha}$ of bacterium contaminated cows is longer than those not (Fredriksson et al., 1985). This might be a feedback mechanism to expel the retained placenta and bacterial toxins (Paisley et al., 1986; Földi et al., 2006), because cows with retained placenta are detected with lower $PGF_{2\alpha}$ in the placentomes (Paisley et al., 1986). The prolonged release of $PGF_{2\alpha}$ contributes to a low concentration of progesterone in the serum, unblocking the inhibitory effects of progesterone on uterine immune system and then favoring clearance of infections (Paisley et al., 1986). Furthermore, $PGF_{2\alpha}$ is an important molecular signal for the uterine immune system because it activates immune responses by enhancing phagocytosis and lymphocyte functions (Singh et al., 2008).

It has been postulated that the difference between clinically healthy cows and those suffering from uterine disease lies in their ability to limit inflammatory

responses during the first week after parturition (Herath et al., 2009). Because in times of inflammation, the immune system of the cows becomes highly active during early postpartum, and it seems that during this period, infections are confined to the uterus instead of spreading into the blood. During 1 or 2 days after parturition cows with retained placenta had greater LPS levels in the uterine lochia (average of 2.24 * $l0^4$ Endotoxin Units (EU)/mL) than those with dystocia or healthy (average of 0.10 and 0.26 EU/mL, respectively), whereas all cows exhibited undetectable levels of LPS in the plasma (Dohmen et al., 2000). Immunoglobulin G anti-LPS antibodies also showed no differences among these three groups of cows immediately after calving but a lower level of anti-LPS IgG in problematic cows after two months (Dohmen et al., 2000).

1.1.6 Influence of uterine infections on postpartum dairy cows

Uterine infections have a major impact on the general wellbeing and health status of dairy cow. Cows with greater incidences of uterine infections were found having poorer body condition scores during dry off. Also, these cows produced less milk in the first 100 days in milk (DIM) and lower milk protein content in the first 21 DIM (Bell and Roberts, 2007). Uterine infections decrease immunity and cows with metritis are 16 times more likely to develop complicated ketosis, and 2.4 times more likely to develop abomasal displacement (Wallace, 1998).

Infections of the uterus also impair reproductive performance of dairy cows and many of them end up being subfertile or infertile and are, therefore, culled from the

herd (Sheldon et al., 2009a). The mechanism(s) by which uterine infections affect reproductive performance are related to delays in uterine involution and resumption of ovarian activity as well as prolongation of the luteal phase once ovulation occurs (Huszenicza et al., 1999; Mateus et al., 2002; Sheldon et al., 2009a) as illustrated in Figure 1-3. It was reported that cows with uterine infections have lower concentration of estradiol in the blood during follicular dominance and also lower concentrations of progesterone during the luteal phase. Indeed, cows with uterine infections exhibit lower conception rate, require more services per conception, and have more days open (Kasimanickam et al., 2004; Sheldon et al., 2009a). According to recent data the culling rate of dairy cows in Canada reached almost 41.7% in 2013 (CanWest DHI and Valacta, 2013). Among all the culling reasons, reproductive failure was rated as number one reason, accounting for 15.4%, which caused a loss of more than 57,600 animals worth $144 million dollars per year.

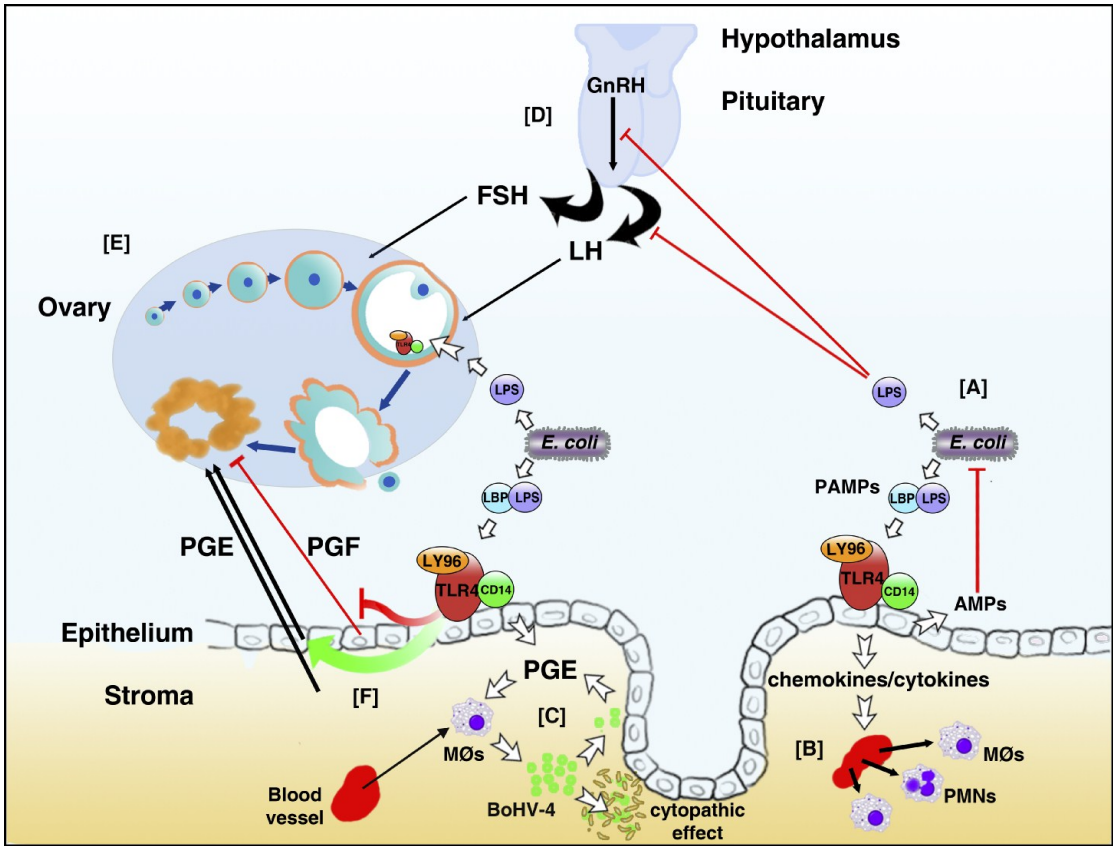

Figure 1-3. Mechanisms underlying infertility associated with uterine infections
(Source: Sheldon et al., 2009a,
reprinted with permission from the Society for the Study of Reproduction)

1.2 Current approaches to treatment of uterine infections

1.2.1 Application of antibiotics in uterine infections

To our best knowledge, there is no known effective treatment(s) or prophylactic medication(s) against uterine infections. Although various intrauterine antibiotics such as oxytetracycline® and cephapirin benzathine® are currently used to treat cows, their efficiency is not proven and concerns about drug residue in milk and carcass, or bacterial acquisition of drug-resistance have limited their widespread use (Lewis, 1997; Galvão, 2011). Presently, there are no intrauterine antibiotics approved for utilization in dairy cows in the US (Galvão, 2011). Only systemic ceftifur® is

approved for treatment of cows with metritis. Although systemic administration of ceftifur® could lower the incidence of metritis, it does not improve the reproductive performance (Risco and Hernandez, 2003). Of note, intrauterine infusion of cephapirin benzathine® has been approved for treatment of clinical endometritis in some countries including Canada, Europe, New Zealand, and Australia although its efficacy in improving reproductive performance seems dependent on the dose (Galvão, 2011). Usually the dose of antibiotics administered via intrauterine route is greater than that of systemic route and, therefore, there is a greater risk of residue in the milk causing milk discard (Azawi, 2008).

1.2.2 Application of antiseptic agents in uterine infections

Infusion of povidone iodine® also has been stopped due to its ineffectiveness, impeding of phagocytic activity of leukocytes in the uterus and triggering of endometrial necrosis (van Dyk and Lange, 1986; Azawi, 2008), which could be detrimental to the fertility of endometritic cows (Nakao et al., 1988).

1.2.3 Application of hormones in uterine infections

Some hormones, such as oxytocin, estradiol, and $PGF_{2\alpha}$, have been applied to simulate expelling of uterine content and eliciting of uterine involution after calving. Although one study showed that using oxytocin within a short period of time (6 h) after calving was effective in lowering the incidence of retained placenta and shortening of the interval between calving and conception (Mollo et al., 1997), this finding could not be reproduced in other investigations (Palomares et al., 2010;

Galvão, 2013). Prepartum use of oxytocin can cause death of the newborn as the cervix is not ripened yet when the fetus is being expelled out. Frequent use of oxytocin also can cause prolonged spastic contractions of the uterus. Moreover the contraction of oxytocin declines rapidly in minutes, thus not efficient for later use.

The efficacy of estradiol is debatable in resolving bacterial uterine contamination (Sheldon, 2004; Sheldon and Dobson, 2004). For instance, intrauterine flushing with estradiol in the gravid horn at 7-10 days postpartum had no positive effects on uterine health and hastening of uterine involution (Sheldon et al., 2003a, 2003b). Moreover, it does not prevent the occurrence of metritis or improve reproductive performance when used in cows with retained placenta (Risco and Hernandez, 2003). Instead, infusion of estradiol into the uterine body at 7-10 days postpartum increased bacterial load on d 14, especially those of *P. melaninogenicus* and *F. necrophorm*, and caused toxemia by eliciting more blood to the uterus (Sheldon, 2004). In addition, infusion of estradiol on day 7 postpartum delayed the interval from calving to the first ovulation (Sheldon, 2004).

Intramuscular $PGF_{2\alpha}$ has been reported as a promising therapy in treating endometritic cows. It has a direct effect on flushing out bacteria from the uterus by stimulating myometrial contractions and enhancing immune responses (Lewis, 2003). Furthermore, $PGF_{2\alpha}$ has an indirect effect in inducing estrus by causing luteolysis in presence of a CL, thus overriding the inhibitory effect of high progesterone on immune response (Lewis, 2003). Both mechanisms are reinforced by endogenous secretion of $PGF_{2\alpha}$ elicited by exogenous $PGF_{2\alpha}$ (Lewis, 2003). However, a

meta-analysis demonstrated only a marginal benefit of $PGF_{2\alpha}$ on reproductive performance (Burton and Lean, 1995).

The current treatments of uterine infections are summarized in Table 1-2.

Table 1-2. Current treatments of uterine infections of dairy cows

Treatment	Limitations	Sources
Antibiotics	Bacterial drug-resistance, drug residual in milk, no benefits on fertility	Lewis, 1997; Risco and Hernandez, 2003; Galvão, 2011
Antiseptic agents	Irritating, causing endometrial necrosis	Van Dyk and Lange, 1986; Azawi, 2008
Hormones	Oxytocin: transient stimulatory effect on uterine contraction	Palomares et al., 2010; Galvão, 2013
	Estradiol: no effect on uterine infections or reproduction, but with risk of increasing bacterial load and delayed time to first ovulation	Risco and Hernandez, 2003; Sheldon, 2004
	$PGF_{2\alpha}$: no consistent benefits on improving fertility	Burton and Lean, 1995

1.3 Probiotics, a new approach to uterine infections

1.3.1 Lactic acid bacteria

Lactic acid bacteria (LAB) are a big group of diverse Gram-positive bacteria that produce lactic acid as the major end product of carbohydrate fermentation and therefore very tolerant to acidic conditions. They are non-spore forming, fastidious, and in the morphology of rod or cocci that can grow under microaerophilic to strictly

anaerobic conditions. Lactic acid bacteria are not named phylogenetically, but based on their fermentation product. Consequently, they represent bacteria from many taxa, but mostly from *Lactobacillales* in the phylum of Firmicutes. The most mentioned genera of LAB are *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Weissella* (LPLW), which are phylogenetically close to each other (Molin, 2003) and usually measured together as *Lactobacillus* group (Walter et al., 2001; Wang et al., 2013).

1.3.2 Application of lactic acid bacteria as probiotics in female urogenital infections

Mounting evidence indicates that women with bacterial vaginosis are at a high risk of being infected by human immunodeficiency virus (HIV) (Sewankambo et al., 1997; Taha et al., 1999) because many of them lack lactobacilli in the vaginal tract (Martin et al., 1999; Alvarez-Olmos et al., 2004). Moreover, bacterial vaginosis is also to be blamed for greater infant mortality and preterm delivery (Reid et al., 2003). Some probiotic bacteria, like *L. rhamnosus* GG, *L. rhamnosus* GR-1, *L. fermentum* RC-14, and *L. acidophilus*, are well-known for their ability to maintain and restore a normal vaginal microflora and therefore have been used to prevent and treat urogenital infections in women (Reid et al., 2001; Gardiner et al., 2002; Reid et al., 2003). *Lactobacillus* strains are able to colonize the vagina when used as suppository and lower the risk of many reproductive tract infections, yeast vaginitis, and bacterial vaginosis (Reid et al., 1995; Cadieux et al., 2002). For instance both *L. rhamnosus* GR-1 (Reid et al., 1994), and *L.* GG have been administered directly in the vagina to treat women with recurrent cystitis and vulvovaginal candidiasis (Hilton et al., 1995).

The results showed that these vaginally administered probiotics attenuated or eliminated symptoms of vaginitis.

Oral administration of probiotics can contribute to the increase of vaginal lactobacilli although the mechanism is not clear. Orally administered *L. acidophilus* by means of yogurt was reported to be associated with increased colonization in the vagina and could be used as prophylactic for candida vaginitis (Hilton et al., 1992). Reid et al. (2001) studied the effects of *L. rhamnosus* GR-1 and *L. fermentum* RC-14 orally administered in a group of women with recurrent yeast vaginitis. The result demonstrated that oral administration of *L. rhamnosus* GR-1 and *L. fermentum* RC-14 made lactobacilli the dominant species in the vaginal tract of the treated subjects independently of the lactobacilli dominance at the beginning of the experiment. This provided strong evidence for potential use of LAB in treating bacterial vaginosis in humans. Later, Reid et al (2003) reported that in healthy women orally administered *L. rhamnosus* GR-1 and *L. fermentum* RC-14 were able to increase the colonization of lactobacilli and decrease that of pathogenic bacteria and yeast in the vaginal tract compared with the placebo subjects.

Most of the research on probiotics has been directed at prevention or treatment of various infectious diseases and, therefore, there is not much research conducted specifically on the dose. However, it is important that consideration should be given to both their effect and efficiency, because they might lose effectiveness after they go through a complicated chemical and physical pathway before they reach and act on the target sites. Currently, probiotics are used mostly at the dose from 10^8~10^{12} cfu per

week. Reid et al. (2001) reported that daily oral intake of 10^9 to 10^{10} of capsulated *L. rhamnosus* GR-1 and *L. fermentum* RC-14, in females with bacterial vaginosis, could restore the vaginal dysbiosis to lactobacilli-dominated one. There is another study stating that vaginal administration once-weekly of a suppository containing 10^9 *L. rhamnosus* GR-1 and *L. fermentum* B-54 for 1 year could lower the occurrence of urinary tract infections (Reid et al., 1995).

During the last decade there is a growing interest to screen beneficial bacteria from their original ecosystems and introduce them back to the host to help maintain a favorable microbiota against pathogenic bacteria. Microbiota in the vaginal tract also has been examined to select the potential probiotic bacteria, which can be used to prevent or treat infections in the reproductive tract. Bacteria with probiotic potential are screened based on their surface characteristics for their adhesive ability (Ocaña and Nader-Macías, 2001; Otero et al., 2004). The ability of surviving through the target ecosystem and production of H_2O_2 has been also tested (Ocaña et al., 1999; Aslim and Kilic, 2006). Besides the utilization of a single strain of probiotics, combination of different strains is also a promising approach. Juárez Tomás et al. (2011) reported that lactobacilli isolated from urogenital tract, which were able to inhibit pathogens, can be combined as different probiotic products based on their compatibility.

1.3.3 Potentiality of lactic acid bacteria for prevention/treatment of uterine infections in dairy cows

With regards to cattle, researchers are also trying to screen probiotic bacteria from

their indigenous niches. For instance, various studies have investigated surface properties such as hydrophobicity, autoaggregation, and bacteriocin-producing activity of LAB from the mammary gland and milk of both healthy and mastitic dairy cows in order to develop probiotic products against mastitis (Espeche et al., 2009; Espeche et al., 2012). Bacteria have been also isolated from fecal samples and oral cavity of calves in order to screen for beneficial ones and utilize them for prevention of calf diseases related to dysbiosis (Nader-Macías et al., 2008; Maldonado et al., 2012). Likewise, bacterial isolates from cattle vaginal samples have been examined on their properties in order to develop probiotics to prevent or treat uterine infections. Nader-Macías et al. (2008) found that LAB strains isolated from vaginal tract have a strong capability of producing H_2O_2. H_2O_2-generating lactobacilli from the vaginal tract of cattle have the potential to be utilized as probiotics, among which *L. gasseri* CRL1421 is reported to have the greatest capacity to inhibit *Staphylococcus aureus* by generating H_2O_2 and lactic acid (Otero and Nader-Macías, 2006). A few strains of LAB (mainly *Lactobacillus fermentum*) isolated from cow's vaginal mucus have been reported to be able to inhibit the growth of *A. pyrogenes* in vitro, a recognized pathogen isolated from metritic cows, which hold great potential to be used as probiotic product to prevent metritis (Otero et al., 2006). *Pediococcus acidilactici* isolated from healthy pregnant dairy cows has exhibited inhibitory effect on *L. innocua* and *E. faecalis* by the production of pediocin (Wang et al., 2013).

In a recent study, our team introduced a cocktail of LAB into the vaginal tract of transition dairy cows, composed of lactobacilli and pediococci isolated from the

vaginal mucus of healthy pregnant dairy cows, which lowered the incidence rate of purulent vaginal discharges in the treated cows (Ametaj et al., 2014).

1.4 Proposed mechanisms for probiotic action

1.4.1 Enhancement of epithelial barrier function

In an in vitro study with T_{84} cells, it was shown the epithelial barrier function and resistance of the colon tissue to *Salmonella* invasion could be enhanced when exposed to a probiotic mixture (VSL#3; composed of bifidobacteria, lactobacilli, and streptococci) (Madsen et al., 2001). Later, other researchers demonstrated that administration of VSL#3 prevented ileitis in mice through modulation of some proteins pertaining to the permeability of epithelium, such as tight junction proteins claudin-2 and occluding (Corridoni et al., 2012). The VSL#3 also prevented the decrease of tight junction protein of a murine colitis model and prevented the epithelial apoptosis (Mennigen et al., 2009). VSL#3 and other lactobacilli were able to increase the expression of mucin, which is the major component of mucus layer and therefore prevent pathogens from approaching the epithelium in in vitro trials (Ohland and Macnaughton, 2010). Although most of the studies on the effect of probiotics on barrier functions have used intestinal epithelium as a model, probiotics have the potential to also improve the barrier functions of vaginal epithelium. Bisanz et al. (2014) reported that the gene expression of caspase 14 which plays a role in epithelial development and barrier functions was greatly increased by intravaginal administration of lactobacilli.

1.4.2 Modulation of immune system

Kummer et al. (1997) observed that intrauterine infusion of lactobacilli induces infiltration of lymphocytes, mast cells, and macrophages in the endometrium. Probiotics have also been shown to increase the production of antibacterial peptides through cross talks with the host immune cells. For instance, lactobacilli stimulated Paneth cells, which are located at the bottom of villous crypt, to produce defensins and / or cathelicidin (Ohland and Macnaughton, 2010).

Wagner and Johnson (2012) reported that *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 suppressed expression of inflammatory cytokines of vaginal epithelial cell induced by *C. albicans*. Knockout mice for IL-10 treated with VSL#3 lowered mucosal production of TNF and IFN-γ and showed an improvement in disease (Madsen et al., 2001). However, another in vitro study demonstrated that oral supplementation of VSL#3 stimulated production of TNF and nuclear factor kappa B (NF_kB) in epithelial cells, suggesting a pro-inflammatory effect of probiotics on the epithelial immune functions (Pagnini et al., 2010). Corridoni et al. (2012) reported that the modulatory effect of VSL#3 on proteins related to permeability of epithelium was dependent on the increase of TNF in mice with ileitis. These inconsistent results suggest that the effect of probiotics on NF_kB and IL-10 might be strain specific and dependent on the host health status (Sherman et al., 2009; Ohland and Macnaughton, 2010).

Isolauri et al. (2001) reported that probiotic bacteria have dual effects on the activity of macrophages depending on the immunologic status of the host. For

instance, in healthy subjects, administration of probiotics stimulates the activation of macrophages; whereas in subjects with allergies, the activation of macrophages can be down-regulated by probiotics. This modulatory role of probiotics on macrophage activity is important considering their role in the innate immunity and activation of the immune system in case of pathogen invasion. Their activation is associated with phagocytosis of invading pathogens and production of pro-inflammatory cytokines, which is essential to alert the whole immune system. However, over-activation of macrophages can be harmful as their major cytokines TNF, IL-1, and IL-6 are pyrogenic and their prolonged production can cause general illness (Vybíral et al., 2005).

Pretreatment with probiotic bacteria has been shown to stimulate production of pathogen specific IgA and total IgA when exposed to pathogens (Isolauri et al., 2001; Ohland and Macnaughton, 2010). The up-regulation of polymeric IgA is said to result from the interaction between probiotic bacteria and the mesenteric lymph node (Walker, 2008). Of note, the IgA stimulating ability and the amount produced is strain specific, not common to all probiotics (Ohland and Macnaughton, 2010).

Altogether, modulatory role of probiotics on the immune functions suggest that probiotic bacteria have a positive impact on the immunologic status of the host, an immunostimulatory effect on healthy subjects but an immunosuppressive effect on subjects with over activated immunity.

1.4.3 Competition for attaching sites

Attachment is the first step of pathogenic bacteria to establish contact with the host tissue, followed by colonization and infection. Given the limited number of available attaching sites, pathogenic bacteria will have lower odds of attaching to the host epithelial tissues if probiotic bacteria have taken those niches. It has been demonstrated that lactobacilli in the vaginal tract are able to adhere to vaginal epithelial cells, which can block receptors and interfere with the adhesion of bacterial pathogens to the epithelium (Boris and Barbés, 2000; Ohland and Macnaughton, 2010; Wagner et al., 2012). Otero and Nader-Macías (2007) reported that *Lactobacillus* spp. isolated from bovine vaginal tract was able to adhere to the vaginal epithelial cells via glycoprotein structure at both pH 4.5 and 7. Interestingly, compounds secreted from probiotic bacteria like *L. helveticus*, called surface layer proteins, can also occupy the binding sites on epithelial cells blocking them from the pathogens (Ohland and Macnaughton, 2010). Such proteins can function as a lining of the epithelial surface; thus, enhancing the epithelial integrity and tight junctions (Sherman et al., 2009). In addition, lactobacilli can competitively exclude the adhesion of pathogens to the epithelium, or even displace the pathogens, which are already bound to the epithelium, due to their greater affinity for the cell receptors (Boris and Barbés, 2000; Ohland and Macnaughton, 2010).

1.4.4 Competition for nutrients

It is believed that the relationship between probiotic and pathogenic bacteria is

competitive (Oelschlaeger, 2010; Boaventura et al., 2012; Hibbing et al., 2010). Bacteria need basic nutrients to grow and proliferate such as nitrogen (N), carbon (C), sulphur (S), and inorganic phosphorous (Pi). Nitrogen and Pi are essential components of nucleic acid and therefore vital for the proliferation of bacteria, especially for those with a short proliferative cycle like *E. coli*. Carbon also is an important component of nucleic acids but also of the energy source from substances like carbohydrates. Sulphur is required for the synthesis of cysteine, a necessary amino acid present in many bacterial proteins. Besides, some microelements like iron, are indispensable for the survival of most microorganisms except for *Lactobacillus* spp. (Oelschlaeger, 2010). Given the shortage of nutrients in the bacterial microenvironment, probiotic bacteria definitely compete for nutrients with pathogenic bacteria limiting their growth potentials.

1.4.5 Production of organic acids

Lactic acid bacteria are well-known for their capability to produce lactic acid from carbohydrate fermentation. Wagner et al. (2012) reported that when cultured with vaginal epithelial cells *L. reuteri* RC-14 and *L. rhamnosus* GR-1 produced lactic acid which inhibited the growth of *C. albicans*. Production of organic acids (lactic acid and acetic acid) lowers the pH of the microenvironment, which can be vital to the growth of most pathogenic bacteria. The maintenance of pH between 4 and 4.5 in the female vaginal tract is attributed to predominance and production of organic acids by lactobacilli (Boris and Barbés, 2000).

1.4.6 Production of hydrogen peroxide

It is believed that lactobacilli predominate microbiota of the female vaginal tract via two major arms, one is the production of organic acids to maintain a low pH; the other one is the production of H_2O_2 (Martín and Suárez, 2010). Wagner et al. (2012) reported that when cultured with vaginal epithelial cells *L. reuteri* RC-14 and *L. rhamnosus* GR-1 produced H_2O_2 which inhibited the growth of *C. albicans*. Studies have shown that lactobacilli isolated from the vaginal tract possess a high capacity of producing H_2O_2, which is highly toxic to adjacent cells including pathogens, in presence of peroxidases (Boris and Barbés, 2000). Nader-Macías et al. (2008) reported that LAB strains isolated from bovine vaginal tract also have a strong capability of producing H_2O_2. H_2O_2-generating lactobacilli from the vaginal tract of cattle have the potential to be utilized as probiotics, among which *L. gasseri* CRL1421 is reported to have the greatest capacity to inhibit *Staphylococcus aureus* by generating H_2O_2 and lactic acid (Otero and Nader-Macías, 2006). Based on the fact that the pH of the bovine vaginal tract is approximately neutral to slightly alkaline, the capacity of producing H_2O_2 probably is a major consideration when screening bacteria for probiotic use.

1.4.7 Production of bacteriocins

Besides generation of organic acids, probiotic bacteria have drawn a lot of attention for their ability to secrete bacteriocins. Bacteriocins are proteinaceous peptides and function by permeating the inner membrane of the targeted

microorganism, causing membrane disruption by pore formation or interfering with the synthesis of cell wall (Ohland and Macnaughton, 2010). Bacteriocins are produced in the primary growth phase, which is different from antibiotics that are chemical compounds produced during secondary metabolism. In addition bacteriocins kill close species, having a narrow antibacterial spectrum, different from the broad antibacterial spectrum of antibiotics. Due to their small size, bacteriocins are heat stable, amphiphilic and can permeate the cellular membrane (Zacharof and Lovitt, 2012). There has been a trend of substituting bacteriocins for antibiotics because bacteriocins can be degraded if orally administered by proteolytic enzymes in the GIT.

Indeed, bacteriocins can be divided into different categories based on the differences of their structures. Class I bacteriocins are small peptides normally less than 5 kDa (Sablon et al., 2000), such as nisin, which is the only bacteriocin officially approved for use in the food industry thus far. The active site of Class I bacteriocins contains lanthionine and therefore they are also known as lantibiotics. Class II bacteriocins are small heat-stable proteins less than 10 kDa (Sablon et al., 2000). Based on the number of peptides and their conformation, class II bacteriocins can further be divided into class IIa – IIe (van Belkum and Stiles, 2000). Pediocin is one of the class IIa bacteriocins which contains only one peptide chain (van Belkum and Stiles, 2000). One of the *Pediococcus* strains used in this project was able to produce pediocin, which could inhibit *E. faecalis* and *L. innocua* (Wang et al., 2013). Class III bacteriocins are large, heat labile proteins greater than 30 kDa (Sablon et al., 2000) and have two subclasses, IIIa and IIIb, due to their different functional mechanisms.

Class IV bacteriocins are complex compounds containing lipid or carbohydrate moieties (Gillor et al., 2007). In contrast to class I, the active sites of class II to IV do not contain lanthionine and therefore are also called non-lantibiotics.

Bacteriocins produced by G- bacteria, mostly *Enterobacteriaceae* are called microcins or colicins. They are synthesized in the ribosome with a low molecular mass and their production increase dramatically under stressful condition, such as a shortage of nutrients (Zschuttig et al., 2012). Microcins can be encoded by chromosomal genes, have a smaller size and the production is not lethal to the producing bacterium; whereas colicins are mostly plasmid encoded, have a large molecular mass and the production is a lethal process to the producing bacterium itself (Gillor et al., 2004; Budič et al., 2011). Both of them interfere with the cytoplasmic membrane and enzymes involved in nucleic acid or protein synthesis of the target bacteria (Gillor et al., 2004; Ohland and Macnaughton, 2010).

The proposed mode of action of LAB is summarized in Table 1-3.

Table 1-3. Proposed modes of action of LAB

Mode of action	Sources
Enhance epithelial barrier function	Bisanz et al., 2014
Stimulate immune system	Kummer et al., 1997; Wagner and Johnson, 2012
Compete for attaching sites	Boris and Barbés, 2000; Otero and Nader-Macías, 2007; Ohland and Macnaughton, 2010; Wagner et al., 2012

Compete for nutrients	Hibbing et al., 2010; Oelschlaeger, 2010; Boaventura et al., 2012
Produce organic acids (acetic acid, lactic acid)	Boris and Barbés, 2000; Wagner et al., 2012
Produce H_2O_2	Otero and Nader-Macías, 2006; Nader-Macías et al., 2008; Boris and Barbés, 2000; Wagner et al., 2012
Produce bacteroicins, e.g. pediocin	Wang et al., 2013

1.5 Research hypothesis and objectives

This project was aimed to test the hypothesis that intravaginal infusion of lactic acid bacteria around calving can lower the incidence rate of uterine infections and improve reproductive performance of postpartum dairy cows.

Therefore the objectives of this study were to evaluate:

1) The effect of intravaginal infusion of LAB on the uterine involution and reproductive performance of postpartum dairy cows, which will be described in chapter 2;

2) The effect of intravaginal infusion of LAB on immune responses and uterine infections of transition dairy cows, which is presented in chapter 3;

3) The effect of intravaginal infusion of LAB on the bacterial composition of vaginal mucus of transition dairy cows, which is addressed in chapter 4; and,

4) The effect of intravaginal infusion of LAB on the metabolite status, milk

composition, and productive performance of transition dairy cows, which will be detailed in chapter 5.

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Chapter 2 Intravaginally administered lactic acid bacteria expedited uterine involution and improved reproductive performance of Holstein dairy cows

ABSTRACT

The objective of this investigation was to evaluate whether intravaginal infusion of lactic acid bacteria (LAB) around parturition could expedite involution rate of the uterus and improve reproductive performance of postpartum dairy cows. One hundred pregnant Holstein dairy cows were assigned to one of the 3 experimental groups as follows: 1) One dose of LAB on wk -2 and -1 and one dose of carrier on wk +1 relative to the expected day of parturition (TRT1); 2) one dose of LAB on wk -2, -1, and +1 (TRT2), and 3) one dose of carrier on wk -2, -1, and +1 (CTR). LAB 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. Uterine involution and ovarian activity was evaluated by transrectal ultrasonography on d 14, 21, 35, and 49 postpartum. Blood samples were collected from a subset of cows to quantify prostaglandin $F_{2\alpha}$ metabolite (PGFM), PGE_2, and progesterone. Results showed that both TRT1 and TRT2 expedited involution of gravid horn and uterine body. Additionally, cows in TRT2 had greater concentration of progesterone in the serum, suggesting an earlier resumption of ovarian cyclicity. Cows in the TRT1 had less days open than those in the CTR (110 vs. 150 d). In addition, both TRT1 and TRT2 increased the concentrations of PGFM at calving week and cows in TRT2 also had greater concentrations of PGE_2 on d 14 and d 21 postpartum relative to CTR. Overall intravaginal infusion of LAB expedited uterine involution, accelerated the resumption of ovarian cyclicity, lowered days open, and modified the concentrations of serum prostaglandins and progesterone.

2.1 Introduction

Uterine infections, which refer to bacterial infections of the uterus, are prevalent in postpartum dairy cows (Sheldon et al., 2006). Normally, during the uterine involution period, the immune system is activated to clear the invading pathogens and most dairy cows self-resolve the infections within 3 weeks after parturition (Bekana et al., 1996; Bondurant, 1999; Sheldon et al., 2006). However, some pathogenic bacteria are not cleared efficiently and persist thereafter causing long-lasting uterine disease.

Infection of the uterus impairs reproductive performance of dairy cows and many of them wind up being subfertile or infertile and are, therefore, culled from the herd (Sheldon et al., 2009a). Although the precise mechanism(s) by which uterine infections influence reproductive performance are not very well understood, they are mostly related to delays in uterine involution, resumption of ovarian activity, and prolongation of the luteal phase once ovulation occurs (Huszenicza et al., 1999; Mateus et al., 2002; Sheldon et al., 2009a). Indeed, cows with uterine infections exhibit lower conception rate, require more services per conception, and have more days open (Kasimanickam et al., 2004; Sheldon et al., 2009a). According to recent statistics the culling rate of dairy cows in Canadian dairy herds reached 41.7% with infertility rated as the number one reason for culling (CanWest DHI, 2013).

To our best knowledge, there is no known efficient treatment or prophylactic medication against uterine infections in dairy cows. Although several intrauterine or intravenous antibiotics have been used to treat cows with uterine infections, their efficacy has been questioned and concerns about drug residue in milk and bacterial

acquisition of antibiotic-resistance have impeded their widespread use (Lewis, 1997; Galvão, 2011). Recently, there is an increasing interest of using lactic acid bacteria (LAB) or probiotics to treat gastrointestinal or vaginal inflammatory conditions (Reid and Bruce, 2003; Ametaj et al., 2014).

Probiotics are live microorganisms that benefit the host in various aspects when administered in adequate amounts, including modifying microbial composition and improving immunity (Reid et al., 2003; Verdu and Collins, 2005; Borchers et al., 2009). Lactic acid bacteria, mainly lactobacilli, have been utilized for treating urogenital infections in humans (Reid and Bruce, 2003). A previous study conducted by our group reported that 6 times treatment around calving with a cocktail of 3 LAB, isolated from the vaginal tract of healthy cows, lowered the incidence of purulent vaginal discharges and improved conception rates of multiparous cows (Ametaj et al., 2014). Based on these results we hypothesized that lowering the number of treatments around calving from 6 times to 2 or 3 treatments might give the same beneficial effects to the cows. Therefore the objectives of this study were to test whether treatment of cows with two doses of LAB (once per week) during the 2 wk before the expected day of calving or treatment with 2 doses of LAB before calving and 1 dose during the first week after calving could improve uterine involution and reproductive performance of postpartum dairy cows.

2.2 Materials and methods

2.2.1 Animals and experimental design

This experiment was conducted at the Dairy Research and Technology Centre (DRTC) at University of Alberta, Canada. 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). One hundred healthy pregnant Holstein cows were blocked according to their parity, body condition score (BCS), and previous lactation milk yield and assigned randomly to one of the following three experimental groups: 1) One dose of LAB on wk -2 and -1 and one dose of carrier (sterile skim milk) on wk +1 relative to the expected day of parturition (TRT1), 2) One dose of LAB on wk -2 and -1 and +1 (TRT2), and 3) One dose of carrier on wk -2, -1, and +1 (CTR). The pre-calving treatment was administered on 14.13 ± 4.67 d and 7.46 ± 4.46 d before the actual calving day, respectively, while the post-calving dose was administered on 7.55 ± 1.29 d after calving. 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. Both LAB and the carrier were stored at -86 ^oC in vials in the form of dry powder, and each vial was reconstituted with 1 mL of sterile 0.9% saline before administration. The LAB or carrier was infused into the vaginal tract gently with individually wrapped sterile infusion tubes (Continental Plastic Corp., Delavan, WI) capped with a 5-mL sterile

syringe (Becton, Dickinson and Company, Franklin Lakes, NJ), and deposited at anterior vagina. Aseptic procedures were maintained during LAB administration.

2.2.2 Determination of uterine involution and resumption of ovarian cyclicity

Uterine involution was blindly evaluated by transrectal ultrasonography. A Sonosite® ultrasound fitted with a 7.5 MHz probe was used to obtain images of the cervix, uterine body and horns, follicles and corpora lutea on ovaries of all cows on a weekly basis beginning on d 7 after calving. However, for expediency, data for d 14.55 ± 1.29, d 21.55 ± 1.29, d 35.55 ± 1.29, and d 49.55 ± 1.29 postpartum were utilized for determination of uterine involution and resumption of ovarian cyclic activity.

The volume of the intrauterine fluid in the uterine body and horns was evaluated and assessed on 3 scales: 1 (little) - small lumen without fluid, 2 (intermediate) - medium lumen with some fluid, 3 (much) - large fluid filled lumen (Mateus et al., 2002). The quality of intrauterine fluid was also assessed on 3 scales based on echogenicity of the lumen of uterus or horns, which reflected the echogenicity of the debris or inflammatory material in the lumen as following: 1 (dark) - anechoic in appearance hence normal, 2 (greyish) - fluid contains some echogenic material hence intermediate, 3 (whitish) - a substantial amount of hyperechogenic particles in the fluid, hence abnormal (McKinnon et al., 1988; Reilas, 2001).

The longest and shortest diameters of a cervical cross-section were measured immediately before the caudal end of the cervix, while that of the uterine body and the

2 horns were measured immediately caudal and cranial to the bifurcation, respectively. As the boundary between myometrium and perimetrium is not so distinguishable as between endometrium and myometrium, 2 perpendicular diameters of the cross-section were measured across the lumen and endometrium to calculate the cross-sectional area of the cervix, uterine body as well as gravid and non-gravid horns (Okano and Tomizuka, 1987; Melendez et al., 2004).

Ovarian structures were examined for presence and sizes of follicles, presence of corpora lutea by ultrasonography to determine the resumption of ovarian cyclicity. If a large follicle (≥ 10 mm in diameter) was followed by a corpus luteum on the same ovary in a 14 d period, or vice versa, then, resumption of ovarian cyclic activity was declared. This was further corroborated by progesterone measurements described below.

2.2.3 Assessment of reproductive performance

Insemination and pregnancy diagnosis records were analyzed retrospectively for all the cows enrolled in this experiment. The reproductive management program at the DRTC utilized the 'Presync+Ovysync' protocol (Ambrose et al., 2006) to time the ovulation on all cows. Therefore, all eligible animals were first inseminated by around 70 d after calving. The median was 69 (66-136) d in TRT 1, 67 (65-97) d in TRT 2, and 68 (66-148) d in the CTR. Pregnancy was declared when the pregnancy check was positive at 60 d after the insemination. Both timed artificial insemination (TAI) and pregnancy check were conducted by the same skilled technician. However, the

technician and management were kept blind about cow treatments during the entire period of the experiment. Reproductive performance was indicated by first service conception rate, cumulative pregnancy rate (up to 5 services), pregnancy rate at 150 DIM, services per conception, and number of days open.

2.2.4 Quantification of hormones

Blood samples were collected from the coccygeal vein once a week with 10-mL vacutainer tubes without anticoagulant (BD Vacutainer Systems, Plymouth, UK) before feeding in the morning from d -14 to d 49 on a weekly basis (actual days: d -14.13 ± 4.67, d -7.46 ± 4.46, d 1.71 ± 1.39, d 7.55 ± 1.29, d 14.55 ± 1.29, d 21.55 ± 1.29, d 28.55 ± 1.29, d 35.55 ± 1.29, d 42.55 ± 1.29, and 49.55 ± 1.29 relative to the real calving day). Blood samples were centrifuged at 3,000 rpm at 4°C for 20 min (Beckman Coulter, Pasadena, California) and serum was stored in pyrogen-free tubes at −20 °C until analysis. A subset of serum samples from 10 cows in each group was used to quantify the concentrations of progesterone, 13,14-dihydro-15-keto-$PGF_{2\alpha}$ (PGFM), indicative of the concentration of $PGF_{2\alpha}$, and PGE_2 with enzyme immuno-assay (EIA) kits following the manufacturer's instructions.

Serum samples collected from d -14 to d 21 on a weekly basis were used to measure the concentration of PGFM with 13,14-dihydro-15-keto Prostaglandin F2α EIA Kits (Cayman Chemical Co., Ann Arbor, MI). Samples were diluted 10-fold with EIA buffer and standards were freshly prepared in serials as instructed in the manufacture book. The limit of detection for PGFM was 13 pg/ml and sensitivity was

120 pg/ml. The quantification of PGE_2 used the same samples following the same procedures as of PGFM with Prostaglandin E2 EIA Kits – Monoclonal (Cayman Chemical Co., Ann Arbor, MI). The limit of detection for PGE_2 was 15 pg/ml and sensitivity was 50 pg/ml. Serum samples collected on d -14, 0, 14, 21, 35, 49 were used to quantify progesterone. Samples were also diluted 10 fold with EIA buffer. The procedures were the same as for PGFM and PGE_2 except that the plate was incubated for 90 min under room temperature instead of 18 hours at 4^oC before the wash using Progesterone EIA Kits (Cayman Chemical Co., Ann Arbor, MI). The limit of progesterone detection was 10 pg/ml and sensitivity was 70 pg/ml. All samples were run in duplicate. The inter- and intra-assay coefficients of variation were less than 10% for all these three hormones.

2.2.5 Statistical Analyses

All data were analyzed with SAS 9.2 software (SAS Institute Inc., Cary, NC). In this study, cows were blocked for parity, BCS, and previous milk yield before being assigned to treatment groups. There was no significant effect of BCS and previous milk yield on the outcome of the results and therefore they were excluded from the statistical model. The health status of the last parity was also tested for differences among the treatment groups and found not to be different. Continuous data, including cross-sectional areas of the cervix, uterine body and horns as well as the concentrations of serum hormones were analyzed using a MIXED model with repeated measurements. For the cross-sectional areas a full model incorporating parity,

treatment, days relative to calving as well as their interaction was firstly tested. There was no significant effect as for the 3-way interaction, the 2-way interaction between parity and group, and that between parity and day. Therefore, the statistical model was reduced as: $Y_{ijkl} = \mu + T_i + D_j + (TD)_{ij} + P_k + e_{ijkl}$, where μ = the overall population mean; T_i = effect of treatment; D_j = effect of days relative to calving; $(TD)_{ij}$ = effect of the interaction between treatment and days relative to calving; P_k = effect of parity; and e_{ijkl} = residual error. The subset of serum samples were randomly selected from multiparous cows, so the statistical model used to analyze the concentrations of serum PGFM and PGE_2 was: $Y_{ijk} = \mu + T_i + D_j + (TD)_{ij} + e_{ijk}$. The covariance structure was modeled using first order autoregressive for the repeated measurements over time.

Categorical data with 3 outcomes, including the volume and quality of intrauterine fluid were analyzed using LOGISTIC procedure incorporating factors parity, treatment, and days relative to calving. Results are presented in odds ratio (OR) to indicate the association between treatment and the likelihood of having a desirable outcome, which means the likelihood of having smaller volume of less echoic intrauterine fluid. Binary data were also analyzed using LOGISTIC procedure. The model for resumption of the ovarian cyclicity incorporated parity, treatment, and days relative to calving. Since no cows resumed cyclicity by d 14, results are presented only for d 21, 35, and 49 postpartum. The models for first service conception rate, cumulative pregnancy rate, and pregnancy rate at 150 DIM incorporated parity and treatment. Non-parametric data on services per conception and days open were analyzed using LIFETEST procedures. Wilcoxon test in the Kaplan-Meier model was

used to test the effect of parity and group.

For all the data, the significance was declared at $P < 0.05$ and tendency at $0.05 \leq P < 0.10$.

2.3 Results

2.3.1 Effect of intravaginal LAB on uterine involution

There was no difference among treatment groups regarding the volume and quality of intrauterine fluid (Table 2-1). The effect of LAB treatment on the cross-sectional areas (CSA) of the uterus is shown in Figure 2-1. The CSA of gravid horns (Figure 2-1A) differed among the 3 treatment groups ($P < 0.05$). Treatment with LAB accelerated the involution of gravid horns as indicated by smaller CSA ($P < 0.05$). In addition, there was a tendency for an interaction between the treatment and day ($P = 0.07$) for CSA. On d 14 postpartum, the CSA of gravid horn, in both TRT1 and TRT2, were smaller than those in the CTR cows. Moreover, on d 21 postpartum, there was a tendency for the CSA of gravid horn of cows in the TRT1 to be smaller than those of cows in the CTR group ($P = 0.07$). Overall, the CSA of gravid horn were decreasing in relation with week ($P < 0.05$). The CSA in both TRT1 and TRT2 plateaued from d 21 through d 49 postpartum, whereas in the CTR group the CSA plateaued from d 35 to d 49. In addition, multiparous cows had a larger gravid horn than primiparous cows ($P < 0.05$, Figure 2-2). The interaction between parity and week exhibited no effect on the uterine involution, and therefore Figure 2 presents the main effect of parity.

The CSA of non-gravid horns decreased over time until d 35 postpartum ($P < 0.01$), but no differences were detected among treatment groups and there was no interaction between the group and day in this study (Figure 2-1B). Multiparous cows had a larger non-gravid horn than primiparous cows ($P < 0.05$, Figure 2-2).

The CSA of uterine body showed differences among the 3 treatment groups ($P < 0.05$, Figure 2-1C). The LAB treatment decreased the CSA of uterine body ($P = 0.01$). There was a significant interaction between treatment and day ($P < 0.05$). On d 14 postpartum, CSA of the uterine body of cows was smaller in TRT1, intermediate in TRT2, and larger in CTR ($P < 0.05$). Overall, CSA of uterine body decreased ($P < 0.01$) over time. The CSA of uterine body in both CTR and TRT1 plateaued from d 35 postpartum, whereas that in the TRT2 tended to plateau from d 21. Multiparous cows had a larger uterine body than primiparous cows ($P < 0.05$, Figure 2-2).

No differences were detected in CSA of the cervix among the 3 treatment groups (Figure 2-1D). The CSA of cervix decreased ($P < 0.01$) gradually over time until d 35 postpartum when it plateaued. No effect of the interaction between the treatment and day was observed on CSA of cervix. Parity did not have an effect on the CSA of cervix (Figure 2-2).

2.3.2 Effect of intravaginal LAB on resumption of ovarian cyclicity

The percentage of cows that resumed ovarian cyclicity differed among the treatment groups ($P < 0.05$), and increased over time ($P < 0.01$) after calving (Figure 2-3). There were less cyclic cows in the TRT1 compared to TRT2 ($P < 0.05$), whereas

no difference was obtained between TRT1 and CTR or TRT2 and CTR cows. By d 49 postpartum, 46.7 ± 6.7% of the cows in TRT1 had resumed ovarian cyclicity compared with 67.8 ± 6.2% in TRT2 and 59.7 ± 6.3% in the CTR cows. Parity had no effect regarding the resumption of ovarian cyclicity of the cows. Moreover, data showed no differences with regards to follicular cyst incidence rate in experimental groups with TRT1, TRT2 and CTR cows having 8.8%, 6.9% and 14.3% of the cows with cysts within 49 days after calving.

2.3.3 Effect of intravaginal LAB on reproductive performance

There were differences among treatment groups in the mean number of days open ($P < 0.05$, Table 2-2). Cows in TRT1 had 40 days shorter than their counterparts in TRT2 (110 vs. 150 d, $P < 0.05$) and the CTR (110 vs. 150, $P < 0.05$). First service conception rate was 44.1 ± 8.5 % in TRT1, 25.0 ± 7.7 % in TRT2, and 38.2 ± 8.3 % in CTR, but did not reach significance. There was no effect of treatment on cumulative pregnancy rate, pregnancy rate at 150 DIM or services per conception.

No interaction between treatment group and parity was observed in this study. Parity was associated with differences of the reproductive performance of dairy cows (Table 2-3). Primiparous cows needed less than 2 services to achieve pregnancy whereas their multiparous counterparts needed around 3 services ($P < 0.05$). Primiparous cows exhibited almost 30% greater pregnancy rate at 150 DIM than multiparous cows (61.5% vs. 90.9%, $P < 0.05$). Cumulative pregnancy rate up to five services tended to be greater in primiparous cows than in multiparous cows

(69.2% vs. 90.9%, $P = 0.06$). However, the differences between primiparous and multiparous cows in terms of first service conception rate and days open did not reach significance.

2.3.4 Effect of intravaginal LAB on reproduction related hormones

Concentrations of serum PGFM were different among the 3 treatment groups ($P < 0.05$) and changed over time ($P < 0.01$, Figure 2-4A). A significant interaction was found between treatment and day ($P < 0.05$). There were no differences in serum PGFM at d -14 and d -7 among treatment groups, and all concentrations were at a low basal level. However, concentrations of serum PGFM increased sharply within the last 7 d before parturition, reached the peak immediately after parturition (d 0), and dropped rapidly to the prepartum level by d 14 postpartum ($P < 0.01$). After parturition (d 0), concentrations of serum PGFM in both TRT1 and TRT2 were greater than in CTR (3,533 ± 328 pg/mL in TRT1, 4,470 ± 372 pg/mL in TRT2, and 2,000 ± 328 pg/mL in CTR, respectively, $P < 0.01$), and TRT2 tended to be greater than TRT1 ($P = 0.06$).

Concentrations of serum PGE_2 differed among the 3 treatment groups ($P < 0.01$, Figure 2-4B). Cows in the TRT2 had greater concentrations of serum PGE_2 than those in the CTR group ($P < 0.05$), and in TRT1 ($P < 0.01$). No differences were found between TRT1 and CTR cows with regards to PGE_2 levels. Overall, concentrations of serum PGE_2 varied over time ($P < 0.01$). It decreased at parturition, and then increased on d 14 and 21 postpartum. The effects of treatment and day were

independent from each other, as their interaction did not reach significance.

The ratio of PGFM/PGE_2 differed among the treatment groups ($P < 0.05$, Figure 2-4C). Both TRT1 (17.18) and TRT2 (17.45) had a greater ratio of PGFM/PGE_2 than the CTR cows (8.59) on d 0. In all the cows, the ratio of PGFM/PGE_2 increased sharply from d -7, reached peak level on d 0 and then gradually decreased to the prepartum level by d 14 ($P < 0.01$). No interaction between treatment and day was observed in terms of the ratio of PGFM/PGE_2.

Concentrations of serum progesterone differed among treatment groups ($P < 0.01$) and varied over time ($P < 0.01$, Figure 2-5). Cows in the TRT2 had greater concentrations of progesterone than those in TRT1 and CTR on d 35 and d 49. There was no difference between cows in the TRT1 and CTR group. There was a sharp drop of progesterone concentrations from greater than 7 ng/mL on d -14 to less than 1 ng/mL at calving, and then remained at a basal level until 21 d after calving when an increase occurred. No differences were detected regarding the interaction between treatment and days related to calving.

2.4 Discussion

In this study we tested the hypothesis that 2 or 3 intravaginal infusions of LAB around calving would expedite uterine involution and improve reproductive performance of dairy cows. The probiotic culture used in this study was a mixture of 3 LAB strains isolated previously from the vaginal tract of healthy pregnant cows (Wang et al., 2013; Ametaj et al., 2014). Indeed data from this study showed that

administration of 2 or 3 LAB doses intravaginally expedited uterine involution of postpartum dairy cows as indicated by smaller CSA of gravid horn and uterine body and earlier involution of gravid horn in cows treated with LAB (both TRT1 and TRT2).

Normally, the postpartum uterus is assumed to complete involution between 4 and 6 wk postpartum (Sheldon, 2004). Results of this study showed that the involution process of the gravid horn was completed within the first 21 d after parturition for the LAB-treated groups of cows. This is supported by the observation that there were no significant changes in the CSA of the gravid horn after 21 d postpartum in LAB-treated cows. Meanwhile CTR cows completed their uterine involution within 35 d from parturition day.

Several factors have been indicated as influential on the uterine involution rate in the postpartum cows such as breed, parity, environment, calving season, milk yield, and diet (Fonseca et al., 1983; Eduvie et al., 1985; Zain et al., 1995). However given that all the cows in this study were of the same breed, shared the same environmental and management conditions, and were fed the same diet during each period those factors can be excluded from our discussion. In addition milk yield from previous year and feed intake and BCS were not different among the three groups of cows in this study (data not presented). Parity showed an effect on the involution rate of gravid and non-gravid horns as well as on uterine body, which has been partitioned from the total effect. Therefore, our search regarding the potential mechanisms underlying the expedited uterine involution was focused on the health condition of the

reproductive tract. Indeed cows treated with LAB had lower incidence of uterine infections compared with cows in the control group.

Kindahl et al. (1999) suggested a relationship between the status of uterine health and $PGF_{2\alpha}$ levels by indicating that pulsatile elevations of $PGF_{2\alpha}$ are negatively correlated with involution time in normal cows and positively correlated in cows with endometritis and retained placenta. $PGF_{2\alpha}$ plays an important role in expediting postpartum uterine involution as well as clearing the fetal remnants and birth canal-ascended bacteria in the reproductive tract due to its efficacy in stimulating myometrial contractions. Consequently, we looked at the concentration of $PGF_{2\alpha}$ in the serum as indicated by the concentration of its metabolite PGFM (Sears et al., 2002; Lewis, 2003). Indeed, cows in both TRT1 and TRT2 had greater concentrations of PGFM relative to the CTR cows immediately after parturition (d 0), which supported our speculation that the expedited involution was probably related to increased secretion of $PGF_{2\alpha}$. These data are in agreement with those of Lindell et al. (1982) who reported that cows with longer duration of the postpartum $PGF_{2\alpha}$ release had relatively shorter involution times. Seals et al. (2002) also showed that low concentrations of PGFM during the first 14 d postpartum seem to be an indicator of cows that are susceptible to uterine infections during the third week or so postpartum. Therefore the finding of lowered uterine infection in LAB-treated cows is in alignment with the greater $PGF_{2\alpha}$ and expedited uterine involution in cows treated with LAB.

Another important prostaglandin involved in the uterine health and reproductive

performance is PGE_2. Cows in the TRT2 exhibited elevated serum PGE_2, which was not observed in cows in the TRT1. PGE_2 has been demonstrated to induce production of progesterone from luteal cells of the ovaries and vice versa progesterone was found to stimulate PGE_2 production by the same cells in vitro (Kotwica et al., 2003). That might account for the greater concentration of progesterone in TRT2 than both TRT1 and CTR cows.

In cattle, $PGF_{2\alpha}$ is luteolytic, it is a strong trigger of uterine motility, and has pro-inflammatory activity; whereas PGE_2 is luteotropic, myorelaxant and has anti-inflammatory activity (Slama et al., 1991; Lewis, 2003). Several reports indicate that bacterial infections switch prostaglandin production from PGFM to PGE_2, which means a lower ratio of PGFM/PGE_2 (Herath et al., 2009; Sheldon et al., 2009b). In this study, the ratio of PGFM/PGE_2 was greater in cows treated with LAB vs CTR ones immediately after parturition, which can be regarded as an indicator of uterine environment less favorable to bacterial infections. Indeed, our clinical data showed that cows treated with LAB had lower incidence of uterine infections (data presented in Chapter 3).

Cows in the TRT2 also were found to have greater numbers with earlier ovarian cyclicity at 21, 35, and 49 d postpartum compared with cows in the TRT1. In addition cows in the TRT2 had numerically greater number of cows that resumed ovarian cyclicity earlier than CTR cows, although the difference did not reach significance. This was corroborated by the greater concentrations of progesterone in the blood of cows in the TRT2. Greater blood progesterone might help cows come in estrus earlier

than those with lower progesterone levels and improve their fertility (Inskeep, 2004). Several investigators reported that the number of cows that do not resume estrous cycle by 60-d postpartum ranges between 6-59% in high-producing dairy herds (Cerri et al., 2004; Santos et al., 2009). Those cows have lower odds to become pregnant and have greater pregnancy losses after the first insemination (Stevenson et al., 2006).

One of the most important findings of this study was that TRT1 lowered the number of days from calving to conception (i.e., days open) by 40 d (110 vs. 150 d) compared with CTR cows; however, TRT2 did not give such benefit to the treated cows. The reason for this difference in days open between the two treatment groups is not clear and deserves further investigation. Greater days open is associated with reduced profitability in dairy herds. Numerous studies have documented that additional days in which cows are not pregnant beyond the optimal time post-calving are costly to the dairy farm (Groenendaal, et al., 2004; Meadows et al., 2005). In this study, cows in the CTR group exhibited a first service conception rate of 38.2%, which is in agreement with a previous report indicating that the overall conception rate to first insemination of dairy herds in Alberta is 38.4% (Ambrose and Colazo, 2007). Of note, no significant effects on first service conception rate were observed among the treatments in this study. Meanwhile, primiparous cows exhibited better reproductive performance than multiparous cows in this study, which is consistent with many other studies reporting that the fertility of dairy cows decreases with age (Tenhagen et al., 2004; Balendran et al., 2008; Bonneville-Hébert et al., 2011).

2.5 Conclusions

Taken together results of this study indicated that both LAB-treated groups expedited involution rates of the gravid horn and uterine body and increased concentrations of PGFM in the serum immediately after parturition. Administration of LAB before calving (TRT1) shortened the number of days open in the treated cows. However, administration of LAB around calving (TRT2) was associated with earlier ovarian cyclicity, and greater concentrations of PGE_2 and progesterone in the serum postpartum. Differences might be related to alterations in the vaginal microbiota composition induced by the two treatments in the vaginal tract of supplemented cows. Overall administration of vaginal LAB could be used to expedite uterine involution and improve reproductive performance of transition dairy cows. However, more research is warranted to better understand the mechanism(s) by which LAB supplemented in the vaginal tract expedite involution rate of the reproductive tract and improve reproductive performance of transition dairy cows.

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Table 2-1. Effect of treatment on intrauterine fluid of dairy cows

	OR[1]				*P* -
Variable	(TRT1[2] vs. CTR[3])	95% CL[4]	(TRT2[5] vs. CTR)	95% CL	value
Volume of intrauterine fluid	1.30	0.74 - 2.28	1.33	0.73 - 2.42	0.56
Quality of intrauterine fluid	1.43	0.79 - 2.58	1.54	0.82 - 2.91	0.33

[1]OR: odds ratio.

[2]TRT1: two prepartum doses of LAB, $n_1 = 34$.

[3]CTR: carrier only, $n_3 = 34$.

[4]95% CL: 95% Wald confidence limits.

[5]TRT2: two prepartum doses plus one postpartum dose of LAB, $n_2 = 32$.

Table 2-2. Effect of treatment on reproductive performance of dairy cows

Variable	TRT1[1]	TRT2[2]	CTR[3]	*P*-value
First service conception rate, %	44.1 ± 8.5	25.0 ± 7.7	38.2 ± 8.3	0.33
Cumulative pregnancy rate[4], %	76.5 ± 7.3	71.9 ± 8.0	73.5 ± 7.6	0.99
Pregnancy rate at 150 DIM, %	70.6 ± 7.8	62.5 ± 8.6	70.6 ± 7.8	0.91
Mean services per conception	2.4 ± 0.3	2.7 ± 0.2	2.3 ± 0.2	0.29
Median services per conception	2.0	3.0	2.0	
25th percentile	1.0	1.5	1.0	
75th percentile	5.0	.	.	
Mean days open, d	110 ± 8 ^{b}	150 ± 11 ^{a}	150 ± 13 ^{a}	0.04
Median days open, d	100	146	138	
25th percentile	70	104	72	
75th percentile	170	215	215	

[1]TRT1: two prepartum doses of LAB, $n_1 = 34$.

[2]TRT2: two prepartum doses plus one postpartum dose of LAB, $n_2 = 32$.

[3]CTR: carrier only, $n_3 = 34$.

[4]Cumulative pregnancy rate: up to five AI.

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

Table 2-3. Effect of parity on reproductive performance of dairy cows

Variable	Multiparous[1]	Primiparous[2]	*P*-value
First service conception rate, %	33.3 ± 5.3	45.5 ± 10.6	0.44
Cumulative pregnancy rate[3], %	69.2 ± 5.5	90.9 ± 6.1	0.06
Pregnancy rate at 150 DIM, %	61.5 ± 5.2 [b]	90.9 ± 6.1 [a]	0.02
Mean services per conception	2.9 ± 0.2 [a]	1.7 ± 0.2 [b]	0.02
Median services per conception	2.0	2.0	
25th percentile	1.0	1.0	
75th percentile	.	2.0	
Mean days open, d	146 ± 8	111 ± 9	0.24
Median days open, d	134	109	
25th percentile	79	70	
75th percentile	215	145	

[1]Multiparous: $n_1 = 78$.

[2]Primiparous: $n_2 = 22$.

[3]Cumulative pregnancy rate: up to five AI.

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

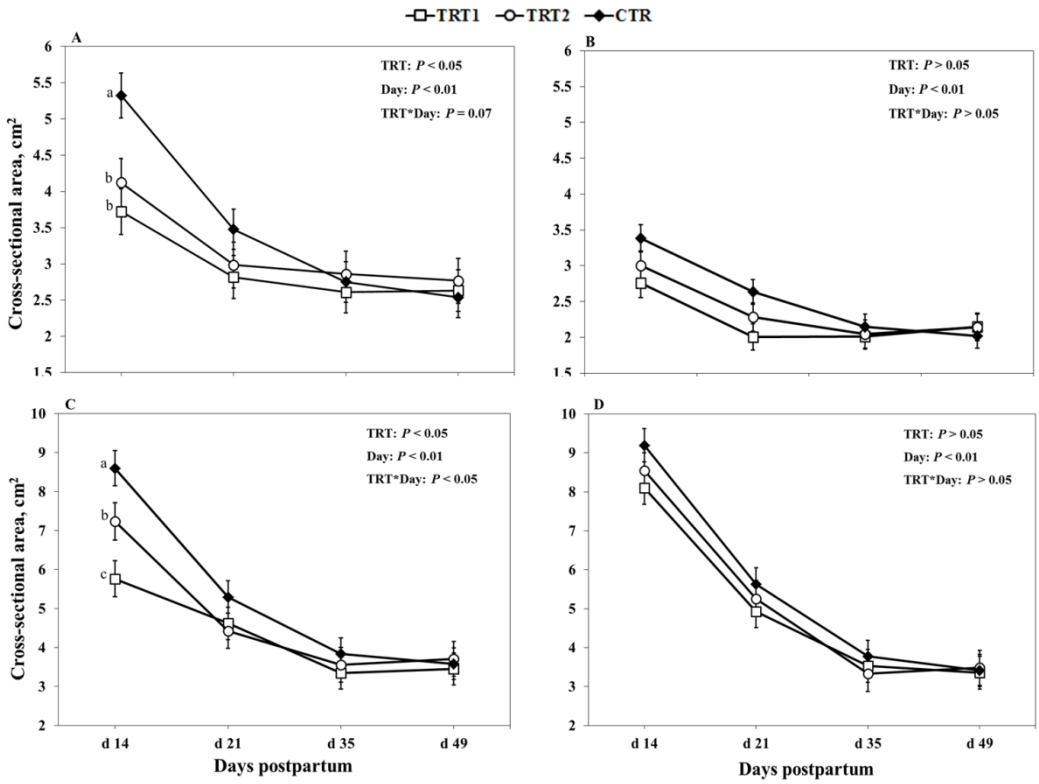

Figure 2-1. Effect of treatment on the uterine involution of postpartum dairy cows (LSM ± SEM. A: gravid horn; B: non-gravid horn; C: uterine body; D: cervix. TRT = effect of LAB treatment; Day = effect of days relative to calving; TRT * Day = effect of the interaction between treatment and day. TRT1: □, two prepartum doses of LAB, $n_1 = 34$; TRT2: ○, two prepartum doses plus one postpartum dose of LAB, $n_2 = 32$; CTR: ♦, carrier only, $n_3 = 34$).

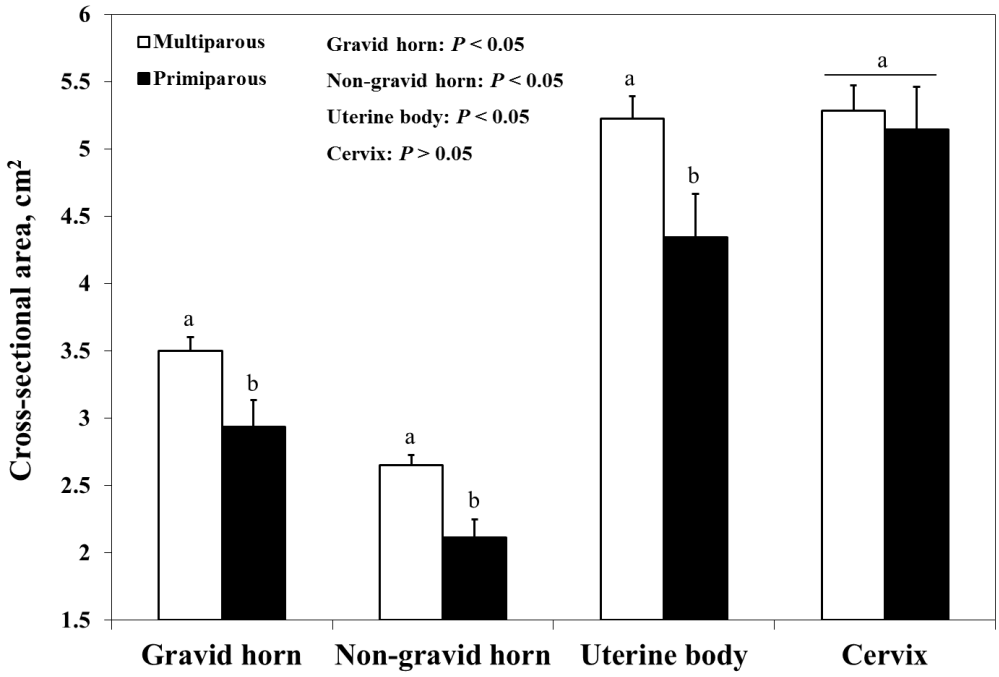

Figure 2-2. Effect of parity on the involution of the uterus of postpartum dairy cows (LSM + SEM. Multiparous: □, $n_1 = 78$; Primiparous: ■, $n_2 = 22$).

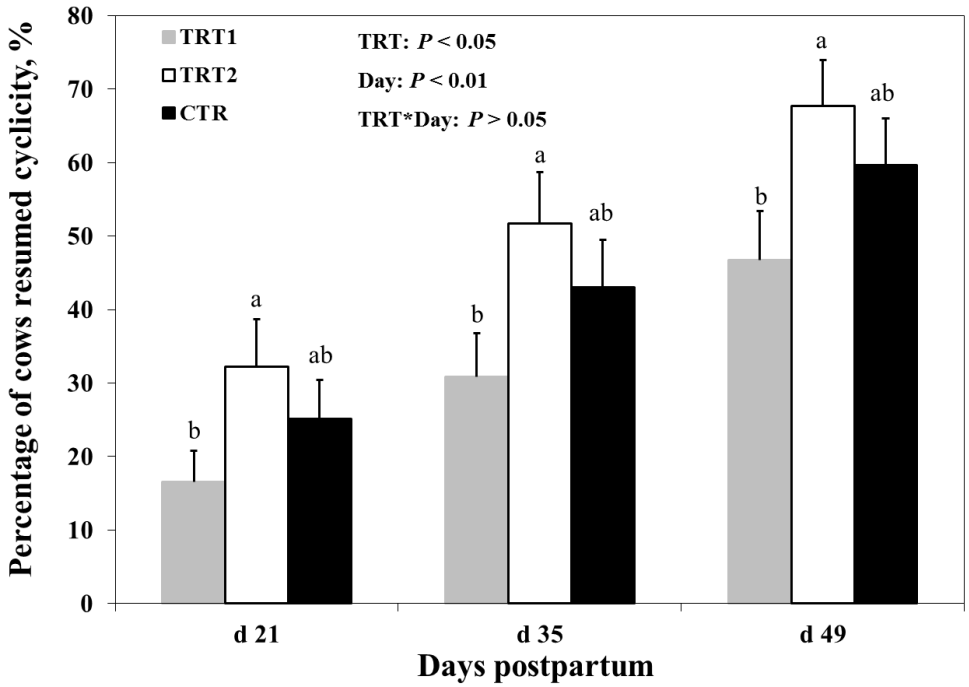

Figure 2-3. Effect of treatment on the resumption of ovarian cyclicity after parturition (predicted probability + standard error. TRT = effect of LAB treatment; Day = effect of days relative to calving; TRT * Day = effect of the interaction between treatment and day. TRT1: ■, two prepartum doses of LAB, $n_1 = 34$; TRT2: □, two prepartum doses plus one postpartum dose of LAB, $n_2 = 32$; CTR: ■, carrier only, $n_3 = 34$).

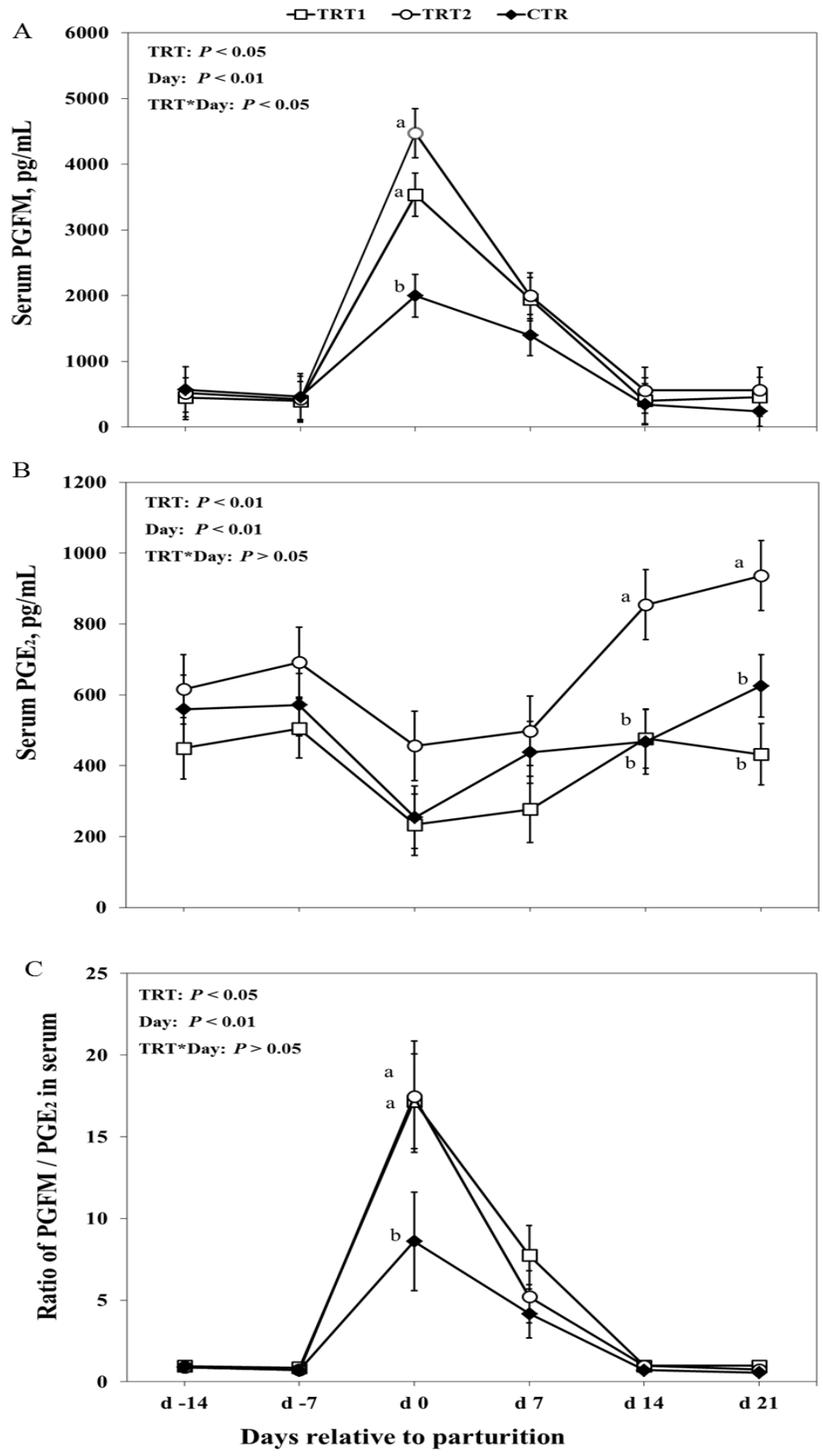

Figure 2-4. Effect of treatment on serum prostaglandins (LSM ± SEM. A: PGFM; B:

PGE_2; C: $PGFM/PGE_2$. TRT = effect of LAB treatment; Day = effect of days relative to calving; TRT * Day = effect of the interaction between treatment and day. TRT1: □, two prepartum doses of LAB, n_1 = 10; TRT2: ○, two prepartum doses plus one postpartum dose of LAB, n_2 = 10; CTR: ♦, carrier only, n_3 = 10).

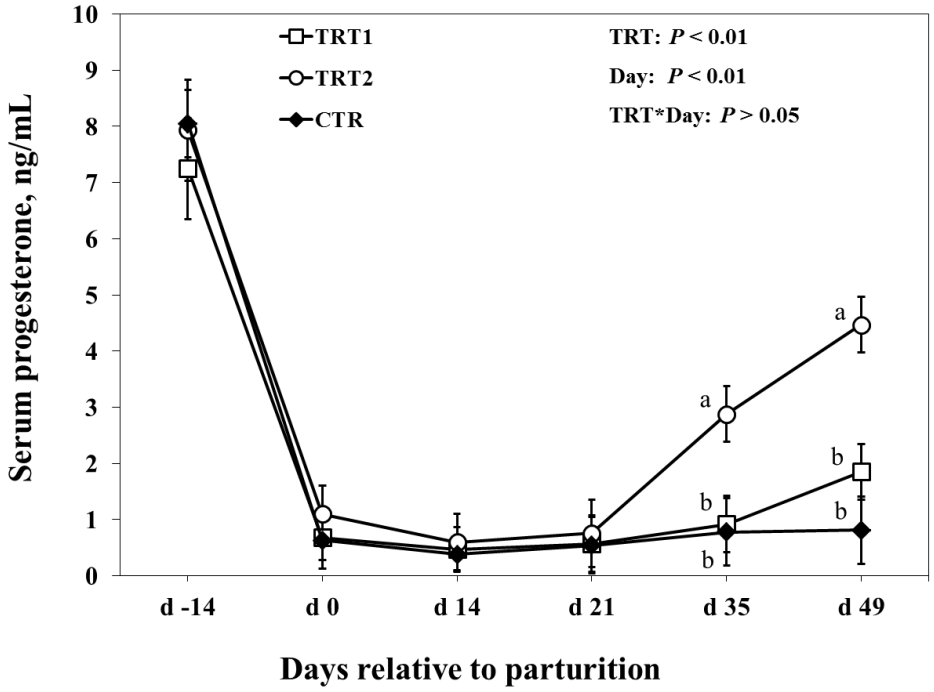

Figure 2-5. Effect of treatment on the concentration of serum progesterone (LSM ± SEM. TRT = effect of LAB treatment; Day = effect of days relative to calving; TRT * Day = effect of the interaction between treatment and day. TRT1: □, two prepartum doses of LAB, n_1 = 10; TRT2: ○, two prepartum doses plus one postpartum dose of LAB, n_2 = 10; CTR: ♦, carrier only, n_3 = 10).

Chapter 3 Intravaginally administered lactic acid bacteria modulated local and systemic immune responses and lowered the incidence of uterine infections in transition dairy cows

ABSTRACT

The objective of this investigation was to evaluate whether intravaginal infusion of a lactic acid bacteria (LAB) cocktail around parturition could influence the immune response, incidence rate of uterine infections, and the overall health status of periparturient dairy cows. One hundred pregnant Holstein dairy cows were assigned to 1 of the 3 experimental groups as follows: 1) one dose of LAB on wk -2 and -1, and one dose of carrier on wk +1 relative to the expected day of parturition (TRT1); 2) one dose of LAB on wk -2, -1, and +1 (TRT2), and 3) one dose of carrier on wk -2, -1, and +1 (CTR). LAB were a lyophilized culture mixture composed of *Lactobacillus sakei* FUA3089, *Pediococcus acidilactici* FUA3138, and *Pediococcus acidilactici* FUA3140 with a cell count of 10^8-10^9 cfu/dose. Blood samples and vaginal mucus were collected once a week from wk -2 to +3 and analyzed for content of serum total immunoglobulin G (IgG), lipopolysaccharide-binding protein (LBP), serum amyloid A (SAA), haptoglobin (Hp), tumor necrosis factor (TNF), interleukin (IL)-1, IL-6, and vaginal mucus secretory IgA (sIgA). Clinical observations including rectal temperature, vaginal discharges, retained placenta, displaced abomasum, and laminitis were monitored from wk -2 to +8 relative to calving. Results showed that intravaginal LAB lowered the incidence of metritis and total uterine infections. Intravaginal LAB also were associated with lower concentrations of systemic LBP, an overall tendency

for lower SAA, and greater vaginal mucus sIgA. No differences were observed for serum concentrations of Hp, TNF, IL-1, IL-6 and total IgG among the treatment groups. Administration with LAB had no effect on the incidence rates of other transition cow diseases. Overall intravaginal LAB lowered uterine infections and improved local and systemic immune responses in the treated transition dairy cows.

3.1 Introduction

Dairy cows undergo an immunosuppressive state around parturition associated with impairment in leukocyte functions (Burton et al., 2001; Herr et al., 2011). Several investigators have reported that although the phagocytic activity of neutrophils remains high, their bactericidal capacity is impaired, especially after parturition (Zerbe et al., 1996; Mateus et al., 2002; Hammon et al., 2006). In addition, concentrations of IgG and IgM in the blood reach the lowest concentrations at calving (Herr et al., 2011). Moreover, there is lower γ-globulin content in the uterine secretions suggesting a decrease in the local bactericidal activity (Hussain, 1989).

The state of immunosuppression in transition cows is associated with high incidence of bacterial infections especially of the uterus (metritis) and mammary gland rendering cows more vulnerable to periparturient diseases. Almost 40% of periparturient dairy cows are affected by clinical metritis during the first 3 wk after calving and another 15-20% by endometritis more than 3 wk after parturition (Sheldon et al., 2009a). Uterine infections predispose dairy cows to impaired reproductive performance and are the number one reason for culling of cows in

Canadian dairy herds (CanWest DHI and Valacta, 2013). Infection of the uterus is accompanied by systemic fluctuations of inflammatory cytokines like TNF, IL-1, IL-6, and APP such as lipopolysaccharide-binding protein (LBP), serum amyloid A (SAA), and haptoglobin (Hp) (Kim et al., 2005; Zeng et al., 2009; Chan et al., 2010). Currently there is no efficient treatment for uterine infections. Although various therapies have been used in the past involving antibiotics, iodine solutions, and hormone treatments, for different reasons, they have not been successful enough to be widely accepted by veterinary practitioners (Burton and Lean, 1995; Lewis, 1997; Azawi, 2008).

On the other hand, a new line of research is growing with the use of probiotic agents as an alternative to antimicrobial compounds. Probiotics are live microorganisms, which confer a health benefit to the host when administered in adequate amounts (WHO/FAO, 2002). They have demonstrated the ability to enhance immune functions such as increasing the number of immune cells and modulating expression of cytokines or antibody production in the host (Cross, 2002; Galdeano and Perdigón, 2006; Madson, 2006). Research conducted in human subjects has indicated that probiotics administered in the vagina have been able to lower the incidence of vaginal infections in women (Reid and Bruce, 2003; Falagas et al., 2006). However, there is a lack of research in dairy cattle regarding the utilization of probiotics to lower the incidence of uterine infections and improve reproductive efficiency. In a recent study, we reported that cows treated intravaginally with 2 prepartum and 4 postpartum doses (on a weekly basis) of a mixture of 3 LAB, isolated

from the vaginal tract of healthy pregnant cows, had lower incidence of purulent vaginal discharges and lower concentration of serum Hp than the control animals (Ametaj et al., 2014). In this study, we hypothesized that a lower number of treatments with LAB might confer the same results on uterine health and immune status of transition dairy cows. Therefore, the objectives of this study were to test whether lowering the treatment frequency of intravaginal administration of LAB to 2-3 doses around calving will enhance local and systemic immune responses and lower the incidence of uterine infections and potentially other periparturient diseases of transition dairy cows.

3.2 Materials and methods

3.2.1 Animals and experimental design

One hundred pregnant Holstein cows were allocated (based on parity, body condition score, and milk yield) to 1 of the 3 experimental groups to receive intravaginal LAB or carrier (sterile skim milk) during the transition period as following: treatment 1 (TRT1) - 2 consecutive LAB doses (on a weekly basis) starting at 2 wk before the expected day of parturition and 1 carrier dose the week after parturition; treatment 2 (TRT2) - 3 consecutive LAB doses (2 doses during the 2 wk before the expected day of parturition and 1 dose the week after parturition); control (CTR) - 3 consecutive carrier doses around parturition starting at 2 wk before the expected day of parturition up to 1 wk after calving. The actual days of pre-calving treatments were on d -14.13 ± 4.67 and d -7.46 ± 4.46 relative to the real calving day

respectively, while the post-calving treatment was administered on d 7.55 ± 1.29. The LAB were composed of *Lactobacillus sakei* FUA3089, *Pediococcus acidilactici* FUA3138 and FUA3140, which were stored in sterile skim milk with a cell count of 10^8-10^9 cfu. Both probiotics and carrier were lyophilized and stored at -86 ^oC in vials, and each vial was reconstituted in 1 mL sterile 0.9% saline before administration. The LAB or carrier were infused into the vaginal tract gently with 18'' individually wrapped sterile drilled infusion tubes (Continental Plastic Corp., Delavan, WI) capped with a 5-mL sterile screw tip syringe (Becton, Dickinson and Company, Franklin Lakes, NJ), and deposited at anterior vagina. All the procedures were maintained aseptic during administration.

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).

3.2.2 Clinical observations and measurements

All cows were monitored clinically from -2 wk before the expected day of parturition and up to +8 wk after parturition. Both ultrasound scanning and clinical evaluation was conducted blindly by the evaluators. Rectal temperature was measured twice a week and fever was declared when it was greater than 39.5 ^oC. Retained placenta was declared if a cow did not expel the placenta within 24 hours after parturition. A Sonosite® (MicroMaxx, SonoSite, Inc., Bothell, Washington) ultrasound fitted with a 7.5 MHz probe was used on +2, +3, +5, and +7 wk to assess

the uterine size and intrauterine fluid. Uterine infections were categorized into different classes. A metritic case was diagnosed if the cow had reddish brown vaginal discharge with fetid odor, together with fever and an abnormally large uterus and decreased feed intake and milk production within 3 wk after parturition (Sheldon et al., 2006). If a cow still had purulent or mucopurulent exudate in the vagina more than 3 wk after parturition, in the absence of systemic illness, she was declared having clinical endometritis (Sheldon et al., 2006). A cow with accumulated purulent materials in the uterine lumen in the presence of a persistent CL was declared having pyometra (Sheldon et al., 2006). Both clinical endometris and pyometra were monitored until wk +7 postpartum. Total uterine infections were defined as the sum of metritis, clinical endometrtis, and pyometra. Displaced abomasum was diagnosed by a veterinary practitioner based on both clinical signs, including abnormal sound over the left side of the abdomen with a stethoscope, and the history of the animal. A displaced abomasum was declared by combining the diagnosis result and the veterinary visit records and treatment records of the barn. Lameness was recorded if a cow stood or walked in an abnormal gait, such as reluctance to bear weight on a hoof, or a noticeable limp with uneven steps, especially when she was observed to have a reddish, swollen, hot foot and retracted her foot when touched on the wall of corium. Then the diagnosis of laminitis was conducted by a skilled veterinary practitioner by checking if the corium was swollen or bleeding and also if pulses in the lower limb arteries were prominent. The diagnosed result was then combined with the veterinary visit records and treatment records of the barn to declare a case of laminitis.

Subclinical mastitis was declared if somatic cell count (SCC) in milk was more than 200,000 cells/mL (Schukken et al., 2003; Sharma et al., 2011).

3.2.3 Sampling and laboratory analyses

Blood samples were collected from the coccygeal vein once a week in the morning before feeding with 10-mL vacutainer tubes without anticoagulant (BD Vacutainer Systems, Plymouth, UK) from -2 to +3 wk around calving on d -14.13 ± 4.67, d -7.46 ± 4.46, d 1.71 ± 1.39, d 7.55 ± 1.29, d 14.55 ± 1.29, and d 21.55 ± 1.29 relative to the real calving day. Blood samples were centrifuged at 2,090 x g and 4 °C for 20 min to separate the serum (Beckman Coulter, Pasadena, California). Serum samples were stored at -20 °C until analysis. Vaginal mucus was sampled using individually wrapped sterile drilled infusion tubes (Continental Plastic Corp., Delavan, WI) capped with a 5-mL sterile screw tip syringe (Becton, Dickinson and Company, Franklin Lakes, NJ), and then gently flushed into a sterile tube with 1 mL 0.9% saline. A subset of samples from 10 cows from each group was randomly selected for laboratory analyses.

Concentrations of lipopolysaccharide binding protein (LBP) in the serum were measured with a commercial sandwich ELISA kit for bovine LBP (Hycult Biotech, Uden, Noord-Brabant, The Netherlands) according to the manufacturer's instructions. Serum samples were diluted 1:100; then, samples and standards were loaded and incubated to allow LBP to be captured by bovine monoclonal antibodies coated on the plate. After washing, detection antibodies labeled with biotin and horseradish

peroxidase (HRP) labeled with streptavidin were loaded to bind the captured LBP. A washing followed each loading. The fixed HRP catalyzed a chromogenic reaction with the subtract 3,3', 5,5'-tetramethylbenzidine (TMB). After adding the stop solution, the plate was read at 450 nm on a microplate spectrophotometer (Spectramax 190, Molecular Devices Corporation, Sunnyvale, CA) within 10 min. The optical density (OD) values were positively correlated with the concentrations of LBP in the sample. The detection range of LBP is between 1.6 and 100 ng/mL. The OD values of samples were within the range of standard curve. All samples were run in duplicate. The inter- and intra-assay coefficients of variation (CVs) were less than 10%.

Concentrations of serum amyloid A (SAA) in the serum were measured with a commercial sandwich bovine ELISA kit (Tridelta Development Ltd., Maynooth County Kildare, Ireland) as per manufacturer's instructions. Serum amyloid A in the samples was captured by both the anti-SAA monoclonal antibodies immobilized on the plate and free anti-SAA monoclonal antibodies labeled with HRP, which catalyzed a chromogenic reaction. The chroma of this enzymatic reaction was proportional to the concentration of SAA in the sample. All serum samples were diluted 1:500 before the assay and the OD values were read on a microplate spectrophotometer (Spectramax 190, Molecular devices Corporation, Sunnyvale, CA) at 450 nm within 10 min after adding the stop solution. The OD values of all diluted samples were within the range of standard curve. According to the manufacturer, the analytical sensitivity of the assay for bovine is 1.5 μg/mL. All samples were run in duplicate.

The inter- and intra-assay CVs were less than 10%.

Concentrations of haptoglobin (Hp) in the serum were measured with a commercially available kit (Tridelta Development Ltd., Maynooth, County Kildare, Ireland). The assay principle is that the free hemoglobin exhibits peroxidase activity, which is inhibited at a low pH. Haptoglobin binds to hemoglobin and preserves its peroxidase activity at a low pH. The preserved peroxidase activity of hemoglobin is proportional to the amount of Hp in the sample. The OD values of this chromogenic enzymatic reaction were read on a microplate spectrophotometer (Spectramax 190, Molecular devices Corporation, Sunnyvale, CA) at 600 nm 5 min after adding the last reagent. According to the manufacturer, the analytical sensitivity is 0.005 mg/ml, and the inter- and intra-assay CV was less than 10%. All samples were run in duplicate.

Concentrations of TNF in the serum were measured with a bovine sandwich ELISA kit (Bethyl Laboratories Inc., Montgomery, TX). Briefly, TNF present in samples and standards is captured by bovine monoclonal antibodies coated on the plate. Then, the captured TNF binds to detection antibodies labeled with HRP. The addition of substrate TMB triggers a chromogenic enzymatic reaction catalyzed by HRP. The color is positively correlated with the concentration of TNF in the sample. The OD values were read at 450 nm with a spectrophotometer (Spectramax 190, Molecular Devices Corporation, Sunnyvale, CA) within 10 min after adding the stop solution. The detection range of TNF is between 0.078 and 5 ng/mL. All samples were run in duplicate. The inter- and intra-assay CVs were less than 10%.

Concentrations of interleukin 1 (IL-1) in the serum were measured with a

competitive inhibition bovine ELISA kit (Cusabio Biotech Co., Ltd, Wuhan, Hubei, China). Interleukin-1 in the serum samples and standards competed with biotin-conjugated IL-1 to bind IL-1 antibodies coated on the plate. The greater the amount of IL-1 in the sample, the less antibodies bound by biotin-conjugated IL-1. Then, the biotin conjugated avidin is combined with HRP, which catalyzes a chromogenic reaction. The chroma of the color develops in opposite to the amount of IL-1 in the sample. The OD values were measured at 450 nm with a spectrophotometer (Spectramax 190, Molecular Devices Corporation, Sunnyvale, CA) within 10 min after adding the stop solution. No significant cross-reactivity or interference was observed. According to the manufacturer, the minimum detectable dose of bovine IL-1 is less than 250 pg/mL. All samples were run in duplicate. The inter- and intra-assay CVs were less than 10%.

Concentrations of IL-6 in the serum were measured with a sandwich ELISA kit for bovine IL-6 (Uscn Life Science Inc., Wuhan, Hubei, China). The principle of the assay is similar with that of IL-1. The assay was done with original serum samples without dilution and the concentrations of all samples were within the range of the standard curve. This assay has high sensitivity and specificity for detection of bovine IL-6. According to the manufacturer, the minimum detectable amount of bovine IL-6 is less than 3.3 pg/mL. All samples were run in duplicate. The inter- and intra-assay CVs were less than 10%.

Concentrations of total IgG in the serum were measured with bovine IgG (total) ELISA kits (Alpha Diagnostic International Inc., San Antonio, TX). The principle of

the assay is similar with that of IL-1. Serum samples were originally diluted 1:100,000 by three dilutions before the assay. The OD values of this chromogenic enzymatic reaction were read on a microplate spectrophotometer (Spectramax 190, Molecular Devices Corporation, Sunnyvale, CA) at 450 nm within 30 min after adding the stop solution. According to the manufacturer, the sensitivity of this assay is 5 ng/ml and has less than 1% cross-reactivity with serum IgG from other animals. All samples were run in duplicate. The inter- and intra-assay CVs were less than 10%.

The concentrations of secretory immunoglobulin A (sIgA) in the vaginal mucus were measured with competitive inhibition bovine ELISA kits (Cusabio Biotech Co., Ltd, Wuhan, Hubei, China). The principle of the assay is similar with that of IL-1. Mucus samples were vortexed and centrifuged at 2,090 x g for 20 min (Beckman Coulter, Pasadena, California), and then the supernatant was collected and diluted 1:100 before the assay. According to the manufacturer, the minimum detectable dose of bovine sIgA is less than 2.4 μg/mL. No significant cross-over or interference between bovine sIgA and analogues was observed. All samples were run in duplicate. The inter- and intra-assay CVs were less than 10%.

3.2.4 Statistical analyses

In this study, cows were blocked by parity, BCS, and previous milk yield before being assigned to the treatment groups. All block factors were tested and found to not be associated with the outcome of the results and therefore were excluded from the model. The reason that parity did not show an effect might be attributed to the

small number of heifers enrolled in this study (22 in total). The health status of the last parity was also tested and found not to be different between the treatment groups.

Data of rectal temperature and serum variables, including concentrations of LBP, SAA, Hp, TNF, IL-1, IL-6, and total IgG, as well as the concentration of sIgA in the vaginal mucus were analyzed using SAS 9.2 software (SAS Institute Inc., Cary, NC). MIXED procedure with repeated measurement was used to test the model as following: $Y_{ijk} = \mu + T_i + W_j + (TW)_{ij} + e_{ijk}$, where μ = the overall population mean; T_i = the effect of treatment; W_j = the effect of week; $(TW)_{ij}$ = the interaction between treatment and week; and e_{ijk} = residual error. The covariance structure for each variable was modeled separately according to the smallest values of the fit statistics based on the Bayesian information criteria. Binary data of diseases were analyzed using procedure FREQ with Fisher's Exact Test to test the effect of treatment. Significance was declared at $P < 0.05$, and tendency at $0.05 \leq P < 0.10$.

3.3 Results

3.3.1 Effect of intravaginal LAB treatment on uterine infections and other periparturient diseases

The results of clinical observation of periparturient diseases are presented in Table 3-1. Data showed that LAB treated cows had lower incidence rate of metritis compared with those in the CTR group ($P < 0.01$). TRT1 and TRT2 lowered the incidence rate of metritis by 23% ($P < 0.05$) and 32% ($P < 0.01$), compared with the CTR group, respectively. Although there were no differences in terms of the incidence

of clinical endometritis and pyometra, LAB-treated cows had a lower incidence of total uterine infections composed of metritis, clinical endometritis, and pyometra, compared with the CTR cows (P = 0.01). Both TRT1 and TRT2 had a numerically lower incidence of retained placenta than CTR cows (3% in TRT1, 6% in TRT2, and 13% in CTR cows). During our experimental period, no cases of displaced abomasum were diagnosed out of 66 LAB-treated cows, but 2 out of 32 cows (6%) in the CTR group. However, this difference did not reach a significant level. There was no difference in the incidence rate of subclinical mastitis indicated by a SCC of > 200,000 cells/mL. No difference was observed also regarding the incidence rates of laminitis among the treatment groups.

Overall there was an increase of approximately 0.3 ^{o}C in the rectal temperature of dairy cows after calving compared with pre-calving (P < 0.01, Figure 3-1). However, the rectal temperature was not affected by the treatment and or the interaction between treatment and week.

3.3.2 Vaginal immune responses to intravaginal LAB treatment

Concentrations of sIgA in the vaginal mucus were affected by LAB treatment (P < 0.01), week (P < 0.01), and the interaction between treatment and week (P < 0.01, Figure 3-2). At wk 0, both TRT1 and TRT2 had greater concentrations of sIgA than cows in the CTR group, but no difference between TRT1 and TRT2 was evidenced. At wk +1, TRT1 had greater concentrations of sIgA than both TRT2 and CTR cows (P < 0.01), whereas cows in the TRT2 had greater sIgA in the vaginal

mucus compared to those in the CTR group ($P < 0.01$). At wk +2, TRT1, but not TRT2, had greater concentrations of sIgA than the CTR groups ($P < 0.01$). However, no differences were detected among treatment groups at wk -1 and +3.

3.3.3 Systemic immune responses to intravaginal LAB treatment

Concentrations of LBP in the serum varied with both treatment ($P < 0.05$) and week ($P < 0.01$, Figure 3-3). Also, there was an interaction between treatment and week ($P < 0.05$). At wk 0, TRT1 had lower (3.8 ± 0.8 vs. 8.7 ± 1.3 μg/mL, $P < 0.01$), and TRT2 tended to have lower (5.9 ± 0.8 vs. 8.7 ± 1.3 μg/mL, $P = 0.08$) concentrations of LBP in the serum compared with the CTR cows. At wk +2, cows in both TRT1 and TRT2 had lower concentrations of LBP relative to those in the CTR group (2.1 ± 0.9, 5.8 ± 0.8, and 9.2 ± 1.4 μg/mL in TRT1, TRT2, and CTR, respectively, $P < 0.05$).

Concentrations of SAA in the serum tended to differ among the treatment groups ($P = 0.07$, Figure 3-4). Week also had an impact on SAA ($P < 0.01$). Moreover treatment tended to interact with week ($P = 0.07$). At wk 0, cows in the TRT1 tended to have lower concentrations of SAA in the serum compared to those in the CTR group (20.1 ± 3.3 vs. 31.4 ± 5.0 μg/mL, $P = 0.06$). No difference was detected among treatment groups at other weeks. Serum SAA declined at wk -1, followed by an increase from wk 0 to wk +2, and then dropped to a level comparable to wk -1 ($P < 0.01$).

Concentrations of Hp in the serum fluctuated with week ($P < 0.01$), but not

among the treatment groups or the interaction between week and treatment (Figure 3-5). Concentrations of Hp in the serum were 182 ± 64 μg/mL, 279 ± 52 μg/mL, and 317 ± 67 μg/mL in TRT1, TRT2, and CTR groups, respectively, ($P > 0.05$).

Concentrations of TNF in the serum showed a difference with the factor week ($P < 0.05$) but not with the treatment (Figure 3-6). Concentrations of TNF were 200 ± 64 pg/mL in TRT1, 123 ± 60 pg/mL in TRT2, and 153 ± 84 pg/mL in the CTR group ($P > 0.05$). Concentrations of IL-1 in the serum did not exhibit differences among the treatment groups, but varied with week ($P < 0.01$, Figure 3-7). There also was an interaction between treatment and week ($P < 0.01$). No differences among treatment groups or in relation with week were observed regarding the concentration of IL-6 in the serum (Figure 3-8). Overall, concentrations of IL-6 in the serum were 23 ± 9.6 pg/mL in TRT1, 22 ± 9.9 pg/mL in TRT2, and 18 ± 11.4 pg/mL in CTR ($P > 0.05$).

Concentrations of total IgG in the serum did not show differences among treatment groups, but varied with week ($P < 0.01$, Figure 3-9). No significant effect with regards to interaction between treatment and week was observed. The concentrations of IgG were 24 ± 0.9, 24 ± 1.1, and 23 ± 0.9 mg/mL in TRT1, TRT2, and CTR, respectively. Concentrations of IgG in the serum decreased slightly at wk -1, and then gradually increased after calving ($P < 0.01$).

3.4 Discussion

This study was conducted to test the hypothesis that intravaginal administration of LAB around calving can lower the incidence of uterine infections,

modulate local and systemic immune responses, and improve the overall health of transition dairy cows. Indeed data showed that cows treated with LAB had lower incidence of metritis and total uterine infections and improved systemic and local innate and adaptive immune responses. However, intravaginally supplemented LAB, at the dose and frequency used in this study, had no effect on the incidence of other periparturient diseases.

One of the most important finding of this study was that the incidence of metritis and total uterine infections were lowered by administration of LAB in the treated cows. Of note, in this study the uterine size was measured by both rectal palpation and ultrasonic imaging. Ultrasonic imaging also was used to monitor the intrauterine fluid and the exudate in the vaginal tract. In addition, rectal temperature and vaginal discharges were taken and observed twice a week to assist diagnosis of uterine infections until 7-wk postpartum. The lower incidence rates of metritis and total uterine infections obtained in this study confirmed our previous finding that intravaginal LAB lowers purulent vaginal discharges in the treated cows (Ametaj et al., 2014). *Lactobacillus* spp., such as *L. rhamnosus* GG, *L. rhamnosus* GR-1, *L. fermentum* RC-14, and *L. acidophilus* are well-known for their ability to maintain and restore a normal vaginal microflora and therefore have been used to prevent and treat urogenital infections in women (Reid et al., 2001; Gardiner et al., 2002; Reid et al., 2003). Other than the oral route, *Lactobacillus* spp. has been administered directly in the vagina attenuating or treating symptoms of vaginal infections (Reid et al., 1994; Hilton et al., 1995). The results of this study imply that intravaginal administration of

LAB confers a health benefit to the reproductive tract against bacterial infections of dairy cows.

Another important finding of this study was that infusion of LAB in the vaginal tract of cows increased concentrations of sIgA in the vaginal mucus. Secretory IgA is recognized as the most important mucosal immunoglobulin of mucosal tissues. The mechanism by which LAB increased sIgA is not fully understood; however, there are reports demonstrating that commensal bacteria can stimulate the production of sIgA with the involvement of local epithelial cells and dendritic cells (DCs) (Thomas and Versalovic, 2010). A great proportion of sIgA in the vaginal mucus originates from local production and not from plasma (Kutteh et al., 1996). The LAB infused in the vagina of the treated cows in our experiment are commensal bacteria identified and isolated from healthy vaginal tracts of cows at our dairy farm as previously described by Wang et al. (2013) and Ametaj et al. (2014). Usually the host recognizes pathogen-associated molecular patterns (PAMP) like LPS, lipoproteins, peptidoglycans, other polysaccharides, and repetitive protein structures from pathogenic bacteria as ‘danger signals’ and responds by various immune mechanisms including production of specific sIgA (Medzhitov and Janeway, 2000). The major function of sIgA is to control the adhesion and uptake of mucosal organisms including commensal bacteria. Therefore, the increased secretion of sIgA in the vaginal mucus induced by administration of LAB can be regarded as strengthening of barrier functions in the vagina. An interesting feature of sIgA is that they are non-inflammatory because they prevent bacterial invasion and colonization by

forming immune aggregation and have no complement-activating capability (Russell et al., 1999; Boullier et al., 2009). In addition, Boullier et al. (2009) found that sIgA was able to dampen the inflammation at mucosal tissues. The LAB-treated cows had greater concentrations of sIgA in the vaginal tract on wk 0 (immediately after calving), +1 and +2. This was probably due to the invasion of pathogenic bacteria into the reproductive tract during this period, as Kaila et al. (1992) found that *Lactobacillus* could promote the development of sIgA specific-antibody producing cells and therefore enhance the secretion of local sIgA in the presence of pathogenic bacteria.

Another important finding of this study was that concentrations of LBP in the serum were lowered by intravaginal administration of LAB. One of the main functions of LBP is to facilitate the transfer of LPS to macrophages or lipoproteins so that it could be cleared from the systemic circulation (Mathison et al., 1993; Wurfel et al., 1994). Lipopolysaccharide binding protein is a positive APP elevated in case of inflammatory conditions in ruminants (Ametaj et al., 2011). Bacterial infections of the uterus are commonly present in the postpartum dairy cows, which are associated with histological lesions and inflammation of the uterine tissue (Sheldon et al., 2006). In the first week postpartum, the inflammation is usually confined within the uterus. However, if the local immune responses are not able to resolve the infection, which is usually the case, bacterial endotoxins and locally produced inflammatory cytokines are absorbed into the blood circulation (Righter et al., 1975; Olsson et al., 1996; Kucharski et al., 2008), triggering systemic inflammatory responses as indicated by elevated concentrations of APP and cytokines in the blood (Chan et al., 2010;

Kasimanickam et al., 2013). The reason for lower concentrations of LBP might be related to the beneficial effects of LAB on improving mucosal immunity of the reproductive tract and lowering of the amount of LPS entering the systemic circulation, hence lessened systemic inflammation.

Meanwhile, concentrations of serum SAA tended to be lower in LAB treated cows; however, the difference did not reach significance. It is important to note that concentrations of SAA in the serum of LAB-treated cows were lower compared to CTR cows on wk 0 (week of parturition). Serum amyloid A is another APP produced by hepatocytes which can increase up to 1000-fold in the blood within hours following infections (Williams et al., 2007). Meanwhile, it is also an apolipoprotein associated with high-density lipoproteins (HDL). High-density lipoproteins exhibit a high binding ability to LPS due to their elevated phospholipid content (Kitchens et al., 2003). Therefore, in the presence of LPS, SAA-containing HDL binds to LPS forming a complex, and then removing it from circulation and disposing it in tissues that express lipoprotein receptor, mainly in the liver (Munford, 2005; Laugerette et al., 2011; Shao et al., 2012). Consequently, LPS is removed from hepatocytes through excretion into the bile (Maitra et al., 1981). The tendency for a lower concentration of SAA in the serum of treated cows is another evidence of a lessened systemic inflammation in the LAB-treated cows.

Haptoglobin is another acute phase protein known for its binding to hemoglobin and preventing iron utilization by bacteria. Although Hp is considered a sensitive APP for bacterial infections of the uterus in ruminants (Ametaj et al., 2011;

Ametaj et al., 2014), the concentrations of Hp in the serum were not different between the LAB treated groups and the CTR group. No differences were detected among treatment groups in terms of concentrations of TNF, IL-1, and IL-6 in the serum of cows. Concentrations of total IgG in the serum also were not affected by the LAB treatment in this study. Moreover, administration of LAB in the vaginal tract of transition cows did not have an effect on the incidence of other periparturient diseases like retained placenta, displaced abomasum, subclinical mastitis, and laminitis.

3.5 Conclusions

The results of this study indicated that intravaginal administration of LAB lowered the incidence of uterine diseases in the treated cows and altered their mucosal and systemic immune responses. Lowered incidence rates of metritis and total uterine infections of postpartum dairy cows were associated with enhanced sIgA production in the vaginal mucus. Cows administered intravaginally with LAB had lower systemic inflammation as denoted by lower concentrations of LBP and SAA in the serum of the treated cows. It can be concluded that intravaginal administration of LAB holds the potential to improve immune status and lower the risk of uterine infections of transition dairy cows.

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Table 3-1. Effect of treatment on periparturient diseases of transition dairy cows

Variable	TRT1[1]	TRT2[2]	CTR[3]	*P*
Metritis, % (case/total)	15 (5/34)[b]	6 (2/32)[b]	38 (12/32)[a]	< 0.01
Clinical endometritis, % (case/total)	6 (2/34)	9 (3/32)	13 (4/32)	> 0.05
Pyometra, % (case/total)	3 (1/34)	6 (2/32)	3 (1/32)	> 0.05
Total uterine infections, % (case/total)[4]	24 (8/34)[b]	22 (7/32)[b]	53 (17/32)[a]	< 0.05
Retained placenta, % (case/total)	3 (1/34)	6 (2/32)	13 (5/32)	> 0.05
Displaced abomasum, % (case/total)	0 (0/34)	0 (0/32)	6 (2/32)	> 0.05
Subclinical mastitis, % (case/total)[5]	40 (8/20)	50 (10/20)	55 (11/20)	> 0.05
Laminitis, % (case/total)	9 (3/34)	9 (3/32)	6 (2/32)	> 0.05

[1] TRT1: two prepartum doses of LAB.

[2] TRT2: two prepartum doses plus one postpartum dose of LAB.

[3] CTR: carrier only.

[4] Total uterine infections comprised metritis, clinical endometritis and pyometra.

[5] Subclinical mastitis was declared when somatic cell count in milk was greater than 200,000/mL within five weeks after parturition.

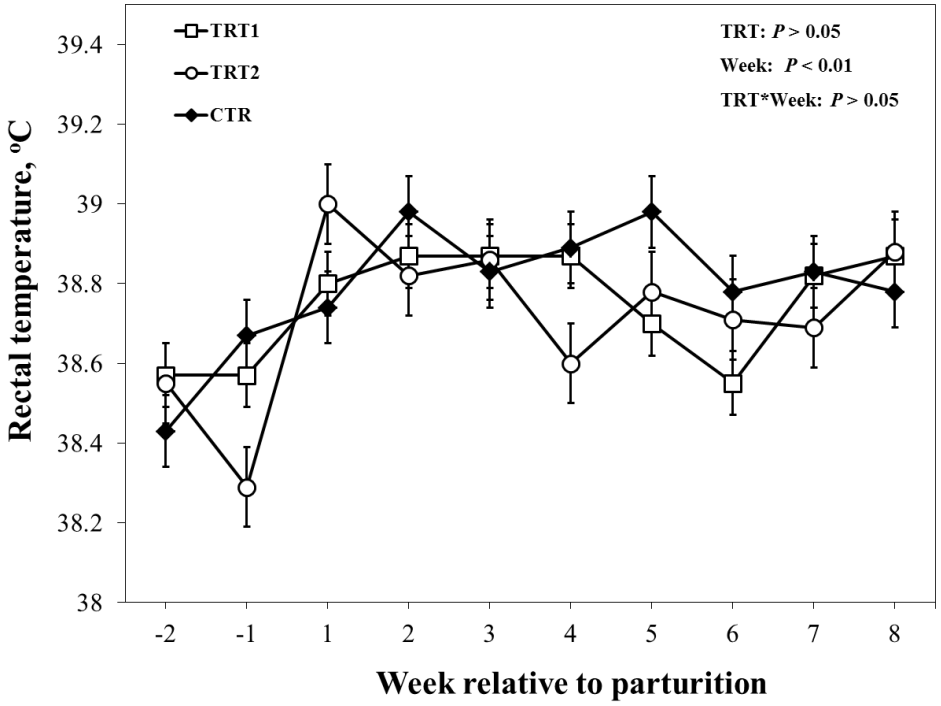

Figure 3-1. Effect of treatment on the rectal temperature of dairy cows (LSM ± SEM. TRT = effect of treatment; Week = effect of week; TRT * Week = effect of the interaction between treatment and week. TRT1: □, two prepartum doses of LAB, n_1=34; TRT2: ○, two prepartum doses plus one postpartum dose of LAB, n_2=32; CTR: ♦, carrier only, n_3=32).

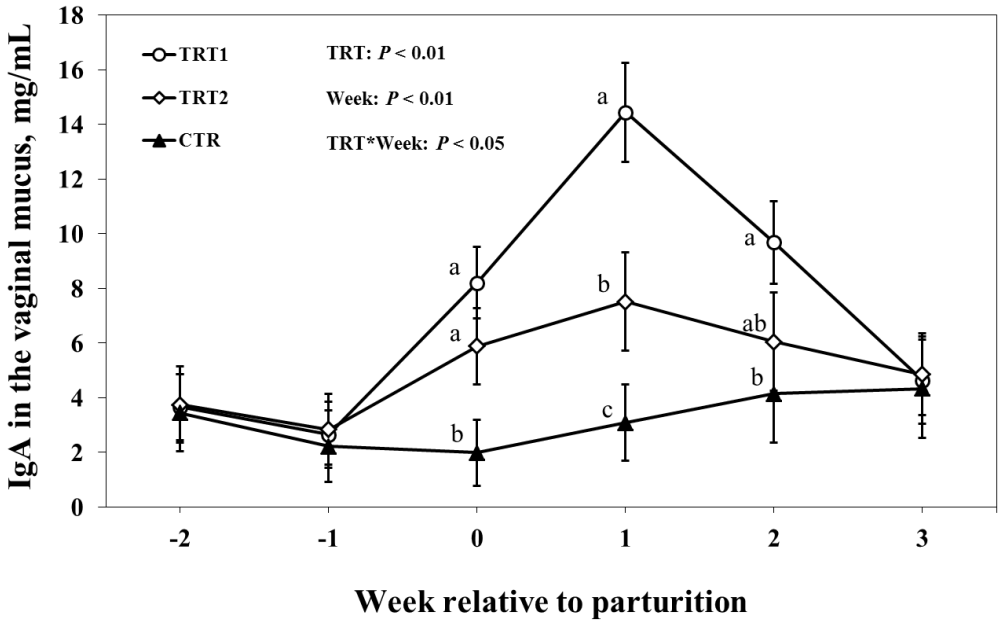

Figure 3-2. Effect of treatment on the concentration of sIgA in the vaginal mucus (LSM ± SEM, n = 10 in each group. sIgA: secretory immunoglobulin A. TRT = effect of treatment; Week = effect of week; TRT * Week = effect of the interaction between treatment and week. TRT1: □, two prepartum doses of LAB; TRT2: ○, two prepartum doses plus one postpartum dose of LAB; CTR: ♦, carrier only).

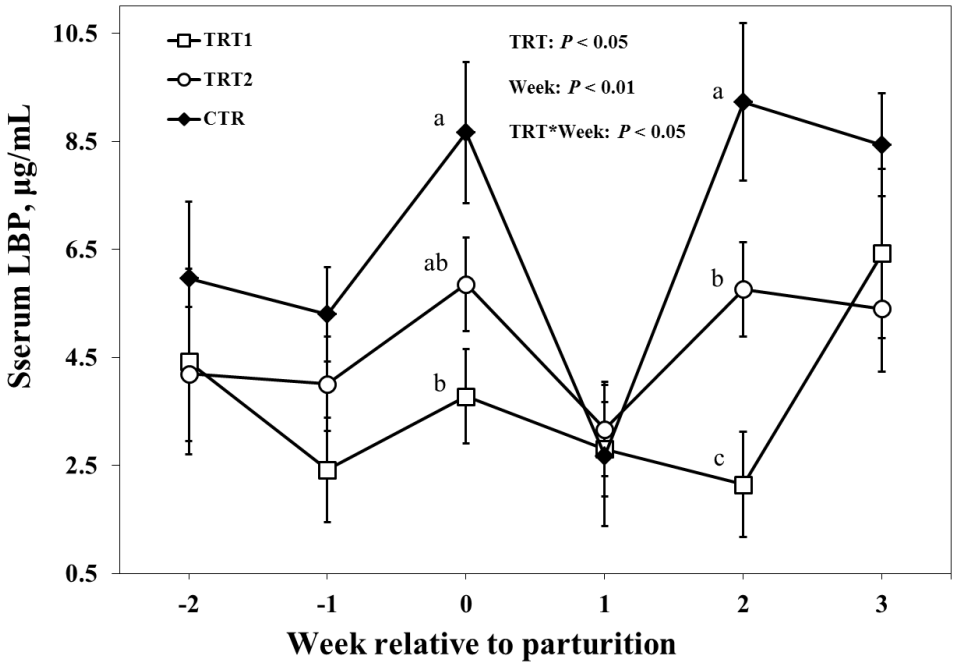

Figure 3-3. Effect of treatment on the concentration of LBP in the serum (LSM ± SEM, n = 10 in each group. LBP: lipopolysaccharide binding protein. TRT = effect of treatment; Week = effect of week; TRT * Week = effect of the interaction between treatment and week. TRT1: □, two prepartum doses of LAB; TRT2: ○, two prepartum doses plus one postpartum dose of LAB; CTR: ♦, carrier only).

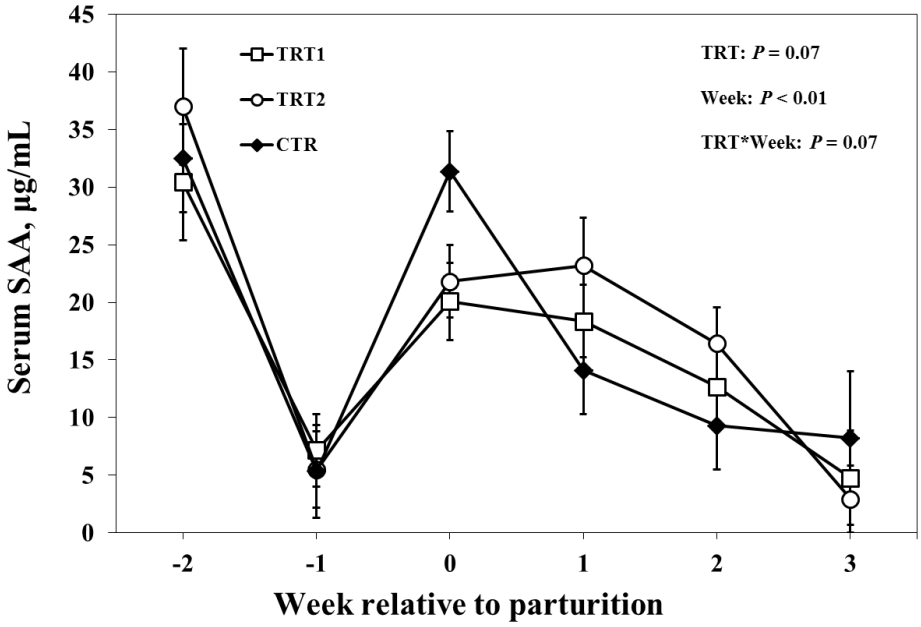

Figure 3-4. Effect of treatment on the concentration of SAA in the serum (LSM ± SEM, n = 10 in each group. SAA: serum amyloid A; TRT = effect of treatment; Week = effect of week; TRT * Week = effect of the interaction between treatment and week. TRT1: □, two prepartum doses of LAB; TRT2: ○, two prepartum doses plus one postpartum dose of LAB; CTR: ♦, carrier only).

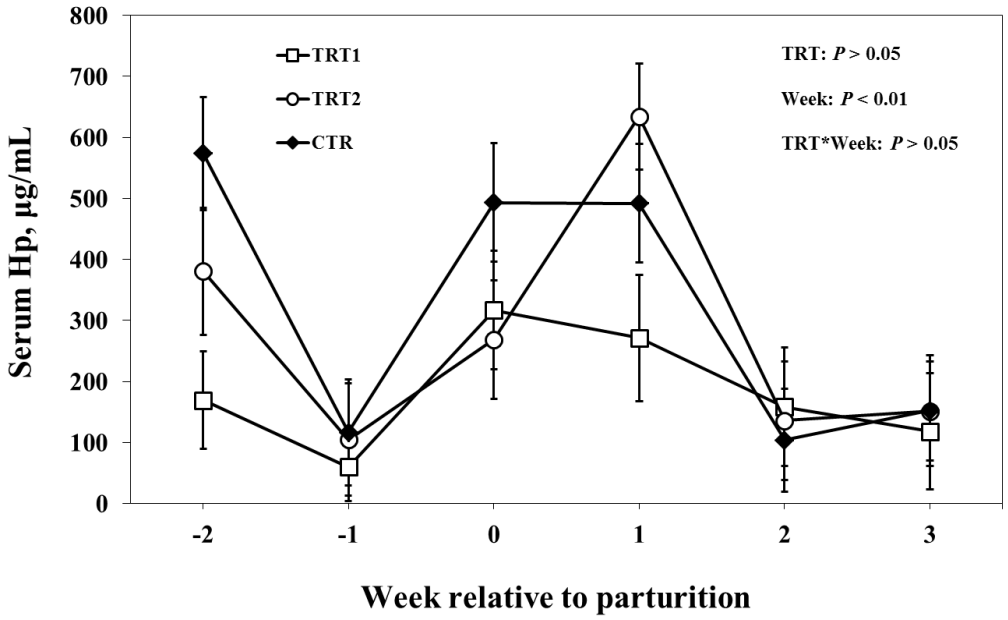

Figure 3-5. Effect of treatment on the concentration of Hp in the serum (LSM ± SEM, n = 10 in each group. Hp: haptoglobin; TRT = effect of treatment; Week = effect of week; TRT * Week = effect of the interaction between treatment and week. TRT1: □, two prepartum doses of LAB; TRT2: ○, two prepartum doses plus one postpartum dose of LAB; CTR: ♦, carrier only).

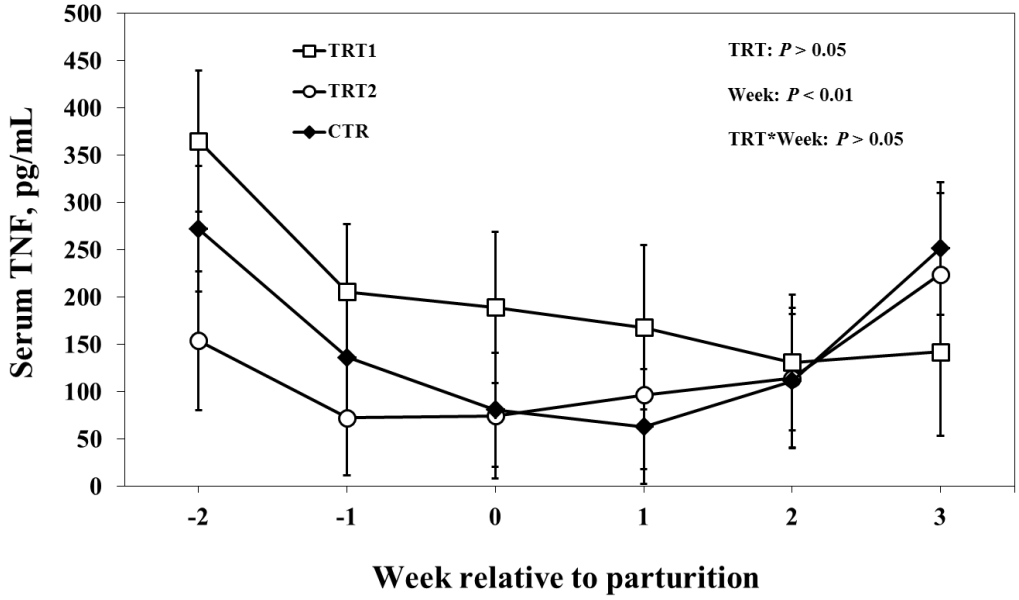

Figure 3-6. Effect of treatment on the concentration of TNF in the serum (LSM ± SEM, n = 10 in each group. TNF: tumor necrosis factor; TRT = effect of treatment; Week = effect of week; TRT * Week = effect of the interaction between treatment and week. TRT1: □, two prepartum doses of LAB; TRT2: ○, two prepartum doses plus one postpartum dose of LAB; CTR: ♦, carrier only).

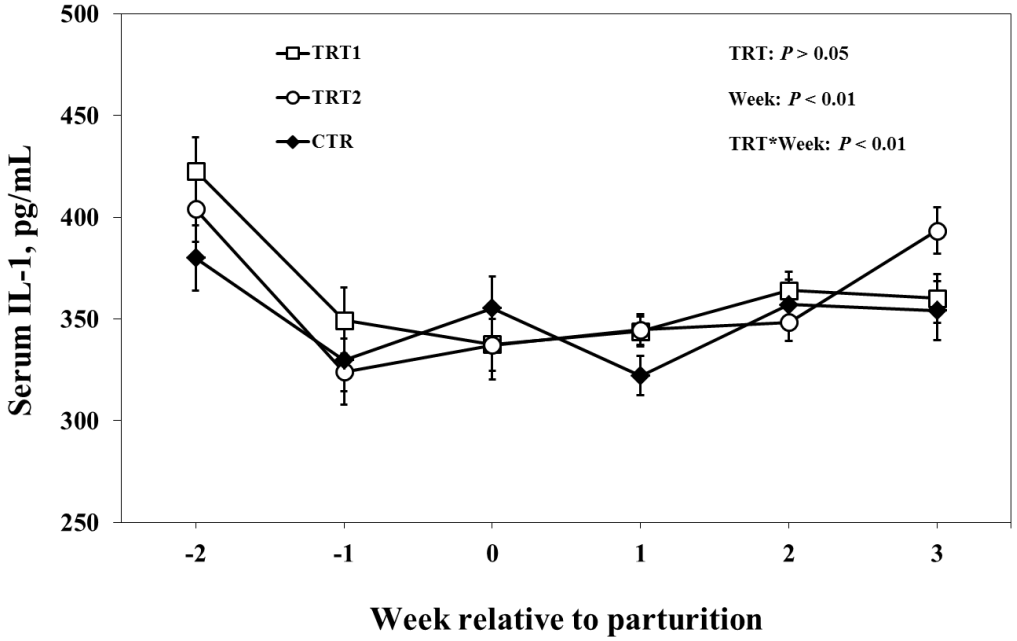

Figure 3-7. Effect of treatment on the concentration of IL-1 in the serum (LSM ± SEM, n = 10 in each group. IL-1: interleukin-1; TRT = effect of treatment; Week = effect of week; TRT * Week = effect of the interaction between treatment and week. TRT1: □, two prepartum doses of LAB; TRT2: ○, two prepartum doses plus one postpartum dose of LAB; CTR: ♦, carrier only).

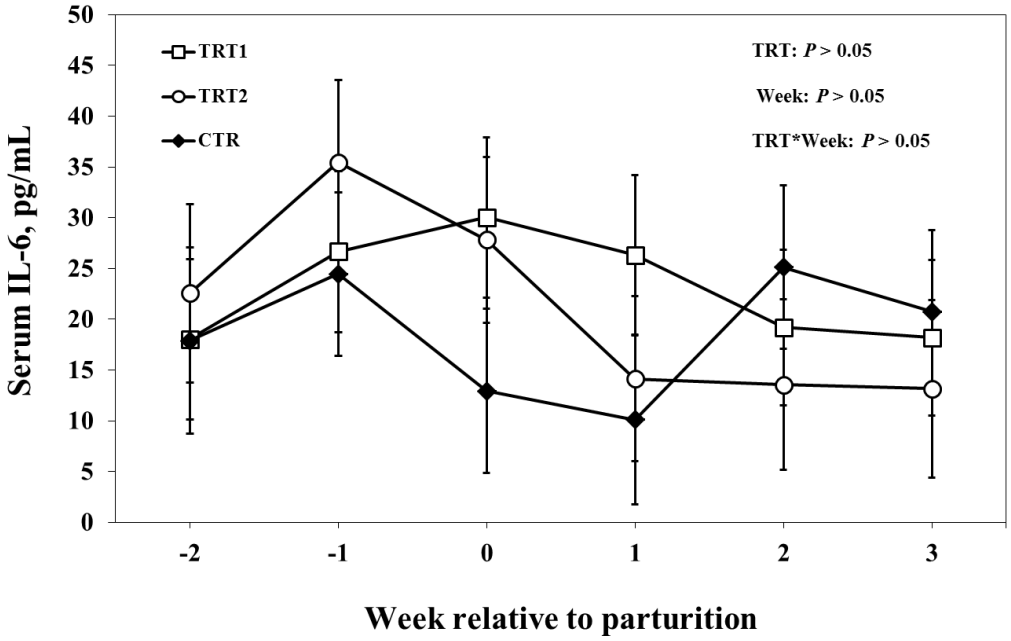

Figure 3-8. Effect of treatment on the concentration of IL-6 in the serum (LSM ± SEM, n = 10 in each group. IL-6: interleukin-6; TRT = effect of treatment; Week = effect of week; TRT * Week = effect of the interaction between treatment and week. TRT1: □, two prepartum doses of LAB; TRT2: ○, two prepartum doses plus one postpartum dose of LAB; CTR: ♦, carrier only).

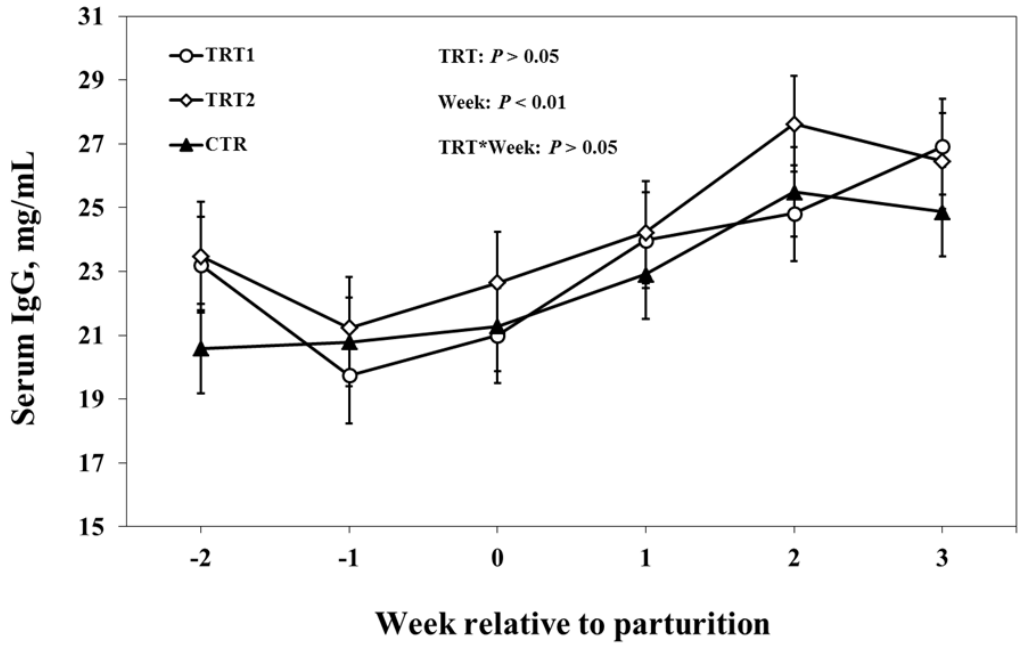

Figure 3-9. Effect of treatment on the concentration of total IgG in the serum (LSM ± SEM, n = 10 in each group. IgG: immunoglobulin G. TRT = effect of treatment; Week = effect of week; TRT * Week = effect of the interaction between treatment and week. TRT1: □, two prepartum doses of LAB; TRT2: ○, two prepartum doses plus one postpartum dose of LAB; CTR: ♦, carrier only).

Chapter 4 Intravaginal administration of probiotics modulated the composition of vaginal microbiota of transition dairy cows

ABSTRACT

The objective of this investigation was to test whether intravaginal infusion of probiotics could modify the composition of vaginal microbiota of periparturient dairy cows. One hundred healthy pregnant Holstein cows were allocated (based on parity, body condition score, and previous 305 d milk yield) to 1 of the 3 experimental groups as following: 1) one dose of probiotics on wk -2 and -1 and one dose of carrier (sterile skim milk) on wk +1 relative to the expected day of parturition (TRT1), 2) one dose of probiotics on wk -2 and -1 and +1 (TRT2), and 3) one dose of carrier on wk -2, -1, and +1 (CTR). The probiotics were lyophilized dry power composed of *Lactobacillus sakei* FUA3089, *Pediococcus acidilactici* FUA3138, and *Pediococcus acidilactici* FUA3140, and administered at a dose of 10^8-10^9 cfu/dose and animal. Vaginal mucus was collected once a week at wk -2, -1, 0, +1, +3, and +8. Vaginal pH was monitored with a portable pH probe inserted into the caudal vagina once a week from wk -2 to +8. Results showed that the Lactobacillus group accounted for a small portion of the microbiota in the vaginal tract of dairy cows. The infused *L. sakei* persisted in the vaginal tract up to 5 wk from the start of probiotic administration in both TRT1 and TRT2. TRT1 also had a greater number of *Lacobacillus* group and *Enterococcus spp.*, which were strongly correlated. At wk +8, TRT1 had smaller numbers of total bacteria and *Escherichia coli*, whereas TRT2 had greater number of lactic acid bacteria (*Lactobacillus* group) in the vaginal mucus. Overall administration

of LAB in the vaginal tract of dairy cows around parturition modified the composition of microbiota in the vaginal mucus and warrants further investigation regarding utilization of LAB in prevention of uterine and vaginal infections in dairy cows.

4.1 Introduction

The vaginal tract of dairy cows harbors various bacteria including aerobes, facultative anaeroboes, and obligate anaeroboes (Wang et al., 2013). Among the aerobic microorganisms *Enterococcus* and *Staphylococcus* are reported as the predominant groups of bacteria followed by *Enterobacteriaceae* and *Lactobacillus* in the vaginal tract of healthy heifers (Otero et al., 2000). Before parturition, the cervix is closed with a mucus plug, isolating the sterile uterine body from the bacterial-harboring vagina. Immediately after parturition the cervix is open to allow the drainage of intrauterine fluid, which also provides an open passage for bacteria to ascend into uterine body via the vaginal tract. The early postpartum uterus is colonized by a wide range of microorganisms originating from the feces, skin, and the environment. Lactic acid bacteria (LAB) like *Enterococcus*, *Lactobacillus*, and *Pediococcus*, *Enterobacteriaceae*, and *Escherichia coli* were detected in both healthy and infected cows with *E. coli* being much more abundant in cows with uterine infection (Wang et al., 2013). Furthermore, the bacterial contamination undergoes a dynamic fluctuation in the first few weeks postpartum (Sheldon and Dobson, 2004).

Recently there is an interest to avoid using antibiotics in treatment of infection diseases in livestock animals and using alternative and healthier choices like

probiotics. Probiotics are live microorganisms that when administered in adequate amounts confer a health benefit on the host (FAO/WHO, 2002). They compete with harmful bacteria for both indispensable nutrients and niches. Lactic acid bacteria produce lactic acid, which keeps the local pH at a low level and therefore inhibit some non-acid tolerant harmful bacteria (Tiwari et al., 2012). In addition, they can produce hydrogen peroxide (H_2O_2) and bacteriocins, which are lethal to other bacteria (Laughton et al., 2006; Spinler et al., 2008). It has been demonstrated that *Staphylococcus aureus*, one of the opportunistic pathogens, is inhibited by H_2O_2 and lactic acid (Otero and Nader-Macías, 2006). Pediocin produced by *Pediococcus acidilactici* strains, used in our experiment, generated inhibition zones against *Enterococcus faecalis* and *Listeria innocua* (Wang et al., 2013). Therefore, we hypothesized that introducing probiotics, composed of *Lactobacillus sakei* and two strains of *Pediococcus acidilactici* isolated from the vaginal mucus of healthy pregnant cows, into the vaginal tract of peripartum dairy cows might modify the bacterial composition in the vaginal tract of dairy cows.

4.2 Materials and methods

4.2.1 Animals and experimental design

One hundred healthy pregnant Holstein cows were allocated (based on parity, body condition score, and previous 305 d milk yield) to 1 of the 3 experimental groups as following: 1) one dose of probiotics on wk -2 and -1 and one dose of carrier (sterile skim milk) on wk +1 relative to the expected day of parturition (TRT1), 2) one

dose of probiotics on wk -2 and -1 and +1 around parturition (TRT2), and 3) one dose of carrier on wk -2, -1, and +1 around parturition (CTR). The pre-calving treatments were administered during d -14.13 ± 4.67 and d -7.46 ± 4.46 relative to the real calving day, respectively, whereas the post-calving treatment was administered on d 7.55 ± 1.29. The probiotic cocktail was composed of *Lactobacillus sakei* FUA3089, *Pediococcus acidilactici* FUA3138, and *Pediococcus acidilactici* FUA3140. Probiotic cultures were prepared by lyophilization in sterile skim milk and were administered at a dose of 10^8-10^9 cfu/treatment. Both probiotics and carrier were stored at -86 ^oC in vials in the form of dry powder, and each vial was reconstituted with 1 mL sterile 0.9% saline before administration. Before infusion, cows were stimulated to urinate by massaging on the perineal area. Then, the external vulvar area of cows was washed with warm water, followed by a 30% iodine spray, and dried with paper towel. The probiotics or carrier was infused into the vaginal tract gently with individually wrapped sterile drilled infusion tubes (Continental Plastic Corp., Delavan, WI) fitted with a 5-mL sterile screw tip syringe (Becton, Dickinson and Company, Franklin Lakes, NJ). While the tubes were inserted in the vagina, the plastic sheath wrapping the tube was retracted to minimize potential induction of contamination with the tube. The probiotics or carrier was then deposited at anterior part of the vagina. 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).

4.2.2 Sample collection and vaginal pH measurement

Vaginal mucus was collected once a week at wk -2, -1, 0, +1, +3 and +8 relative to the expected day of parturition and sampling was done before administration of treatments. Briefly, an individually wrapped sterile drilled infusion tube (Continental Plastic Corp., Delavan, WI) fitted with a 5-mL sterile screw tip syringe (Becton, Dickinson and Company, Franklin Lakes, NJ) was gently inserted into the anterior-middle part of the vaginal tract and mucus was sucked into the tube while the syringe cylinder was pulled back. Then, the mucus sample was flushed into a sterile plastic tube with 1 mL sterile 0.9% saline. The weight difference before and after sampling was calculated as the weight of mucus. Mucus samples were kept under -20 ^{o}C for microbiological analysis. Aseptic procedures were maintained with the same procedure as LAB administration.

Vaginal pH was monitored with a portable pH probe inserted into the caudal vagina once a week on a weekly basis from wk -2 to +8 except the calving week, during d -14.13 ± 4.67, d -7.46 ± 4.46, d 7.55 ± 1.29, d 14.55 ± 1.29, d 21.55 ± 1.29, d 28.55 ± 1.29, d 35.55 ± 1.29, d 42.55 ± 1.29, and d 49.55 ± 1.29 relative to the real calving day.

4.2.3 DNA extraction and bacterial quantification

A subset of mucus samples, from 9 healthy cows per group, were selected for quantification of bacteria. Total bacterial DNA of mucus samples was extracted using a QIAamp DNA Stool Mini Kit (Qiagen Inc., Mississauga, Ontario, Canada) after

pre-processing. Approximately 0.5 g mucus sample was lysed with 50 μL sodium dodecyl sulfate (SDS, 10% w/v) and 10 μL proteinase K (20 mg/mL) in TN150 buffer at 55^{o}C for 1 hr. Then, all the lysate was transferred to a 2-mL screw tube with 0.5 g zirconia bead, and homogenized on a bead beater 3 times for 30 sec. After that, the tubes were incubated at 95^{o}C for 15 min followed by the procedures on the manufacturer's instructions of QIAamp DNA Stool Mini Kit. Quantity and quality of DNA were checked on a NanoDrop spectrophotometer system ND-1000, software version 3.3.0 (Thermo Fisher Scientific Inc., Wilmington, USA). Before PCR analysis, samples were diluted to contain comparable DNA concentrations.

Real-time qPCR was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The primers and probes were designed to target total bacteria, *Bacteroides-Prevotella-Porphyromonas* spp. (*Bacteroides* group), *E. coli*, *Enterobacteriaceae*, *Enterococcus* spp., *Staphylococcus* spp., *Bacillus* spp., *Lactobacillus-Pediococcus-Leuconostoc-Weissella* spp. (*Lactobacillus* group) and *Lactobacillus sakei*. Pediocin structural gene pedA was also amplified to specifically quantify pediocin structural gene pedA harboring bacteria. Primer pairs used are listed in Table 4-1.

TaqMan probes (Invitrogen, Burlington, ON, Canada) were used for the quantification of pediocin structural gene pedA and *Lactobacillus sakei*. In both cases, each probe was labeled with 5'-FAM reporter and 3'-TAMRA quencher. The total reaction mixture contained 12.5 μL TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 2.0 μL template DNA, 0.4 mM of each primer,

and 0.2 μM of probe, and Nuclease-free H_2O (Integrated DNA Technologies, Inc., Coralville, IA) to a final volume of 25 μL. SYBR green assays were used for all the remaining primer pairs. The total reaction mixture contained 12.5 μL Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA), 0.4 mM of each primer, and 2.0 μL template DNA, and Nuclease-free H_2O (Integrated DNA Technologies, Inc., Coralville, IA, USA) to a final volume of 25 μL. Standards were prepared using purified PCR products and diluted serially (10^{-3} to 10^{-8}) to generate standard curves. For all the targets, R^2 was greater than 0.99 and PCR efficiency was between 90-110%. Samples were analyzed in duplicate in MicroAmp Fast Optical 96-well reaction plates covered with MicroAmp Optical Adhesive Film (Applied Biosystems, Foster City, CA, USA). Amplification was conducted generally as following: an initial denaturation at 95°C for 5 min for 1 cycle, 40 cycles of denaturation at 95°C for 15 sec, annealing at temperatures listed in Table 4-1 for 30 sec, and extension at 72°C for 30 sec.

4.2.4 Statistical analyses

The blocking factors including BCS, previous milk yield, and parity were tested for effects on the results but were found not associated with the outcome and therefore were excluded from the model. The difference among treatment groups in terms of bacterial numbers and vaginal pH was analyzed using SAS 9.2 software (SAS Institute Inc., Cary, NC). MIXED PROC of SAS with repeated measurement was used to test the effect of treatment, week, and the interaction between treatment

and week. The covariance structure for each variable was modeled separately according to the smallest values of the fit statistics based on the Bayesian information criteria.

Linear discriminant analysis and principal component analysis were done using JMP 9.0.0 software (SAS Institute, Inc. Cary, NC). The canonical score plot shows how the first two canonical functions, which reflect the most variance in discriminant model, classify observation between groups. The loading plot signifies the amount of variation and the correlation among bacterial populations and vaginal pH in terms of the first two principal components, which was further analyzed with Spearman's correlation test using JMP 9.0.0 software (SAS Institute Inc., Cary, NC).

4.3 Results

4.3.1 Effect of probiotic treatment on bacterial populations

The effect of treatment on the number of gene copies of vaginal microbiota based on real time - qPCR is shown in Figure 4-1. No difference was detected at wk -2 before the treatment among groups (Figure 4-1A). At wk -1, TRT1 had increased numbers of both *Lactobacillus* group ($P < 0.01$) and *Enterococcus* spp. ($P < 0.01$), whereas TRT2 also showed an increased in the number of *Enterococcus* spp., compared with the control group (Figure 4-1B). At wk 0, probiotic treated cows had a higher number of *L. sakei* ($P < 0.01$), but a lower number of *Enterococcus* spp. ($P < 0.05$) than the control cows (Figure 4-1C). Meanwhile, TRT2 also had a lower number of *Staphylococcus* spp. than the CTR group ($P < 0.05$). At wk +1, TRT1 had a higher

number of *Lactobacillus* group than both TRT2 and CTR ($P < 0.01$) with no difference btween TRT2 and CTR (Figure 4-1D). Meanwhile, the number of *Enterococcus* spp. in TRT1 was also detected higher than the CTR group ($P < 0.05$), but not different from TRT2. Both TRT1 and TRT2 had a tremendously higher number of *L. sakei* than the CTR group ($P < 0.01$). By wk +3, the number of *L .sakei* maintained higher in TRT1 and TRT2 than the CTR group ($P < 0.05$, Figure 4-1E). TRT1 had a pronounced higher number of *Lactobacillus* group and *Enterococcus* spp. than both TRT2 and CTR ($P < 0.01$). Interestingly, by wk +8 (Figure 4-1F), compared with CTR, TRT1 had a lower number of total bacteria ($P < 0.01$), *Bacillus* spp. ($P < 0.01$), and *E. coli* ($P < 0.01$), whereas TRT2 had a higher number of *Lactobacillus* group ($P < 0.01$) and *Staphylococcus* spp. ($P < 0.05$). The numbers of most bacterial populations in the vaginal mucus varied with week, such as *Lactobacillus* group ($P < 0.01$), *L. sakei* ($P < 0.01$), *Staphylococcus* spp. ($P < 0.01$), *Enterococcus* spp. ($P < 0.05$), *Enterobacteriaceae* ($P < 0.05$), and *Bacteroides* group ($P < 0.05$). In addition, gene copy numbers of both *E. coli* ($P = 0.08$) and total bacteria ($P = 0.06$) also tended to vary with week. The difference between treatment groups over time is also shown in Figure 4-2 as relative proportion of each bacterial population in the vaginal mucus.

4.3.2 Weekly changes of bacterial populations

The weekly change of bacterial populations is presented in Table 4-2. There was a significant drop of *Lactobacillus* group at calving week (wk 0) and then gradual recovery in all the three experimental groups. With regards to *L. sakei*, the gene copy

number dropped sharply at calving week in the CTR group and remained at a low level until wk +8 ($P < 0.001$). However, probiotic treatment maintained the number of *L. sakei* throughout wk -2 to +3 and +8 without significant change ($P > 0.05$). Pediocin gene pedA demonstrated a drastic decrease at calving week and then recovered from wk +1 in all the three experimental groups ($P < 0.001$). The number of *E. coli* remained constant throughout the experimental period in LAB treated groups ($P > 0.05$), but tended to be greater at calving week in the CTR group ($P = 0.08$). The gene copy number of total bacteria had an increase in all the groups at calving week and wk +1 ($P < 0.01$). TRT1 had a slight decline of *Bacteroides* group but an increase of *Enterobacteriaceae* during wk 0 and +1, whereas none of TRT2 or CTR showed any fluctuation regarding these two bacterial populations. The number of *Enterococcus* dropped at calving week in TRT1 ($P < 0.001$) and showed the same tendency in TRT2 ($P = 0.06$) but didn't change in the CTR. The number of *Staphylococcus* didn't change much in TRT1 but dropped in TRT2 at wk 0 ($P < 0.001$) and in the CTR at wk +1 ($P < 0.05$). The number of *Bacillus* remained constant in TRT1 and the CTR but decreased at calving week in TRT2 ($P < 0.05$).

4.3.3 Effect of probiotic treatment on vaginal pH

No difference was observed among treatment groups regarding the vaginal pH of perparturient dairy cows (Figure 4-3). The vaginal pH values were 7.55 ± 0.05 in TRT1, 7.46 ± 0.06 in TRT2, and 7.49 ± 0.05 in CTR, respectively ($P > 0.05$). The vaginal pH changed with week ($P < 0.01$). Overall, it remained at around 7.13 before

calving and increased to 7.84 by wk +6, and then dropped to 7.44 by wk +8.

4.3.4 Classification of observations

The linear discriminant model classified observations at wk +3 into 3 clusters with no intersecting over their 95% confidence intervals (Figure 4-4), which means the 3 treatment groups were well discriminated by vaginal pH and bacterial populations measured in this study. The canonical score plot also demonstrated that the gene copy numbers of *Lactobacillus* group discriminated best for TRT1, whereas those of *L. sakei* and *Bacillus* spp. discriminated best for TRT2. In contrast, bacterial composition of CTR was relatively random as it wasn't discriminated by any bacterial populations determined in this study.

4.3.5 Correlations among bacterial populations and vaginal pH

The loading plot displays correlations among bacterial populations and vaginal pH based on the first two greatest eigenvalues (Figure 4-5). The first principle component (PC1) explained 30.8% of the total variation, and the second principle component (PC 2) explained 21.4%. The cluster located on the upper right quadrant included *Lactobacillus* group, *Enterococccus* spp., *Staphylococcus* spp., *Enterobacteriaceae* and *E. coli*. This cluster was positively influenced by both PC1 and PC2. *Lactobacillus sakei* and vaginal pH formed another cluster in the upper left quadrant, whereas the vaginal pH was mainly explained by PC2. A third cluster was comprised of total bacteria, *Bacillus* spp., *Bacteroides* group and pediocin gene pedA in the lower right quadrant, which was negatively correlated with the cluster in upper

left quadrant.

The associations between the variables were further analyzed with Spearman's correlation analysis to determine the direction and strength of the correlation, and the major correlations are listed in Table 4-3. Total bacteria, *Bacteroides* group, *Bacillus* spp., and pediocin gene pedA were positively and strongly correlated with each other ($r > 0.50$, $P < 0.01$). *Lactobacillus* group was strongly and positively correlated with *Enterococcus* spp. ($r > 0.80$, $P < 0.01$), as was *Enterobacteriaceae* with *E. coli* ($r = 0.80$, $P < 0.01$). *Enterobacteriaceae* were also positively correlated with the total bacteria, *Lactobacillus* group, and *Enterococcus* spp. ($r < 0.50$, $P < 0.05$). *Escherichia coli* were also positively correlated with total bacteria ($r < 0.50$, $P < 0.05$). Total bacteria exhibited a tendency to be positively correlated with *Lactobacillus* group ($r < 0.50$, $P = 0.08$) and *Enterococcus* spp. ($r < 0.50$, $P = 0.07$). *Staphylococcus* spp. was positively correlated with both the *Lactobacillus* group and *Bacillus* spp. ($r < 0.50$, $P < 0.05$). A negative correlation was evidenced between *L. sakei* and total bacteria ($r < -0.30$, $P < 0.05$), vaginal pH and *Bacteroides* group ($r < -0.30$, $P < 0.05$). *Lactobacillus sakei* tended to have a negative correlation with *Bacteroides* group ($r < -0.30$, $P = 0.06$), and the same tendency was also observed between *Bacillus* spp. and vaginal pH ($r < -0.30$, $P = 0.09$).

4.4 Discussion

The postpartum uterus of transition dairy cows is temporarily contaminated by a broad spectrum of bacteria ascending via the vaginal tract with dynamic fluctuation

within the first few weeks after calving (Sheldon and Dobson, 2004). This random contamination is usually resolved by the host immunological responses within the first 3 wk postpartum, in most of the cases (Bekana et al., 1996; Bondurant, 1999; Sheldon et al., 2006). However, depending on the composition of microbiota in the vaginal tract, some pathogenic bacteria persist and cause vaginal and uterine infections, impairing subsequent fertility of transition dairy cows. We hypothesized that infusing LAB probiotics in the vagina of periparturient cows will beneficially modify the composition of bacteria in the vaginal mucus. Indeed results of this study showed that administering LAB in the vaginal tract of dairy cows modulated composition of their vaginal mucus microbiota as indicated by the 3 clusters discriminated by bacterial populations with no intersection over their 95% confidence intervals.

The probiotics used in this study were a mixture composed of *L. sakei* and two *P. acidilactici* strains isolated from vaginal mucus of healthy pregnant cows, as described previously (Wang et al., 2013; Ametaj et al., 2014). Intravaginal administration of probiotics resulted in an increased number of *L. sakei* in the vaginal mucus microbiota, which persisted until 3 wk after calving. Various *Lactobacillus* strains have different adhesive capability to the epithelial cells or mucus at mucosal tissues (Jacobsen et al., 1999; Collado et al., 2006), which is related to their physical and antimicrobial characteristics (Jacobsen et al., 1999). Some *Lactobacillus* strains have a hydrophobic cell surface with a thick protein layer, which enables them to adhere to the surface of epithelial cells (Sherman et al., 2009). Kummer et al. (1997)

infused lactobacilli suspension into the uterus of cyclic dairy cows and observed that the infused lactobacilli were able to colonize in the uterus for at least 12 days (Kummer et al., 1997). Although we have not tested the adhesive ability of the LAB strains used in this study, it seems that our lactobacilli were able to establish their niches in the vaginal mucus of the treated cows. In support of this observation is the fact that despite the flow of large amounts of lochia, immediately after calving, *L. sakei* was able to persevere in the vaginal mucus of the treated cows up to 5 wk from the administration of LAB cocktail.

On the other hand, the abundance of pediocin structural gene pedA was not different between the treatment groups at 3 wk postpartum. This might be indicative of a weak colonizing ability of these *Pediococcus* strains in the vagina. The less persistent capacity of *Pediococcus* spp. compared with *Lactobacillus* spp. has also been reported in a study on pigs via oral route (Gardiner et al., 2004). The supportive finding was that little amount of pediocin gene pedA was detected at calving week, which suggested that pediocin-producing bacteria inhabited the vaginal tract with weak adhesion and were not able to withstand the flush of uterine content after calving.

Another main finding of this study was that the number of *Enterococcus* spp. was highly and positively correlated with that of *Lactobacillus* group. Although the probiotic culture used in this study did not include *Enterococcus* spp., the results showed that there was an increase in the *Enterococcus* spp. associated with that of *Lactobacillus* group induced by the treatment. Actually, both *Lactobacillus* spp. and

Enterococcus spp. are ubiquitously present in the native microbiota of both human and animals, such as in the intestine and feces. *Enterococcus* spp. isolated from animals and humans have been identified to have probiotic properties and have been used as a co-culture with *Lactobacillus* spp. (Strompfová et al., 2004; Fan et al., 2006; Strompfová and Lauková, 2009). In addition, it was reported that in the presence of *Lactobacillus* spp., *Enterococcus* spp. exhibit stronger survival ability (Fraenkel et al., 2011). Consequently, it can be speculated that the infusion of lactobacilli might have boosted the enterococci in the vaginal tract of dairy cows.

As anticipated the LAB treatment had a long lasting effect on the bacterial composition until 8 wk after calving as indicated by the difference in the numbers of multiple bacterial populations such as total bacteria, *Bacillus spp.*, *Lactobacillus* group, *Enterobacteriaceae*, *Staphylococcus spp.*, and *E. coli*, between the treated cows and those of the CTR group, but not of the *L. sakei* or pediocin structural gene pedA carrying bacteria, suggesting more frequent probiotic administrations to further maintain high levels of LAB in the vaginal tract. Our data are in agreement with another study, which reported that the recovering rate of supplemented probiotics declined with time after the cessation of the administration and none of the administered *Lactoabacillus* or *Pediococcus* were detected at 66 d post-administration (Gardiner et al., 2004). The latter authors also reported a dynamic change of the micobiota after the cessation of probiotic administration. In Chapter 2, we have shown that probiotic treatment affected the production of progesterone and prostaglandin E_2 (PGE_2). It has been recognized that both progesterone and PGE_2 affect the microbial

activities of microbiota either directly or indirectly by influencing the immune cells (Miller and Morse, 1977; Agard et al., 2013; García-Gómez et al., 2013). Altogether, these results indicate that infusion of LAB in the vaginal tract of dairy cows had both short-term and long-term effects on the composition of native microbiota.

The total bacteria in the vaginal mucus of dairy cows were detected at 10^8, in terms of the gene copies per gram, which confirmed our previous findings (Wang et al., 2013). In accordance with our previous report, *Bacteroides* group, *Bacillus* spp., *Enterobacteriaceae*, and *E. coli* accounted for the largest proportion of microbiota in the vaginal mucus of postpartum dairy cows, whereas *Lactobacillus* group represented a relatively small group of bacteria by gene copy numbers (Wang et al., 2013). This might explain the difference of the pH in the vaginal tract of humans and dairy cows, which are acidic (Rönnqvist et al., 2006) and neutral (Dhaliwal et al., 2001; Wehrend et al., 2003; Beckwith-Cohen et al., 2012), respectively. *Lactobacillus* has been reported as the dominant population in female vaginal tract contributing to urogenital health by keeping the microenvironment acidic (Rönnqvist et al., 2006). Hence, the neutral microenvironment in the vaginal tract of dairy cows might be mainly attributed to the low numbers of *Lactobacillus* group. *Enteroccoccus* spp. in the vagina originates mostly from feces and usually inhabits vagina as a commensal microorganism. They are able to produce extracellular superoxide, which is harmful to pathogenic microorganisms (Tendolkar et al., 2003). The presence and abundance of *Enterococcus faecalis* was also reported to be associated with vaginal pH changes in human subjects (Jahić et al., 2006). In this study, it was noticed that there was an

increase of pH after parturition compared with the prepartum one. Therefore, the lack of a low pH could also explain at least partly the ubiquitous bacterial invasion in the reproductive tract of postpartum dairy cows (Beckwith-Cohen et al., 2012). Of note, in the control group, *Lactobacillus* group, *L. sakei* and bacteria harboring pediocin gene pedA underwent a dramatic drop whereas the number of total bacteria increased and *E. coli* tended to increase at wk 0. The intravaginal administration of LAB curbed the drop of *L. sakei* and prosperity of *E. coli* although not of others at wk 0. In this study, prepartum administration of probiotics (TRT1) boosted the number of *Lactobacillus* group and *Enterococcus* spp., which were strongly correlated and both persisted until 3 wk after calving. However, the increment in *Lactobacillus* group and *Enteroccoccus* spp. was not sufficient to lower the vaginal pH in acidic values. In addition, *Enterobacteriaceae* was highly correlated with *E. coli*, which indicates that *E. coli* is the major member of *Enterobacteriaceae* in the vaginal mucus of dairy cows (Wang et al., 2013).

4.5 Conclusions

Intravaginal infusion of a cocktail of LAB around calving increased the abundance of LAB in the vaginal mucus. In a short-term at 3 wk after calving, prepartum administration of LAB (TRT1) increased the number of *L. sakei*, *Lactobacillus* group, and *Enterococcus* spp. postpartum, whereas prepartum plus postpartum infusion of LAB (TRT2) increased the number of *L. sakei* only. In a long term at 8 wk after calving, TRT1 had lower number of total bacteria and pathogenic

bacteria (*E. coli*), whereas TRT2 had greater number of *Lactobacillus* group.

4.6 Acknowledgements

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Table 4-1. Primers used for quantitative PCR in this study

Target	Primer/probe sequence (5'→3')	Annealing temperature (°C)	References
Lactobacillus-Pediococcus-Leuconostoc-Weisella group (341)	**Lac1:** AGC AGT AGG GAA TCT TCC A **Lab667r:** CAC CGC TAC ACA TGG AG	62	Wang et al., 2013
Bacillus (995)	**BacF:** GGG AAA CCG GGG CTA ATA CCG GAT **BacR:** GTC ACC TTA GAG TGC CC	58	Garbeva et al., 2003; Wang et al., 2013
Enterococcus (144)	**Ent-F:** CCC TTA TTG TTA GTT GCC ATC ATT **Ent-R:** ACT CGT TGT ACT TCC CAT TGT	60	Wang et al., 2013
Staphylococcus (370)	**TStaG422:** GGC CGT GTT GAA CGT GGT CAA ATC **TStaG765:** TIA CCA TTT CAG TAC CTT CTG GTA A	58	Martineau et al., 2001; Wang et al., 2013
Enterobacteriaceae (195)	**Enterobac-F:** CAT TGA CGT TAC CCG CAG AAG AAG C **Enterobac-R:** CTC TAC GAG ACT CAA GCT TGC	63	Wang et al., 2013
E. coli (544)	**ECP79F:** GAA GCT TGC TTC TTT GCT **ECP620R:** GAG CCC GGG GAT TTC ACA T	54	Wang et al., 2013
Bacteroides-Prevotella-Porphyromonas group (140)	**BfraF:** GGT GTC GGC TTA AGT GCC AT **BfraR:** CGG AYG TAA GGG CCG TGC	60	Rinttila et al., 2004; Koleva et al., 2012
Lactobacillus sakei (78)	**SakF:** GAT AAG CGT GAG GTC GAT GGT T **SakR:** GAG CTA ATC CCC CAT AAT GAA ACT AT **SakS:** FAM-GCC CAT TGT ACC AAT TT-MGB	63	Martín et al., 2007
Pediocin structural gene pedA (100)	**pedA2RTF:** GGC CAA TAT CAT TGG TGG TA **pedA2RTR:** ATT GAT TAT GCA AGT GGT AGC C **TqM-pedA:** FAM-ACT TGT GGC AAA CAT TCC TGC TCT GTT GA-TAMRA	60	Mathys et al., 2007; Wang et al., 2013
Total bacteria	**TotalBac-F785:** GGA TTA GAT ACC	54	Wang et al.,

(727)	CTG GTA GTC **TotalBac-R1512r:** TAC CTT GTT ACG ACT T	2013

Table 4-2. Weekly changes of bacterial populations in the vaginal mucus (log_{10} gene copies/g)

Bacterial population	group	-2	-1	0	+1	+3	+8	P value
Lactobacillus group	TRT1	2.54±0.83	6.00±0.52	2.42±0.55	4.93±0.52	5.84±0.52	2.39±0.55	< 0.001
	TRT2	4.17±0.68	4.02±0.55	1.50±0.55	3.35±0.55	3.35±0.55	5.49±0.55	< 0.05
	CTR	3.90±0.68	3.65±0.74	1.73±0.52	2.36±0.52	2.52±0.52	2.18±0.52	< 0.05
L. sakei	TRT1	4.50±0.88	4.84±0.83	2.83±0.83	4.67±0.79	2.08±0.83	2.74±0.83	> 0.05
	TRT2	3.93±0.94	4.52±0.83	3.14±0.83	3.91±0.83	3.16±0.83	4.43±0.83	> 0.05
	CTR	4.44±0.67	5.70±0.67	1.16±0.64	1.20±0.64	0.50±0.64	3.85±0.64	< 0.001
Pediocin gene pedA	TRT1	2.83±0.41	3.34±0.36	< 0.01±0.37	3.67±0.34	3.87±0.34	2.84±0.49	< 0.001
	TRT2	2.98±0.18	3.42±0.18	< 0.01±0.17	3.59±0.18	3.23±0.18	2.79±0.18	< 0.001
	CTR	3.01±0.19	3.05±0.19	< 0.01±0.18	3.77±0.18	3.53±0.18	2.62±0.19	< 0.001
Bacillus spp.	TRT1	3.83±0.30	4.24±0.30	4.10±0.32	4.00±0.30	4.73±0.30	3.52±0.32	> 0.05
	TRT2	5.00±0.39	4.54±0.39	3.45±0.37	4.46±0.41	4.23±0.39	5.10±0.39	< 0.05
	CTR	4.22±0.30	4.26±0.30	4.64±0.28	4.28±0.28	4.56±0.28	4.96±0.28	> 0.05
Enterococcus spp.	TRT1	1.93±0.46	3.68±0.46	1.69±0.48	3.92±0.48	4.03±0.46	2.27±0.48	< 0.001
	TRT2	2.75±0.47	2.86±0.47	1.44±0.44	2.97±0.47	2.41±0.47	3.48±0.47	= 0.06
	CTR	2.86±0.50	1.43±0.55	2.94±0.50	2.56±0.50	1.60±0.50	2.88±0.50	> 0.05
Staphylococcus spp.	TRT1	2.20±0.55	3.32±0.55	2.73±0.58	1.69±0.55	3.38±0.58	2.53±0.58	> 0.05
	TRT2	3.71±0.46	3.58±0.46	1.15±0.44	2.33±0.46	1.87±0.46	3.78±0.46	< 0.001
	CTR	3.56±0.55	3.21±0.58	3.49±0.55	1.17±0.55	2.17±0.55	2.74±0.55	< 0.05
Enterobacteriaceae	TRT1	4.88±0.59	4.71±0.59	6.83±0.62	7.91±0.76	6.39±0.59	4.30±0.62	< 0.01
	TRT2	5.78±0.69	5.33±0.69	5.75±0.69	6.35±0.69	5.67±0.69	7.54±0.69	> 0.05
	CTR	4.46±0.73	5.02±0.77	5.04±0.73	6.12±0.73	4.62±0.73	6.18±0.69	> 0.05
E. coli	TRT1	4.91±0.44	4.76±0.50	5.67±0.47	5.18±0.44	5.57±0.50	4.81±0.47	> 0.05
	TRT2	5.09±0.47	4.39±0.47	5.17±0.44	4.82±0.47	4.97±0.50	5.53±0.47	> 0.05
	CTR	5.46±0.47	4.39±0.44	5.88±0.44	5.56±0.44	4.06±0.44	5.73±0.44	= 0.08
Bacteroides group	TRT1	7.32±0.53	6.00±0.53	5.93±0.55	6.92±0.68	6.72±0.53	5.90±0.55	< 0.05
	TRT2	6.34±0.60	5.98±0.60	5.13±0.57	6.64±0.64	6.26±0.81	7.09±0.60	> 0.05
	CTR	5.85±0.54	6.10±0.54	6.38±0.54	7.16±0.51	7.56±0.61	7.03±0.54	> 0.05
Total bacteria	TRT1	6.50±0.35	7.37±0.35	7.44±0.37	7.82±0.35	7.93±0.35	6.15±0.37	< 0.01
	TRT2	6.96±0.36	7.46±0.36	7.20±0.34	8.00±0.36	7.33±0.36	7.39±0.36	< 0.01
	CTR	6.50±0.38	6.83±0.38	8.20±0.36	8.33±0.36	7.73±0.36	7.21±0.36	< 0.01

Table 4-3. Correlations among bacterial populations (log_{10} gene copies/g) in the vaginal mucus and vaginal pH assessed by Spearman's correlation test (correlation coefficients r/*P* value)

	Total bacteria	*Bacteroides* group	*Lactobacillus* group	*Bacillus*spp.	*Enterobacteriaceae*
Total bacteria	1.000	0.685/0.001**	0.327/0.084	0.724/0.001**	0.415/0.025*
Bacteroides group	0.685/0.001**	1.000	0.044/0.820	0.761/0.001**	0.089/0.648
Lactobacillus group	0.327/0.084	0.044/0.820	1.000	0.300/0.114	0.422/0.023*
Pediocin gene pedA	0.560/0.002**	0.575/0.001**	0.224/0.243	0.739/0.001*	0.184/0.340
L. sakei	-0.375/0.045*	-0.356/0.058	0.018/0.926	-0.186/0.334	0.022/0.912
Enterococcus spp.	0.341/0.070	-0.008/0.966	0.879/0.001**	0.222/0.246	0.447/0.015*
Staphylococcus spp.	-0.013/0.947	-0.002/0.993	0.398/0.033*	0.401/0.031*	0.163/0.400
E. coli	0.399/0.032*	0.069/0.723	0.282/0.139	0.195/0.312	0.800/0.001**
Vaginal pH	-0.198/0.304	-0.369/0.049*	0.200/0.300	-0.321/0.089	0.230/0.230

*Coefficients with $P < 0.05$.

**Coefficients with $P < 0.01$.

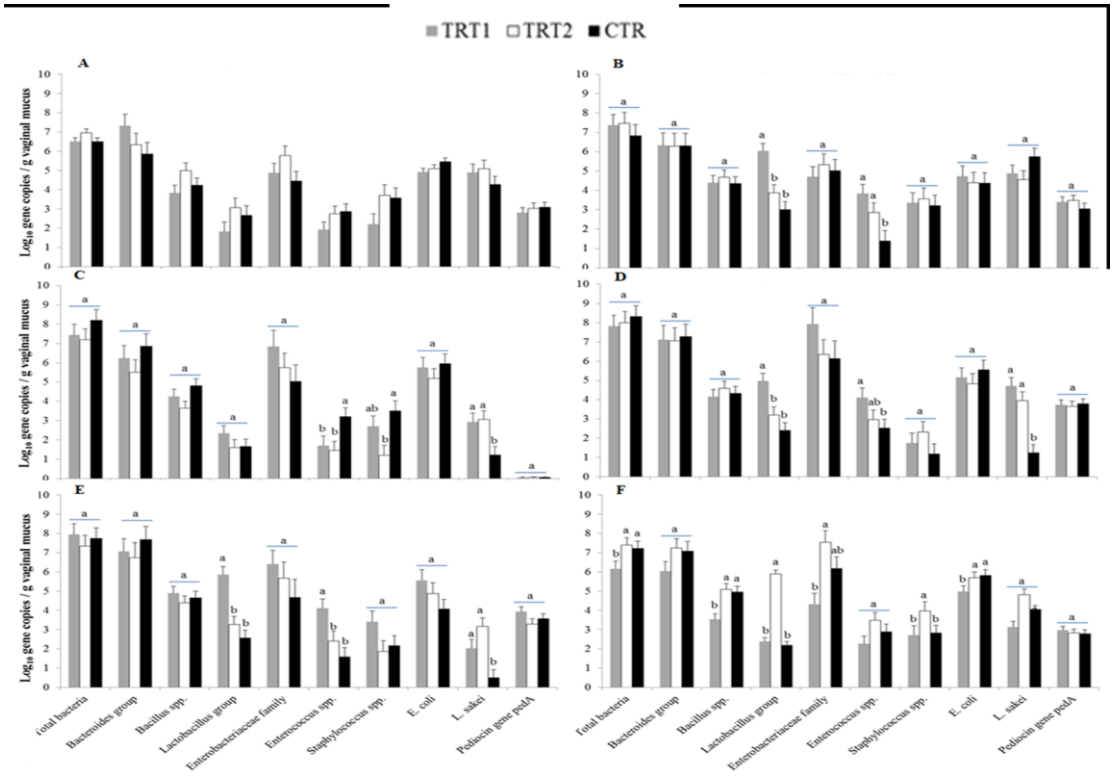

Figure 4-1. Numbers of bacterial populations in the vaginal mucus of dairy cows over time (values are the Least Square Mean (LSM) of Log_{10} gene copies/g vaginal mucus with vertical bars showing the Standard Error of the Mean (SEM) at wk -2 (A), -1 (B), 0 (C), +1 (D), +3 (E) and +8 (F); values obtained with the same primer pairs with different letters were significantly different ($P < 0.05$); TRT1: ■, two prepartum doses of probiotics, TRT2: □, two prepartum plus one postpartum doses of probiotics. CTR: ■ , carrier only. n = 9 in each group)

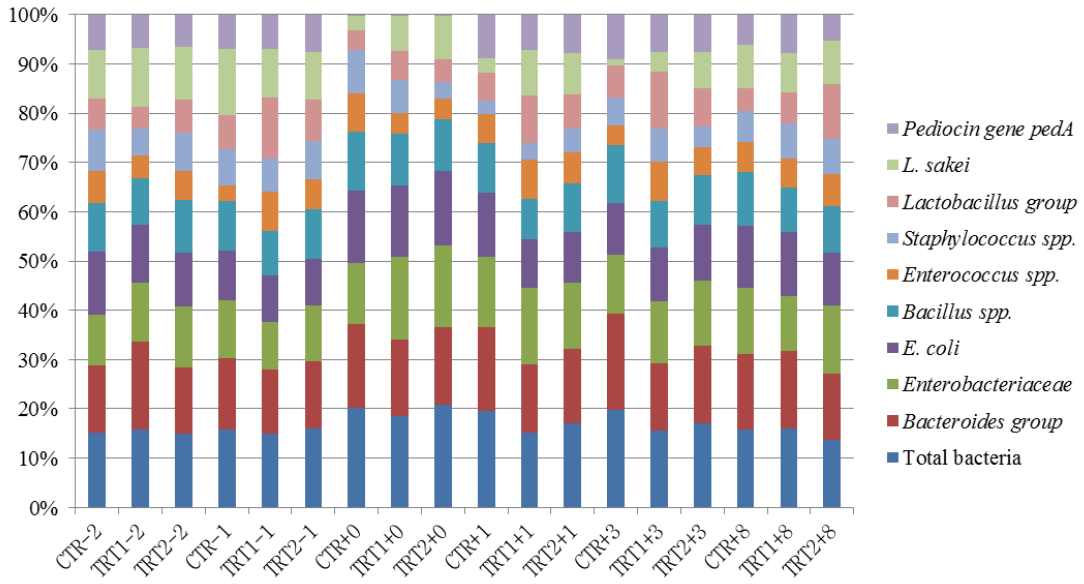

Figure 4-2. Relative proportion of each bacterial population in the vaginal microbiota over time (values are relative proportions of each population based on $\log_{10}$ gene copies/g vaginal mucus with X-axis showing the changes of treatment groups over time relative to calving day. TRT1: ▒, two prepartum doses of probiotics, TRT2: □, two prepartum plus one postpartum doses of probiotics. CTR: ■ , carrier only. n = 9 in each group. -2: 2 weeks before calving, +0: calving week, +1: 1 week after calving)

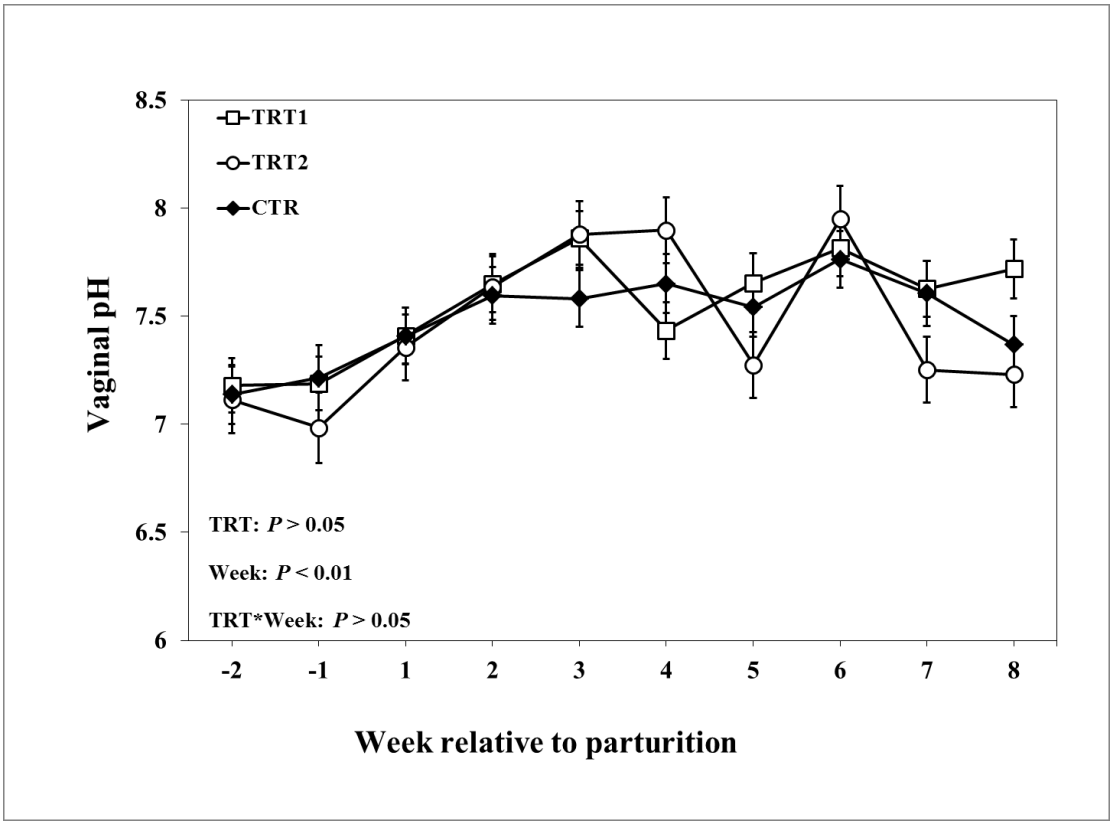

Figure 4-3. Effect of probiotic treatment on vaginal pH of dairy cows (LSM + SEM. TRT = effect of probiotic treatment; Week = effect of week; TRT * Week = effect of the interaction between treatment and week. TRT1: □, two prepartum doses of probiotics; TRT2: ○, two prepartum plus one postpartum doses of probiotics; CTR: ♦, carrier only. n = 10 in each group)

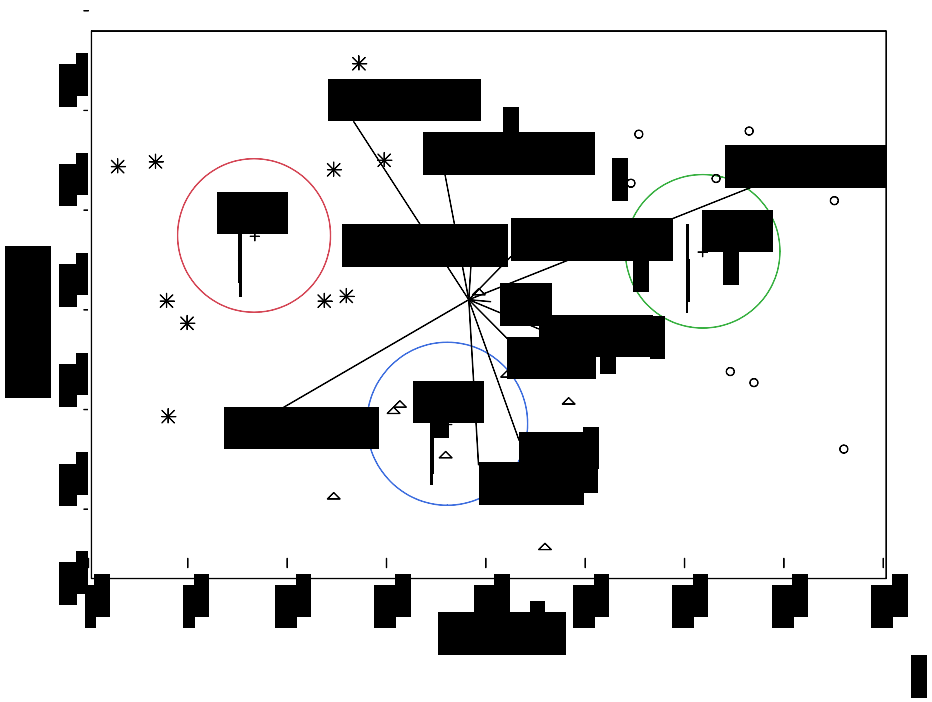

Figure 4-4. Linear discriminant analysis of the number of bacterial populations and vaginal pH (CTR: *, carrier only; TRT1: ○, two prepartum doses of probiotics; TRT2: Δ, two prepartum plus one postpartum doses of probiotics)

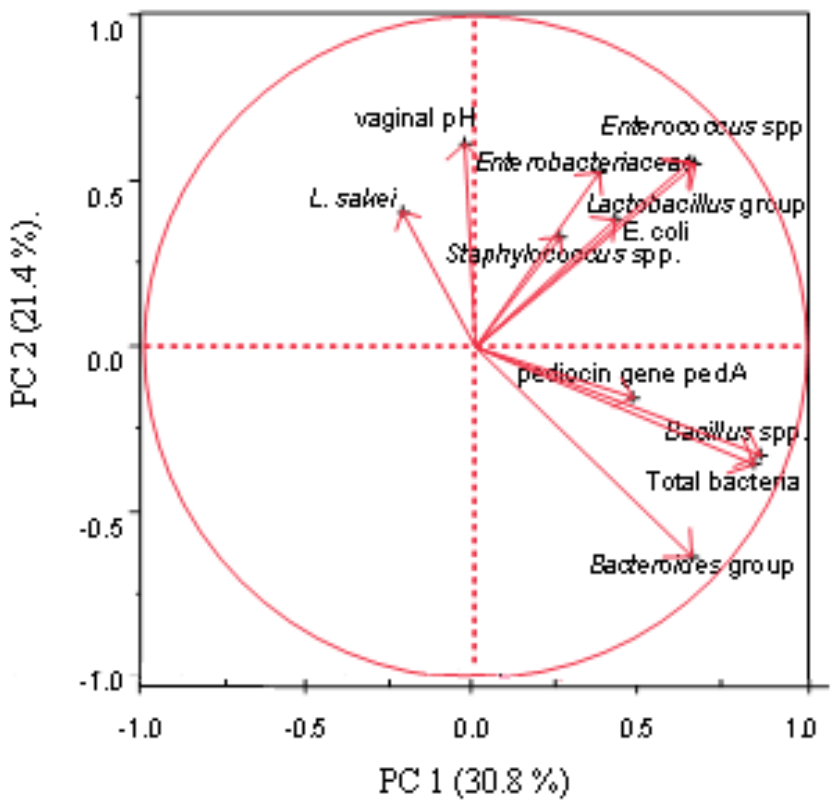

Figure 4-5. Loading plot of the first two principal components showing the correlations among gene copy numbers of bacterial populations and vaginal pH

Chapter 5 Intravaginal lactic acid bacteria modulated metabolic status and improved productive performance of transition dairy cows

ABSTRACT

The objective of this investigation was to evaluate whether intravaginal infusion of a lactic acid bacteria (LAB) mixture around parturition influences metabolic status and improves productive performance of transition dairy cows. One hundred pregnant Holstein dairy cows were assigned to 1 of the 3 experimental groups receiving intravaginal infusion of probiotics or carrier once a week at wk -2, -1, and +1 relative to calving as follows: TRT1 - 2 consecutive LAB and 1 carrier dose before parturition; TRT2 - 3 consecutive LAB doses around parturition; CTR - 3 consecutive carrier doses around parturition. LAB were a lyophilized culture mixture composed of *Lactobacillus sakei* FUA3089, *Pediococcus acidilactici* FUA3138 and FUA3140 with a cell count of 10^8-10^9 cfu/dose. Blood was sampled from wk -2 to +3 and milk on the 3rd day in milk (DIM) and from wk +1 to +5 on a weekly basis. Feed intake and milk production was monitored until wk +8. Results showed that TRT2 had a lower concentration of NEFA in the serum. LAB treatment increased concentrations of cholesterol and lactate in the serum. TRT1 had the greatest, TRT2 intermediate, and CTR the lowest concentrations of IgG in the milk. Meanwhile, both TRT1 and TRT2 had lower haptoglobin in the milk compared with the CTR group cows. However, the concentrations of milk amyloid A (MAA) were greater in the TRT1 than TRT2 and CTR cows, whereas no difference between TRT2 and CTR was obtained. TRT1 had greater milk protein content and TRT2 had greater lactose content compared with cows in the CTR group. In both multiparous and primiparous cows, LAB treatment (both TRT1 and TRT2) exhibited greater milk production and feed efficiency. In conclusion, intravaginal infusion of LAB modulated concentrations of selected serum metabolites and milk components, and improved the productive performance of transition dairy cows.

5.1 Introduction

Transition dairy cows experience major metabolic alterations around calving due to parturition, initiation of lactation, and dietary changes introduced during this time period. Long selection of dairy cows for high milk yield is associated with massive energy flow toward mammary gland even with inadequate feed intake resulting in negative energy balance (NEB) after parturition. Two of the hallmarks of NEB, non-esterified fatty acid (NEFA) and β-hydroxy butyric acid (BHBA), have been associated with high incidence of multiple diseases such as retained placenta, displaced abomasum, ketosis, mastitis, and milk fever (Schröder and Staufenbiel, 2006; Duffield and LeBlanc, 2009; LeBlanc, 2010). Body condition score (BCS), an indicator of overall energy status, is also closely associated with postpartum disorders and reproductive performance (Bewley and Schutz, 2008). Meanwhile, feed intake is greatly influenced by cow's health status and both milk production and composition fluctuate in association with blood metabolites, especially those related to energy metabolism (van Knegsel et al., 2007).

Various dietary functional components like probiotics have been tested in the past in order to modulate the metabolic status in general of transition dairy cows and also to improve their milk yield and composition. Recent research indicates that dairy cattle fed lactic acid bacteria (LAB) show increased milk yield and feed intake as well as improved feed efficiency (Krehbiel et al., 2003; Weinberg, 2003). Probiotic bacteria including LAB also have been used in feedlot cattle with beneficial effects with respect to feed efficiency (Krehbiel et al., 2003). More recently, another report

from our group showed that treating dairy cows intravaginally with LAB for 6 consecutive weeks (once per week) was associated with alterations in blood metabolites and innate immunity indicators as well as increased feed intake and milk production in transition dairy cows (Iqbal et al., 2010a; 2010b).

In this study, we hypothesized that lowering the frequency of intravaginal treatment with LAB to 2 or 3 times around parturition may influence the metabolic status and improve productive performance of transition dairy cows. Therefore the objective of this study was to evaluate whether intravaginal infusion of LAB around parturition with 2-3 LAB doses during 2 wk before and 1 wk after the expected day of parturition would affect the metabolic status and milk production and composition in transition dairy cows.

5.2 Materials and methods

5.2.1 Animals and experimental design

One hundred healthy pregnant Holstein cows were assigned (based on parity, BCS, and milk yield) to 1 of the 3 groups at 2 weeks before the expected day of parturition. Cows received intravaginal probiotics or carrier (sterile skimmed milk) once a week at wk -2, -1, and +1 relative to calving as following: treatment 1 (TRT1) - 2 consecutive LAB doses and 1 carrier dose; treatment 2 (TRT2) - 3 consecutive LAB doses; control (CTR) - 3 consecutive carrier doses. The pre-calving treatment was administered on d -14.13 ± 4.67 and d -7.46 ± 4.46 relative to the real calving day, respectively, whereas the post-calving treatment was administered on d 7.55 ± 1.29.

Probiotics used in this study were a lyophilized culture mixture of 3 lactic acid bacteria (LAB) composed of *Lactobacillus sakei* FUA3089, *Pediococcus acidilactici* FUA3138 and FUA3140, which were infused at 10^8-10^9 cfu/ dose. The carrier was sterile skim milk. 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 carrier were stored at -86 ^{o}C in vials in the form of dry powder, and each vial was reconstituted in 1mL sterile 0.9% saline before administration. After blood sampling, cows were stimulated to urinate by massaging the perineal area. Then, the external genital area of cows was washed with warm water and soap, dried with paper towel, and followed by 30% iodine spay before treatment with LAB. The LAB or carrier was infused into the vaginal tract gently with individually wrapped sterile drilled infusion tubes (Continental Plastic Corp., Delavan, WI) capped with a 5-mL screw tip sterile syringe (Becton, Dickinson and Company, Franklin Lakes, NJ), and deposited at anterior vagina. Aseptic procedures were maintained during LAB or carrier administration. Cows were fed with prepartum diet before calving and early lactation diet after calving until +8 wks (Table 5-1). All cows were housed in individual tie stalls with free access to water, fed once daily at 0800 h, and milked twice a day at 0500 and 1600 h.

5.2.2 Sampling and clinical monitoring

Blood samples were collected from the coccygeal vein once a week before the

morning feeding (0800) with 10-mL vacutainer tubes without anticoagulant (BD Vacutainer Systems, Plymouth, UK) from wk -2 to +3 before the expected day of parturition, which was done on d -14.13 ± 4.67, d -7.46 ± 4.46, d 1.71 ± 1.39, d 7.55 ± 1.29, d 14.55 ± 1.29, and d 21.55 ± 1.29 relative to the real calving day. Blood samples were centrifuged at 2,090 × g and 4 °C for 20 min (Beckman Coulter, Pasadena, California) to separate serum. Serum samples were stored at -20 °C until analyses.

Milk samples were collected on the 3rd day after parturition (3 DIM) as well as from wk +1 to +5 postpartum, on a weekly basis. For each milk sample, two sub-samples from the same cow were collected at 0500 and 1600 h, respectively, and then mixed half and half to form one sample. Daily milk production was monitored from wk +1 to +8 postpartum. Individual daily feed intake was calculated as the difference between feed given and orts from wk -2 to +8.

Body condition score was evaluated at wk -3, -1, +3, +6, and +9 on a 5-point scale at an interval of 0.25, referring to the judgment of BCS in Dairy Cattle Production (342-450A, McGill University and Elanco Animal Health, Indianapolis, Indiana, U.S).

5.2.3 Laboratory analyses

A subset of serum samples from 30 cows (10 per group) were randomly selected to evaluate concentrations of glucose, insulin, NEFA, BHBA, cholesterol, and lactate. A subset of milk samples from 60 cows (20 per group) collected on +1 to +5 wk were

randomly selected to be analyzed at Central Milk Testing Laboratory (Edmonton, Alberta, Canada) for milk fat, protein, lactose, milk urea nitrogen (MUN), total solid (TS) contents, and somatic cell count (SCC) using mid-infrared spectroscopy (MilkoScan 605, A/S N Foss Electric, Hillerød, Denmark). A subset of milk samples from 30 cows (10 per group) collected on 3 DIM, 14 DIM, and 35 DIM were used to determine the concentrations of milk amyloid A (MAA), haptoglobin (Hp), and total immunoglobulin G (IgG). All samples were tested in duplicate. Both the inter- and intra-assay coefficients of variation (CVs) were less than 10%.

Concentrations of glucose in serum were quantified by an enzymatic method with a commercial kit (Genzyme Diagnostics P. E. I. Inc. Charlottetown, Prince Edward Island, Canada). Briefly, glucose in samples is first phosphorylated into glucose-6-phosphate. Then the oxidation of glucose-6-phosphate leads to production of NADH, which produces a color proportional to the glucose concentration in the sample. The serum glucose was then determined by reading on a microplate spectrophotometer (Spectramax 190, Molecular Devices Corp., Sunnyvale, CA) at 340 nm.

Concentration of insulin in the serum was measured using a bovine insulin ELISA kit (Mercodia, Sylveniusgatan, Uppsala, Sweden) based on a sandwich technique. The insulin in the samples is bound by both the anti-insulin monoclonal antibody coated on the plate and peroxidase-conjugated anti-insulin monoclonal antibody. The enzyme catalyzes 3,3’,5,5’-tetramethylbenzidine (TMB) reaction giving a blue color, which is converted into yellow when acid is added to stop the reaction

and read spectrophotometrically at 450 nm.

Concentrations of NEFA in the serum were determined by an enzymatic colorimetric method using a commercially available kit (Randox Laboratories Limited, Crumlin, County Antrim, UK). Coenzyme A is acylated by fatty acids in the sample in presence of acyl-CoA synthetase and then produces hydrogen peroxide in presence of acyl-CoA oxidase. Hydrogen peroxide is oxidized by peroxidase in the presence of 3-methyl-N-ethyl-N-β-hydroxy ethyl-O-aniline (MEHA) with 4-aminoantipyrine to form a purple adduct, which is proportional to the concentration of NEFA in the sample. The optical density was measured at 550 nm on a microplate spectrophotometer (Spectramax 190, Molecular Devices Corp.).

Quantitation of serum BHBA was done using a commercially available kit (Stanbio Laboratory, Boerne, TX USA). Briefly BHBA is converted to acetoacetate and NADH at pH 8.5 by β-hydroxybutyrate dehydrogenase in presence of NAD^+, resulting in production of NADH, which reacts with 2-p-iodophenyl-3-p-nitrophenyl-5-phenyltetrazolium chloride (INT) in presence of iaphorase and produces a color proportional to the concentration of BHBA in the sample. The plate was finally read at 505 nm on a microplate spectrophotometer (Spectramax 190, Molecular Devices Corp.).

The concentration of serum cholesterol was determined by using an enzymatic colorimetric method with a commercial kit (Genzyme Diagnostics P.E.I. Inc. Charlottetown, Prince Edward Island, Canada). The cholesterol esters are hydrolyzed to free cholesterol by cholesterol esterase (CE). The free cholesterol is then oxidized

by cholesterol oxidase (CO) to cholesten-3-one with the simultaneous production of hydrogen peroxide. The hydrogen peroxide produced couples with 4-aminoantipyrine and phenol, in the presence of peroxidase, to yield a chromogen with maximum absorbance at 505 nm.

Concentration of serum lactate was measured using a kit (Biomedical Research Service Center of University at Buffalo, Buffalo, NY, USA) based on the reduction of the tetrazolium salt 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) in a NADH-coupled enzymatic reaction to formazan, which is water soluble and exhibits an absorption maximum at 492 nm. The intensity of the color is in proportional to the lactate concentration.

Milk Amyloid A (MAA) was measured using the Tridelta Mast ID range MAA kits (Tridelta Development Limited, Maynooth, Co., Kildare, Ireland). Milk samples were diluted 50-fold prior to testing. The intensity of the color produced is proportional to the concentration of MAA present in the sample. Milk haptoglobin (Hp) was measured using a bovine haptoglobin ELISA test kit (Life Diagnostics Inc., West Chester, PA). Milk samples were diluted 20-fold prior to testing. Concentrations of total IgG in the milk were measured with bovine IgG ELISA kits (Alpha Diagnostic Intl. Inc., San Antonio, TX). Milk samples were originally diluted 1:100,000 by 3 dilutions prior to testing. For those samples whose concentration was still out of the standard range, samples were diluted more until it fell within the range. The principle of all these three assays is a solid phase sandwich ELISA similar to that of insulin (not described here).

5.2.4 Statistical analyses

The blocking factors including BCS and previous milk yield were tested but found not to be associated with the outcome and therefore excluded from the model. Feed efficiency was calculated as the ratio of milk production to feed intake from +1 to +8 wk. All data, including blood variables, milk components, milk production, feed intake, feed efficiency and BCS were analyzed using SAS 9.2 software (SAS Institute Inc., Cary, NC) with a repeated design structure in a MIXED model incorporating treatment, week, and parity. However, parity was tested to have no effect on the blood variables and milk components and therefore the model used was the following: $Y_{ijk} = \mu + T_i + W_j + (TW)_{ij} + e_{ijk}$, where μ = the population mean; T_i = the effect of i^{th} level of treatment; W_j = the effect of j^{th} level of week; $(TW)_{ij}$= the interaction between i^{th} level of treatment and j^{th} level of week; and e_{ijk} = the residual error. The covariance structure of the repeated measurements for each variable was modeled separately according to the smallest values of the fit statistics based on the Bayesian information criteria. Significance was declared at $P < 0.05$ and tendency at $0.05 \leq P < 0.10$.

5.3 Results

5.3.1 Effect of LAB treatment on metabolic status

Concentrations of NEFA in the serum differed among treatment groups ($P < 0.01$, Figure 5-1). Cows in the TRT2 had lower concentrations of NEFA in the serum than both TRT1 ($P < 0.01$) and CTR cows ($P < 0.01$), especially at wk +1. TRT2 also had lower concentrations of serum NEFA at wk +2 than TRT1 ($P < 0.01$), and tended to be

lower than cows in the CTR group ($P = 0.07$). There was no difference between TRT1 and CTR cows with regards to serum NEFA. Serum NEFA also varied over time ($P < 0.01$). It increased significantly after calving compared with before calving and reached peak values at wk +1. There was no significant interaction between treatment and week.

Intravaginal LAB treatment exerted a significant effect on the concentration of cholesterol in the serum of dairy cows ($P < 0.01$, Figure 5-2). Both TRT1 and TRT2 had greater cholesterol than cows in the CTR group, especially at wk +2 and +3. TRT2, but not TRT1, also had greater serum cholesterol at wk -1 and +1 than cows in the CTR group. All cows had increased concentration of cholesterol after calving compared with pre-calving levels ($P < 0.01$). No significant interactions were observed between treatment and week.

LAB treatment increased concentration of lactate in the serum compared with CTR group ($P < 0.01$, Figure 5-3). Cows in the TRT1 had greater concentrations of lactate than cows in the CTR group at wk -1, +1 and +3 ($P < 0.01$). At wk 0, there was a tendency for greater concentrations of lactate in TRT1 than CTR cows ($P = 0.07$). Concentrations of lactate also was greater in cows in the TRT2 than CTR cows at wk -1 ($P < 0.01$), wk 0 ($P < 0.01$) and wk +1 ($P < 0.05$). At wk +3, TRT2 tended to have a greater concentration of lactate than CTR one ($P = 0.09$). No difference was observed between TRT1 and TRT2. There was no effect of week or interaction between treatment and week on concentration of lactate.

Concentrations of BHBA in the serum was not affected by LAB treatment ($P >$

0.05), but varied over time ($P < 0.01$, Figure 5-4). Overall, concentrations of BHBA in the serum increased greatly after calving compared with pre-calving. There was no interaction between treatment and week.

No difference was observed among treatment groups in terms of concentration of glucose (Figure 5-5A) and insulin in the serum (Figure 5-5B). Serum glucose decreased gradually from wk -2 to wk +3 ($P < 0.01$), whereas serum insulin decreased and reached nadir point at wk 0, and then remained at a stable level thereafter ($P < 0.01$). No interaction between group and week was detected with regards to both of these two variables.

5.3.2 Effect of LAB treatment on immunoglobulin and acute phase proteins in milk

The concentration of total IgG in the milk differed among treatment groups ($P < 0.01$) and varied over time ($P < 0.01$, Figure 5-6). Treatment and DIM interacted regarding total IgG ($P < 0.05$). At 3 DIM, cows in the TRT1 had the greatest concentration of IgG, whereas CTR cows had the lowest concentration of total IgG in the milk, whereas TRT2 was positioned in the middle ($P < 0.01$). However, no difference was observed among treatment groups at 14 and 35 DIM. In the LAB-treated groups, total IgG in the milk decreased from 3 to 14 DIM ($P < 0.01$) and stabilized at 35 DIM, but there was no differences from between 3 to 35 DIM in the milk of cows from the CTR group.

The concentration of Hp in the milk also was differed among treatment groups ($P < 0.01$) and varied over time ($P < 0.01$, Figure 5-7). There was also an interaction

between treatment and week with regards to milk Hp ($P < 0.01$). At 3 DIM, cows in the CTR group had greater concentration of milk Hp than both TRT1 and TRT2 ($P < 0.01$), whereas no difference between TRT1 and TRT2 was observed. At 14 DIM, milk Hp in the CTR cows was greater than TRT1 ($P < 0.05$), and tended to be greater than TRT2 ($P = 0.08$). No difference in concentration of Hp in milk was detected among treatment groups at 35 DIM. In the CTR group, milk Hp decreased sharply over time ($P < 0.01$), whereas Hp in the milk of cows in TRT2 decreased gradually over time ($P < 0.05$). No difference in milk Hp was observed in the TRT1 between 3 to 35 DIM.

The concentration of MAA was also affected by LAB treatment ($P < 0.05$) and days in milk ($P < 0.01$, Figure 5-8). The interaction between treatment and days in milk had no significant effect on MAA. Overall, TRT1 had greater concentrations of MAA than both TRT2 ($P < 0.01$) and CTR cows ($P < 0.05$), especially at 3 DIM. However, TRT2 was not different from the CTR group with regards to MAA. There was a decrease of MAA over time, from 3 to 35 DIM.

5.3.3 Effect of LAB treatment on productive performance

The average daily milk production during the first 8 wk after calving was affected by both LAB treatment ($P < 0.01$) and parity ($P < 0.01$, Figure 5-9). There was also a significant interaction between treatment and parity regarding milk production ($P < 0.01$). Among multiparous cows, those in the TRT1 and TRT2 had greater milk production than cows in the CTR group ($P < 0.01$ and $P < 0.05$, respectively); TRT1

cows also produced greater amounts of milk compared with cows in the TRT2 ($P < 0.05$). However, among primiparous cows, those in the TRT2 had greater milk production than the CTR cows ($P < 0.05$), whereas there were no differences between TRT1 and TRT2 or TRT1 vs CTR cows. In all the 3 treatment groups, multiparous cows exhibited greater milk production than primiparous cows ($P < 0.01$).

The LAB treatment ($P < 0.01$), parity ($P < 0.01$), and their interaction ($P < 0.01$) affected the average daily feed intake during the first 8 wk after calving (Figure 5-10). However, there was no difference among treatment groups in all multiparous cows with regards to feed intake. Among primiparous cows, those in the TRT1 had lower feed intake than TRT2 ($P < 0.01$) and CTR ($P < 0.01$); however, feed intake in the TRT2 was not different from that of CTR cows. In all the 3 treatment groups, multiparous cows exhibited a greater feed intake than primiparous cows ($P < 0.01$).

Feed efficiency, presented as the ratio of milk production to feed intake, was influenced by LAB treatment ($P < 0.01$), parity ($P < 0.01$), and their interaction ($P < 0.01$, Figure 5-11). Among multiparous cows, LAB treatment (both TRT1 and TRT2) enhanced feed efficiency ($P < 0.05$), compared with the CTR group, but no differences between TRT1 and TRT2 groups was evidenced. With respect to primiparous cows, TRT1 had the greatest feed efficiency, whereas the CTR group had the lowest and TRT2 was positioned in the middle, and all of them were different from each other ($P < 0.01$). In addition, multiparous cows in the TRT2 ($P < 0.01$) and CTR groups ($P < 0.01$) demonstrated greater feed efficiency than primiparous ones; however, no such effect on feed efficiency was observed for multiparous cows in the

TRT1.

Body condition score was affected by both parity ($P < 0.01$) and week ($P < 0.01$), but not by LAB treatment ($P > 0.05$, Figure 5-12). Multiparous cows had lower BCS than primiparous cows. In all the cows, BCS deceased from wk -1 to +9. Apart from treatment, no two-way interactions or three-way interaction exerted any effect on BCS.

5.3.4 Effect of LAB treatment on milk composition

The effect of LAB treatment on milk composition is shown in Table 5-2. Both lactose ($P < 0.01$) and protein ($P < 0.05$) content differed among treatment groups. The content of lactose in in TRT2 was greater than that in TRT1 ($P < 0.01$) and tended to be greater than that in the CTR group ($P = 0.05$). Milk from cows in the TRT1 had a greater content of protein than the CTR group cows ($P < 0.05$), but none of them was different from TRT2. There was no effect of treatment on milk fat content, the ratio of milk fat to protein, milk urea nitrogen, total solid of the milk or somatic cell count, although all of these indicators varied with week except milk urea nitrogen. There was no interaction between treatment and week in terms of all these milk components.

5.4 Discussion

It was our hypothesis that 2 or 3 intravaginal infusions of LAB around calving can modulate the metabolic status and improve productive performance of transition dairy cows. Indeed, the results of this study demonstrated that treatment with

intravaginal LAB affected several blood variables related to carbohydrate and lipid metabolism, milk production, and milk composition as will be discussed in more detail below.

Results of this study showed that intravaginal infusion of LAB was associated with lowered concentrations of NEFA in the serum in cows in the TRT2; whereas TRT1 had no effect on this plasma variable. NEFA is a lipid metabolite that has been reported repeatedly to rise in blood immediately after parturition. Moreover, enhanced plasma NEFA has been associated with greater risk of metritis and the incidence of other multiple diseases (Sordillo et al., 2009; LeBlanc, 2010; Giuliodori et al., 2013). Because probiotics in our experiment were infused in the vaginal tract and not orally the only possible explanation for lower plasma NEFA is that LAB lower the incidence of uterine infections. Indeed our data showed that intravaginal infusion of LAB lowered the incidence of uterine infection of the treated cows and boosted local production of sIgA in the vaginal mucus (data presented in Chapter 3). Other research has shown that infection of the uterus is associated with translocation of endotoxin (Mateus et al., 2003) and cytokines into the blood circulation of dairy cows (Kucharski et al., 2008). Zu et al. (2009) demonstrated that endotoxin stimulates lipolysis of adipose tissue and elevation of NEFA in the blood. Therefore, it is likely that the NEFA lowering effect of intravaginally administered LAB resulted from less endotoxin translocated into the systemic circulation. Our data challenge the concept that NEB triggers mobilization of lipids around parturition and support the idea that inflammation and infection during peripartum period might modulate lipid responses

of transition dairy cows (Ametaj et al., 2010). The reason why TRT1 did not have similar effects on plasma NEFA might be related to the differences in the number of doses used for each treatment with TRT1 using 2 rather than 3 doses of LAB.

Another important finding of this study was that cows infused with LAB in the vaginal tract exhibited greater concentrations of cholesterol in the serum compared to CTR cows. It has been recognized that infection and inflammation are associated with a variety of alterations in lipid metabolism (Feingold et al., 1992), including a lowering of plasma concentration of total cholesterol, HDL cholesterol, and LDL cholesterol (Pruzanski et al., 2000). These changes in the plasma lipid composition affect the host immune response, due to the ability of lipoproteins to bind several molecules that activate toll-like receptors through pathogen-associated molecular patterns, including endotoxin (Read et al., 1995) and the ability to serve as a scavenger for such molecules (Feingold et al., 1995). Lower cholesterol in the plasma of CTR cows might be related to the suppressive effects of endotoxin translocated into the blood circulation on reverse cholesterol transport.

Another metabolite affected by LAB treatment was lactate. Cows in both LAB-treated groups maintained concentrations of lactate in the serum around calving whereas those in the CTR group showed an almost 30% decrease in the concentration of lactate at -1 wk before parturition and remained at lower levels during the remaining experimental period. Previously we reported that presence of fatty liver in transition dairy cows is negatively correlated with concentrations of lactate in the blood (Ametaj et al., 2005). Fatty liver affects 50% of dairy cows and has been related

to endotoxemia and expedited removal of LPS-high density lipoprotein complexes to clean endotoxin from circulation (Ametaj et al., 2005). It is speculated that although infusion of LAB has no direct relationship to metabolism of lactate it is possible that LAB-treated cows might have suffered less from fatty liver. In addition greater blood lactate is correlated with milk production and with energy availability (Coffey et al., 1982; Lalman et al., 2000). Consequently, greater lactate levels in the blood of treated cows probably served as a supportive energy source for the increased milk production and lactose content observed in the LAB-treated cows. The positive association between glucogenic energy source (i.e., lactate) and milk lactose content as well as the negative association between glucogenic and lipogenic energy source (i.e., NEFA) is in alignment with the findings of previous studies (van Knegsel et al., 2007).

LAB-treated cows had lower concentrations of Hp and greater concentrations of IgG in the milk (i.e., colostrum) at 3 DIM compared with their CTR counterparts. Lower milk haptoglobin is indicative of a better mammary gland health in LAB-treated cows compared with CTR cows. Haptoglobin is transported from blood into the milk, but also originates from milk immune cells and epithelial cells in the mammary gland (Hiss et al., 2004). Additionally greater milk IgG in the LAB-treated cows is important with respect to the health of newborn calves providing them better protection against bacterial infections in the early days of life. Cows in the TRT1 had greater MAA at 3 DIM only. Serum amyloid A is secreted by hepatocytes and, in addition, the mammary gland epithelium also secretes a mammary gland-specific isoform known as MAA (Eckersall et al., 2001). Of note, cows in the TRT1 had

numerically more cases of subclinical mastitis (3 vs 2) during the first week only (when MAA was greater) compared to the CTR group. Gerardi et al. (2009) indicated that MAA could be used as an indicator of subclinical mastitis in dairy cows.

Another important finding of this study was that LAB treatment improved the productive performance of transition dairy cows as indicated by increased milk production and feed efficiency in the treated cows. In a previous study, we observed that intravaginal infusion of the same cocktail of LAB for 6 consecutive weeks around calving increased milk production (Ametaj et al., 2014). Although the number of doses administered to cows in this study was only 2-3 per cow we still observed greater milk production from both primiparous and multiparous cows. Most probiotic studies in the past have demonstrated beneficial effects on milk yield, feed intake, and feed efficiency, however, they were mostly administered via the oral route (Krehbiel et al., 2003; Weinberg, 2003). In this study, the probiotic mixture was applied intravaginally and still exhibited the same benefits with respect to feed efficiency and milk production. One of the potential mechanisms of how probiotics increased milk production in the current study could be related to prevention of uterine infections (data presented in Chapter 3) as well as prevention of translocation of endotoxin into the blood circulation, which is known for its inhibitory effects on the release of prolactin and the decrease in milk production (Smith and Wagner, 1984). Therefore, it is likely that the differences in milk yield and milk composition among the LAB-treated cows and CTR ones are related to the overall improvement in the health status and metabolic profiles of those cows (Markusfeld et al., 1997; Kida, 2003; Bell

and Roberts, 2007).

5.5 Conclusions

Taken together, results of this study demonstrated that intravaginal infusion of LAB around calving modulated concentrations of selected metabolites in the serum of transition dairy cows as indicated by lower NEFA and greater cholesterol and lactate. LAB treatment also was associated with increased total IgG, lactose, and protein content in the milk as well as lower milk Hp. Moreover, cows treated with LAB exhibited greater milk production and improved feed efficiency. It will be interesting to study the mechanisms by which intravaginal LAB influence milk production and metabolic status of transition dairy cows. Also, intravaginal probiotics might be an unexploited tool to increase milk production in dairy cows.

5.6 Acknowledgements

We acknowledge the financial support of the funding agencies including Alberta Livestock and Meat Agency Ltd. (ALMA), Alberta Milk, and Natural Sciences and Engineering Research Council of Canada (NSERC) for this project. We acknowledge the contribution of Drs. Michael G. Gänzle and Yvonne Wang for preparation of LAB cocktail. We also are grateful to the technical staff of the Dairy Research and Technology Centre, University of Alberta for their help and care to the cows.

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Table 5-1. Ingredients and chemical composition of the diets for cows during experimental period

	Prepartum diet	Early lactation diet
Ingredients, % of DM		
Alfalfa hay	12.28	10.00
Barley silage	61.79	41.19
CUD grain[1]	25.93	-
Dairy supplement[2]	-	48.81
Chemical composition		
Dry matter, %	49.45	57.98
NE lactation, Mcal/kg	1.55	1.84
TDN, % of DM	68.95	-
NS carbohydrate, % of DM	26.79	35.22
Forage DM, % of DM	77.29	52
NDF, % of DM	44.99	33.82
ADF, % of DM	28.73	21.79
Crude protein, % of DM	15.45	18.15
SIP, % of CP	46.49	39.02
RUP, % of CP	29.56	33.39
RDP, % of CP	70.44	66.61
RLY : RMT	3.06	2.59
BV Met, % of DM	0.19	0.33
Calcium, % of DM	0.96	1.06
Phosphorus, % of DM	0.43	0.47
Magnesium, % of DM	0.37	0.36
Sodium, % of DM	0.09	0.24
Potassium, % of DM	1.68	1.46
Salt, % of DM	-	0.24
Copper, ppm	51.62	-
Manganese, ppm	90.33	-
Selenium, ppm	0.65	-
Zinc, ppm	154.86	-
Cat/An balance, mEq/100g	24.79	33.77
VA, KIU/kg	15.49	8.73
VD, KIU/kg	3.10	1.53
VE, IU/kg	232.29	60

[1]CUD contains: 55.0% rolled barley grain, 7.5% canola meal, 6.2% Dairy dry cow micro-premix, 8.7% limestone, 15.7% Animate (primary source of anionic salts (chloride and sulfur), with supplemental protein, minerals, trace minerals and vitamins), 0.9% molasses, 4.1% canola oil, 1.7% yeast.

[2]Dairy supplement contains: 0.056% ADE Vit, 0.10% Ruminant TM Pak, 0.07% Selenium, 0.06% Custom TM Complex, 1.25% Di-calcium phosphate, 10% Co-op alantic Corn Dist, 25% corn ground, 30% corn rolled, 0.015% Vit D-10,000KIU/kg; 0.14% Diamond V XPC, 1.00% Magalac/Enteria, 2.00% Fermenten, 1.5% limestone, 0.37% Mag Ox-56%, 15.5% canola meanl, 2.75% Hi bypass soy, 6.5% soy bean meanl-47.5%, 1% sodium bicarbonate, 0.113% salt, 2.45%

cattle tallow, 0.007% Biotin 2%, 0.015% ADM Vit E 405 Vegetable source.

Table 5-2. Effect of intravaginal LAB treatment on milk composition

Variable	TRT[1]				Week[5]	TRT*week
	TRT1[2]	TRT2[3]	CTR[4]	*P*-value	*P*-value	*P*-value
Lactose, %	4.39±0.02[b]	4.53±0.03[a]	4.44±0.03[ab]	< 0.01	< 0.01	> 0.05
Protein, %	2.99±0.04[a]	2.93±0.05[ab]	2.82±0.05[b]	< 0.05	< 0.01	> 0.05
Fat, %	4.00±0.18	4.37±0.22	3.99±0.20	> 0.05	< 0.01	> 0.05
Fat : protein	1.34±0.07	1.49±0.09	1.43±0.08	> 0.05	< 0.01	> 0.05
MUN[6], mg/dL	15.57±0.93	15.01±1.14	16.06±1.04	> 0.05	> 0.05	> 0.05
TS[7], %	12.46±0.20	12.89±0.24	12.27±0.22	> 0.05	< 0.01	> 0.05
SCC[8], *1000/mL	57.20±9.21	68.42±10.99	66.10±10.36	> 0.05	< 0.01	> 0.05

[1]TRT: intravaginal treatment of LAB or carrier.

[2]TRT1: two prepartum doses of LAB, $n_1 = 20$.

[3]TRT2: two prepartum doses plus one postpartum dose of LAB, $n_2 = 20$.

[4]CTR: carrier only, $n_3 = 20$.

[5]Week: from wk+ 1 to +5 on a weekly basis.

[6]MUN: milk urea nitrogen.

[7]TS: total solid.

[8]SCC: somatic cell count.

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

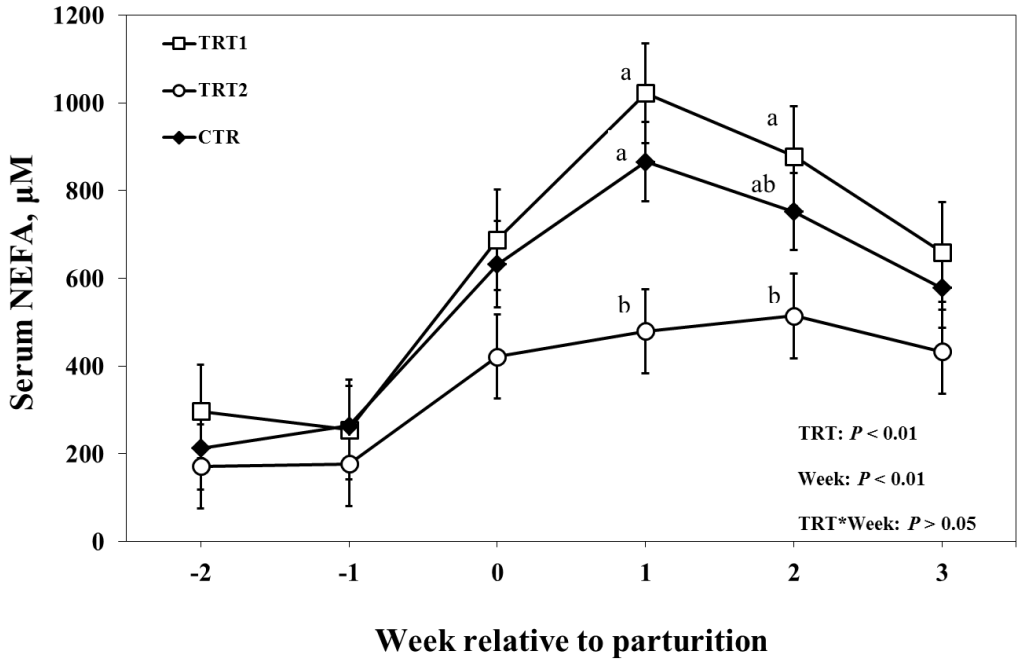

Figure 5-1. Effect of intravaginal infusion of LAB on concentrations of NEFA in the serum (LSM ± SEM, n = 10 in each group. NEFA: non-esterified fatty acid. TRT = effect of probiotic treatment; Week = effect of week; TRT * Week = effect of the interaction between treatment and week. TRT1: □, two prepartum doses of probiotics; TRT2: ○, two prepartum doses plus one postpartum dose of probiotics; CTR: ♦, two prepartum doses plus one postpartum dose of carrier only).

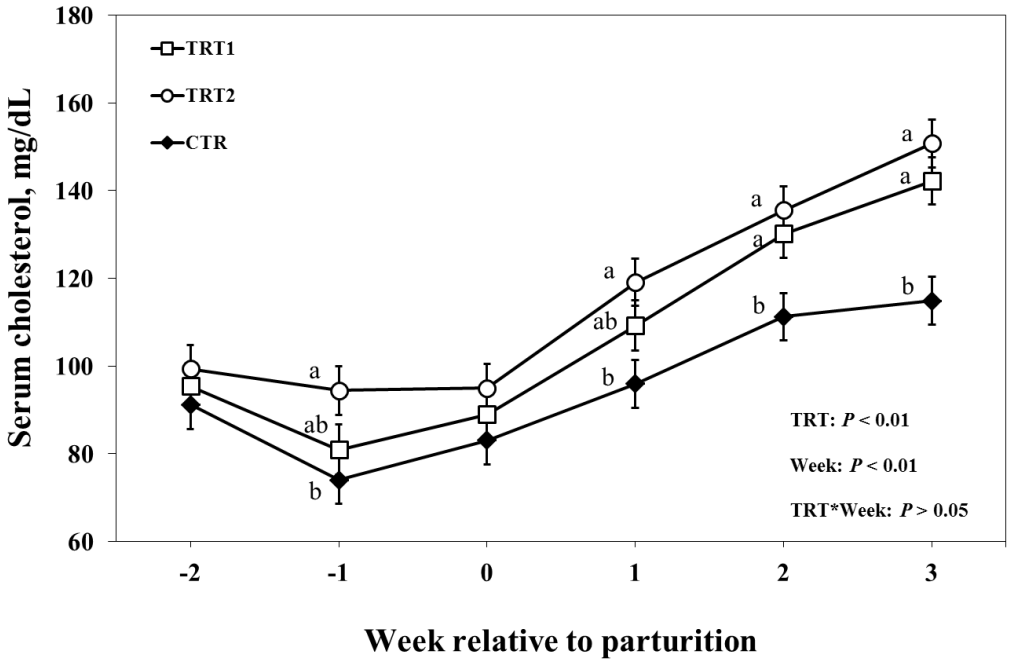

Figure 5-2. Effect of intravaginal infusion of LAB on concentrations of cholesterol in the serum (LSM ± SEM, n = 10 in each group. TRT = effect of probiotic treatment; Week = effect of week; TRT * Week = effect of the interaction between treatment and week. TRT1: □, two prepartum doses of probiotics; TRT2: ○, two prepartum doses plus one postpartum dose of probiotics; CTR: ♦, two prepartum doses plus one postpartum dose of carrier only).

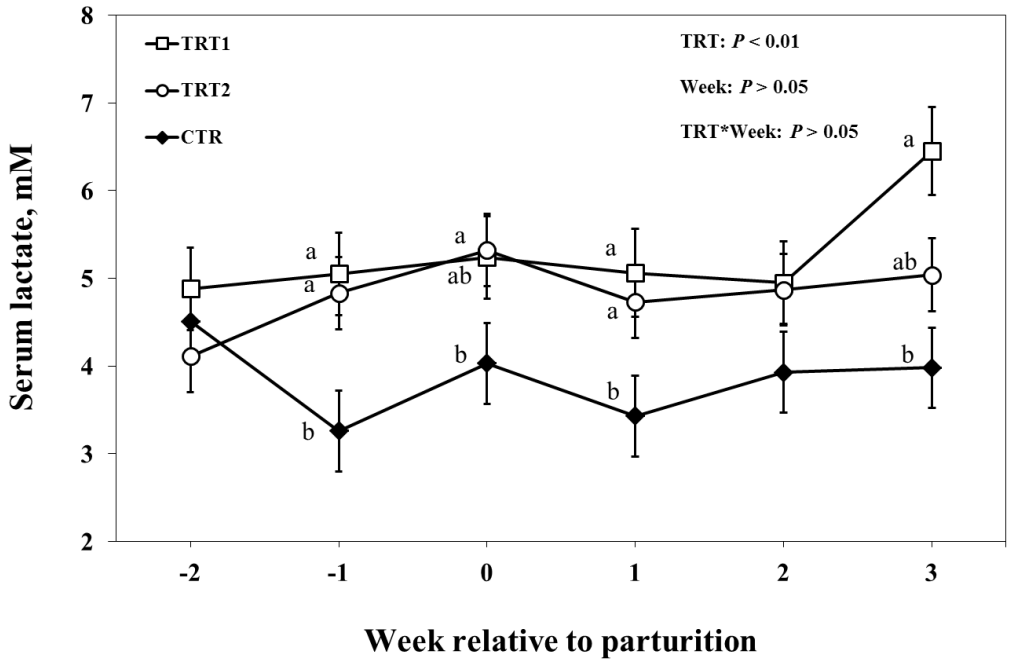

Figure 5-3. Effect of intravaginal infusion of LAB on concentrations of lactate in the serum (LSM ± SEM, n = 10 in each group. TRT = effect of probiotic treatment; Week = effect of week; TRT * Week = effect of the interaction between treatment and week. TRT1: □, two prepartum doses of probiotics; TRT2: ○, two prepartum doses plus one postpartum dose of probiotics; CTR: ♦, two prepartum doses plus one postpartum dose of carrier only).

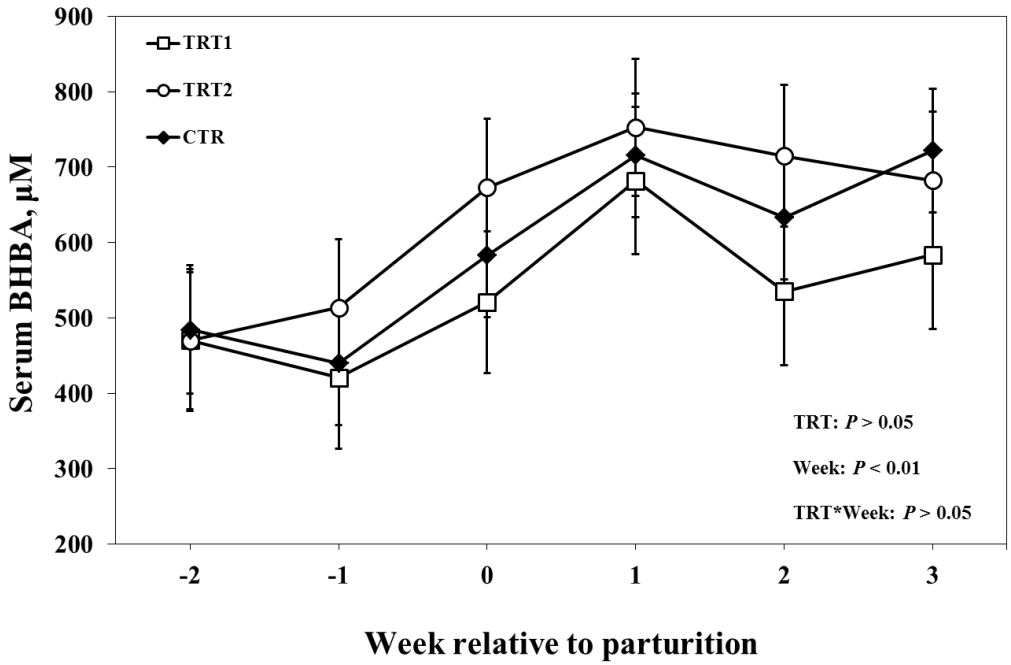

Figure 5-4. Effect of intravaginal infusion of LAB on concentrations of BHBA in the serum (LSM ± SEM, n = 10 in each group. BHBA: β-hydroxy butyric acid. TRT = effect of probiotic treatment; Week = effect of week; TRT * Week = effect of the interaction between treatment and week. TRT1: □, two prepartum doses of probiotics; TRT2: ○, two prepartum doses plus one postpartum dose of probiotics; CTR: ♦, two prepartum doses plus one postpartum dose of carrier only).

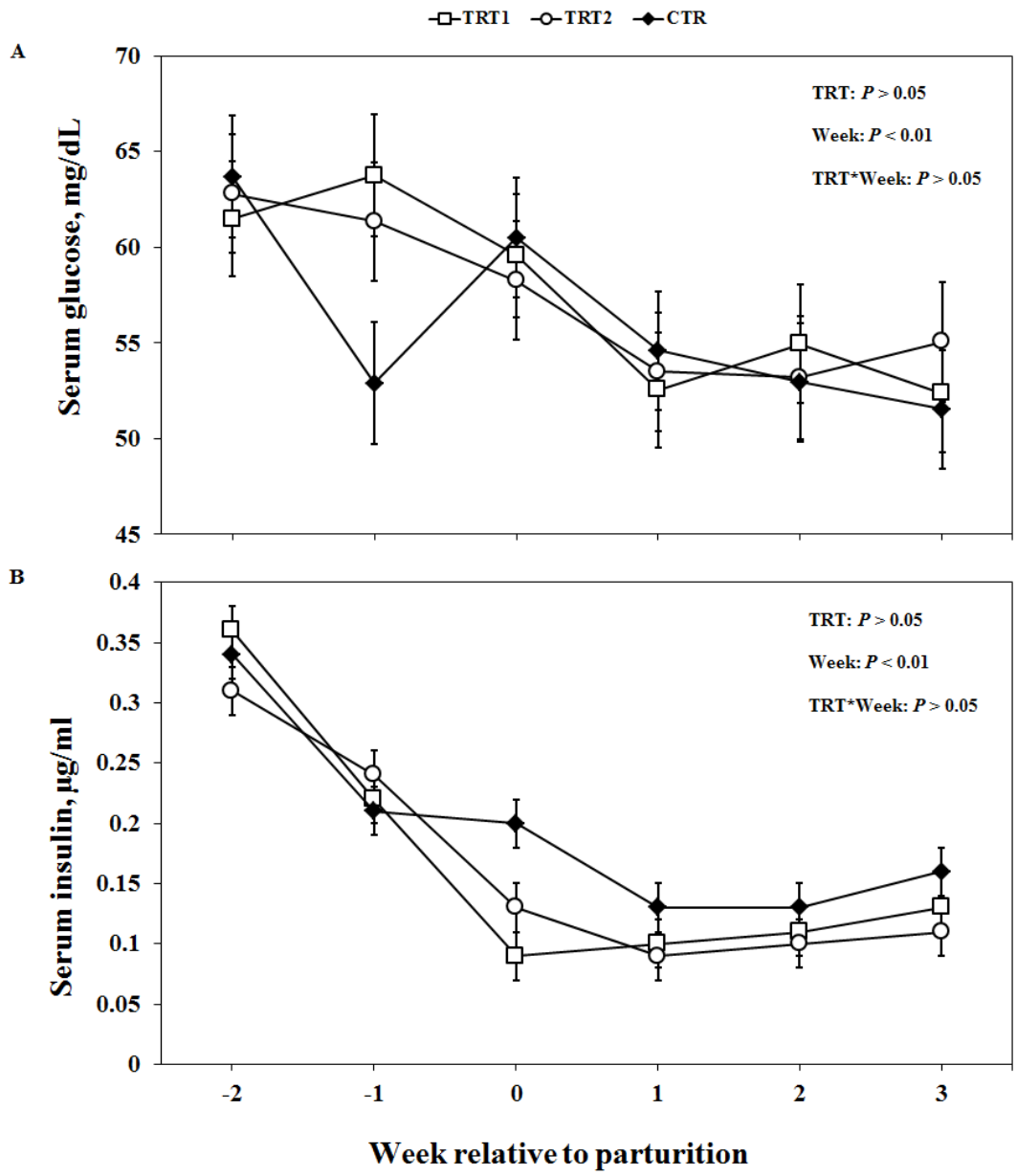

Figure 5-5. Effect of intravaginal infusion of LAB on concentrations of glucose (A) and insulin (B) in the serum (LSM ± SEM, n = 10 in each group. TRT = effect of probiotic treatment; Week = effect of week; TRT * Week = effect of the interaction between treatment and week. TRT1: □, two prepartum doses of probiotics; TRT2: ○, two prepartum doses plus one postpartum dose of probiotics; CTR: ♦, two prepartum doses plus one postpartum dose of carrier only).

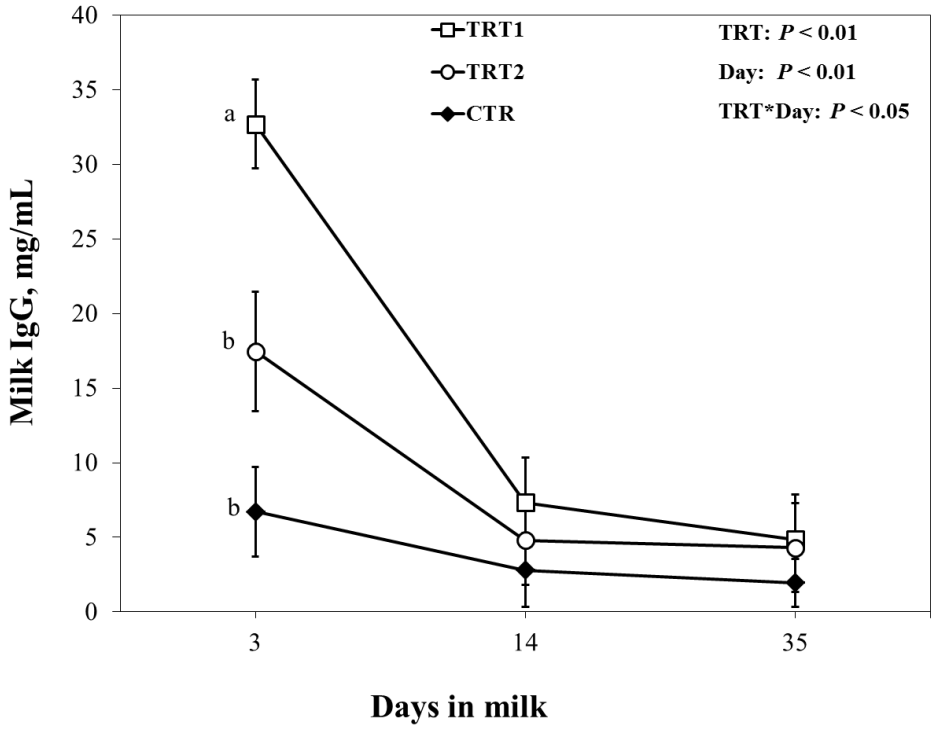

Figure 5-6. Effect of intravaginal infusion of LAB on concentrations of total IgG in the milk (LSM ± SEM, n = 10 in each group. IgG: immunoglobulin G. TRT = effect of probiotic treatment; Week = effect of week; TRT * Week = effect of the interaction between treatment and week. TRT1: □, two prepartum doses of probiotics; TRT2: ○, two prepartum doses plus one postpartum dose of probiotics; CTR: ♦, carrier only).

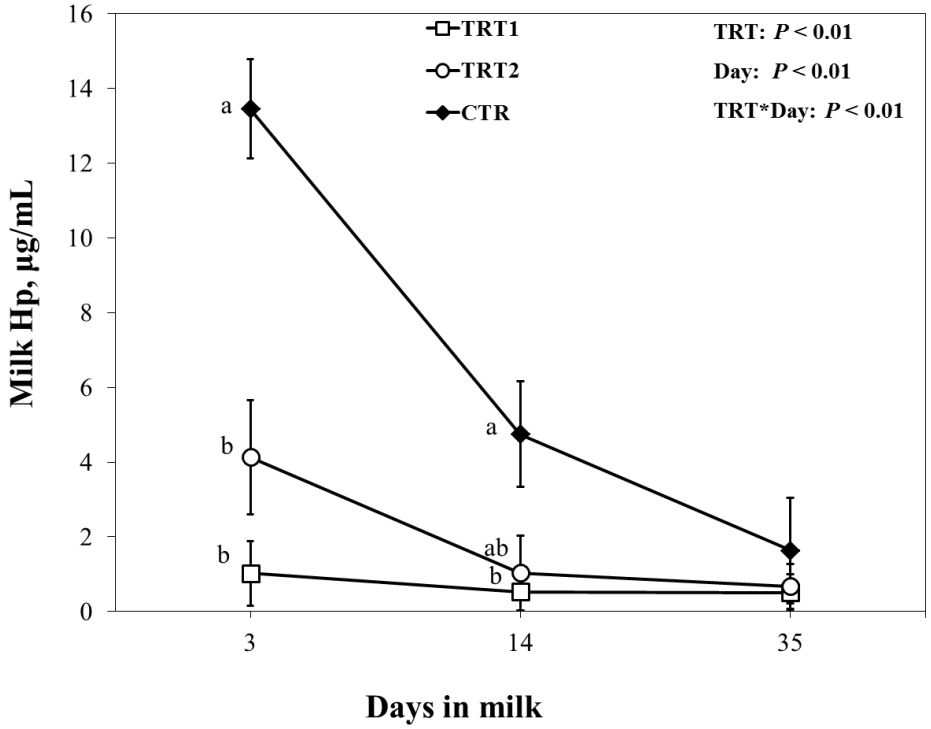

Figure 5-7. Effect of intravaginal infusion of LAB on the concentrations of haptoglobin (Hp) in the milk (LSM ± SEM, n = 10 in each group. Hp: haptoglobin. TRT = effect of probiotic treatment; Week = effect of week; TRT * Week = effect of the interaction between treatment and week. TRT1: □, two prepartum doses of probiotics; TRT2: ○, two prepartum doses plus one postpartum dose of probiotics; CTR: ♦, two prepartum doses plus one postpartum dose of carrier only).

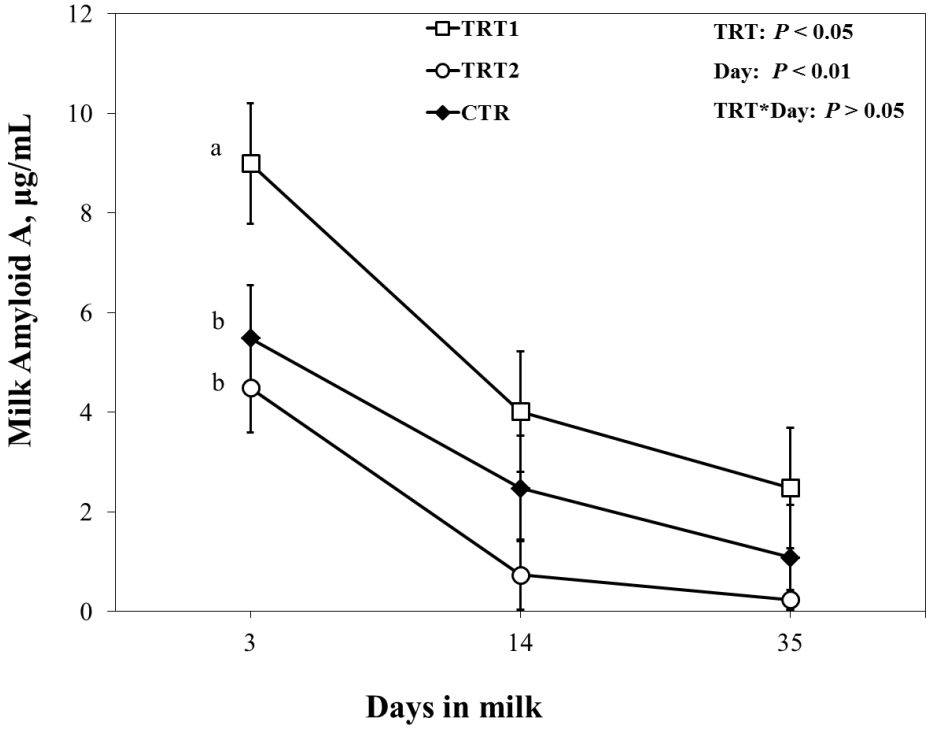

Figure 5-8. Effect of intravaginal infusion of LAB on concentrations of milk amyloid A (MAA; LSM ± SEM, n = 10 in each group. MAA: milk amyloid A. TRT = effect of probiotic treatment; Week = effect of week; TRT * Week = effect of the interaction between treatment and week. TRT1: □, two prepartum doses of probiotics; TRT2: ○, two prepartum doses plus one postpartum dose of probiotics; CTR: ♦, two prepartum doses plus one postpartum dose of carrier only).

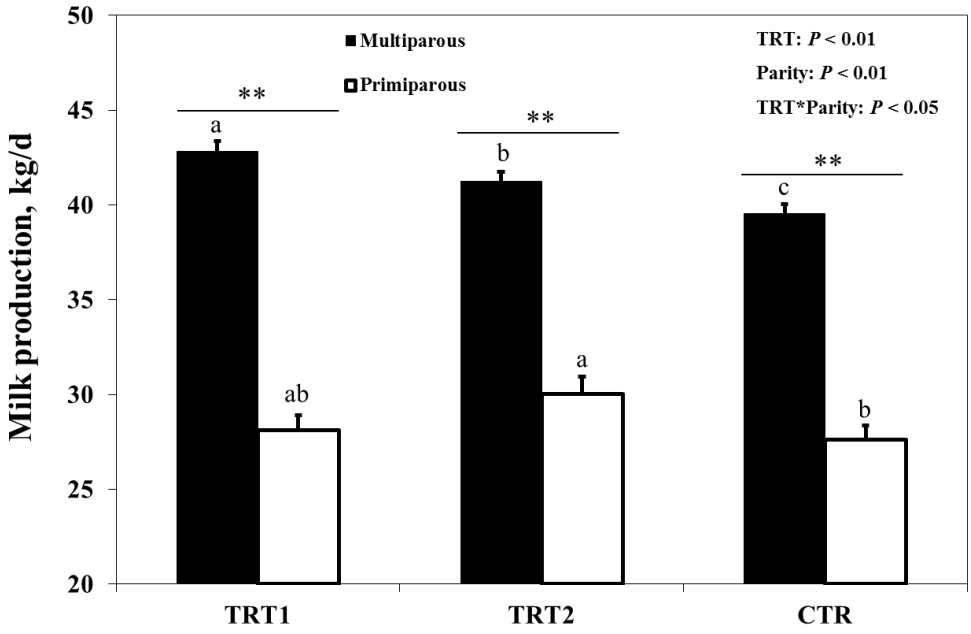

Figure 5-9. Effect of intravaginal infusion of LAB on average daily milk production during the first 8 wk postpartum (LSM ± SEM. Multiparous: $n_1 = 30$; primiparous: $n_2 = 12$. TRT = effect of probiotic treatment; Parity = effect of parity; TRT * Parity = effect of the interaction between treatment and parity. TRT1: □, two prepartum doses of probiotics, $n_1 = 14$; TRT2: ○, two prepartum doses plus one postpartum dose of probiotics, $n_2 = 14$; CTR: ♦, two prepartum doses plus one postpartum dose of carrier only, $n_3 = 14$. a - c: different letters denote significant difference between treatment groups of the same parity. **: denote significant difference between parity in the same treatment group).

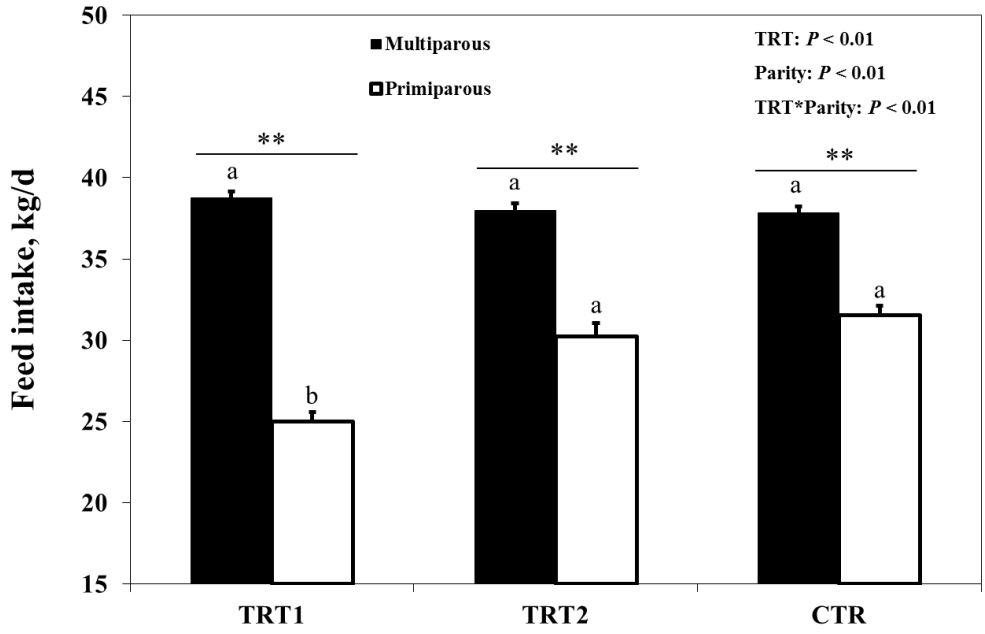

Figure 5-10. Effect of intravaginal infusion of LAB on average daily feed intake during the first 8 wk postpartum (LSM ± SEM. Multiparous: $n_1 = 30$; primiparous: $n_2 = 12$. TRT = effect of probiotic treatment; Parity = effect of parity; TRT * Parity = effect of the interaction between treatment and parity. TRT1: □, two prepartum doses of probiotics, $n_1 = 14$; TRT2: ○, two prepartum doses plus one postpartum dose of probiotics, $n_2 = 14$; CTR: ♦, two prepartum doses plus one postpartum dose of carrier only, $n_3 = 14$. a - b: different letters denote significant difference between treatment groups of the same parity. **: denote significant difference between parity in the same treatment group).

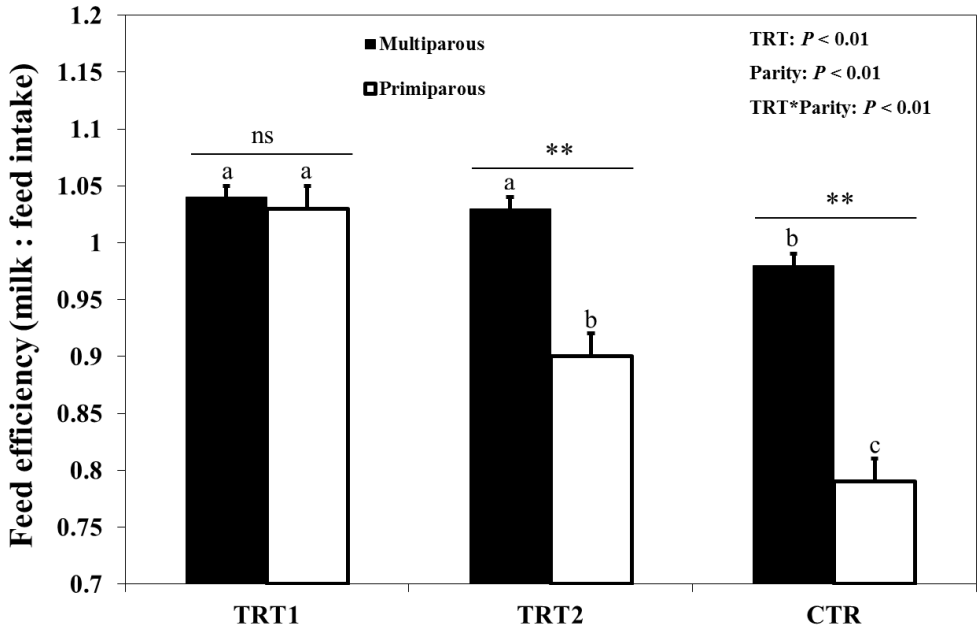

Figure 5-11. Effect of intravaginal infusion of LAB on feed efficiency during the first 8 wk postpartum (LSM ± SEM. Multiparous: $n_1 = 30$; primiparous: $n_2 = 12$. TRT = effect of probiotic treatment; Parity = effect of parity; TRT * Parity = effect of the interaction between treatment and parity. TRT1: □, two prepartum doses of probiotics, $n_1 = 14$; TRT2: ○, two prepartum doses plus one postpartum dose of probiotics, $n_2 = 14$; CTR: ♦, two prepartum doses plus one postpartum dose of carrier only, $n_3 = 14$. a - c: different letters denote significant difference between treatment groups of the same parity. **: denote significant difference between parity in the same treatment group; ns: not significant).

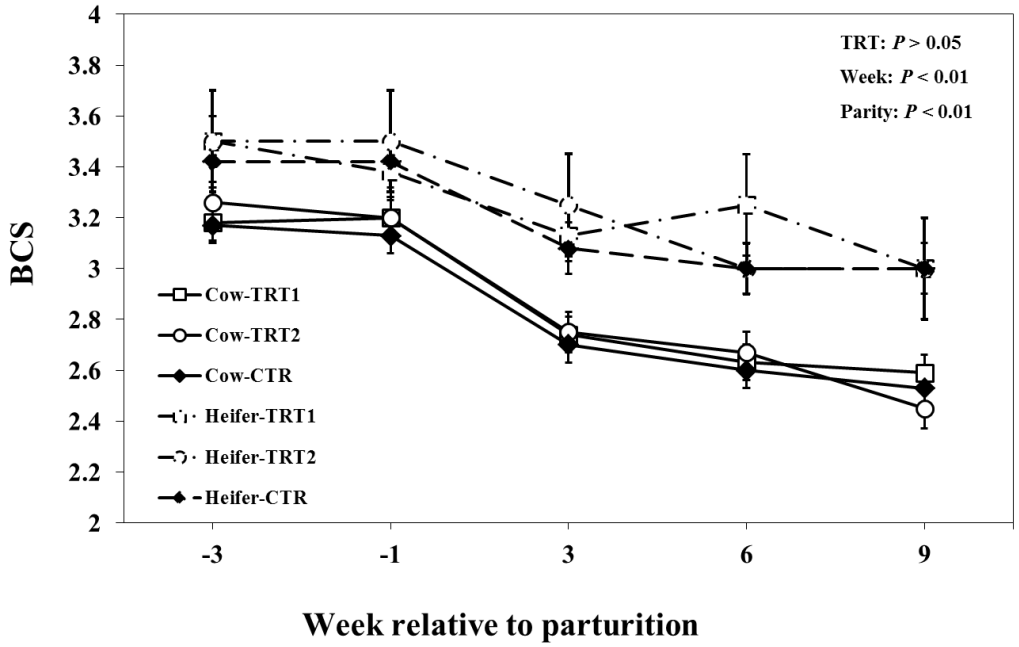

Figure 5-12. Effect of intravaginal infusion of LAB on the body condition score (BCS) (LSM ± SEM. Multiparous: $n_1 = 30$; primiparous: $n_2 = 12$. TRT = effect of probiotic treatment; Parity = effect of parity; TRT * Parity = effect of the interaction between treatment and parity. TRT1: □, two prepartum doses of probiotics, $n_1 = 14$; TRT2: ○, two prepartum doses plus one postpartum dose of probiotics, $n_2 = 14$; CTR: ♦, two prepartum doses plus one postpartum dose of carrier only, $n_3 = 14$.).

Chapter 6 Overall discussion

6.1 Beneficial effects of LAB on reproductive and productive performance of transition dairy cows

6.1.1 Health benefits

The main hypothesis of this investigation was that intravaginal administration of LAB would lower the incidence rate of uterine infections in postpartum dairy cows. Indeed cows treated with LAB showed health benefits as indicated by lower incidence rates of metritis and total uterine infections, which were associated with modified microbiota and greater sIgA in the vaginal tract. To our best knowledge this is the first time that such a benefit is documented for LAB in both human and animal experimentation.

In a previous study we infused transition dairy cows intravaginally with 6 doses of LAB at 10^{10}-10^{12} cfu/dose around calving, on a weekly basis starting at 2 wks before and ending at 4 wks after parturition (Ametaj et al., 2014). The results of that study showed lower incidence of vaginal purulent discharges in the treated cows. The present study confirms health benefits of using intravaginal LAB in dairy cows. Preventing development of uterine infections is a great benefit to the dairy industry given the fact that dairy cows subjected to uterine infections are prone to subfertility or infertility. Reproductive failure is the number one reason for culling of cows in Canada and beyond. Given the fact that antimicrobial compounds and antibiotics are

inefficient in the treatment or prevention of uterine infections and are associated with milk loss the benefits of using probiotics are quite exciting and encouraging.

Being used as probiotics, administration of LAB in the vaginal tract of transition cows modulated the composition of vaginal microbiota of dairy cows. The gene copy number of *L. sakei* remained higher in the vaginal mucus of LAB treated cows even 3 wks after calving compared with their control counterparts, which means the infused probiotic bacteria persisted for 5 wks after the first administration. In addition, the intravaginal infusion of LAB before calving led to an increase in the number of *Lactobacillus* group by almost 4 log at 3 wks after calving. More interestingly, the impact that LAB administration exerted to the microbiotia of the vaginal tract lasted till 11 wks since the first administration. At 8 wks after calving, cows received LAB before calving had a lower number of total bacteria, *Bacillus* spp., and *E. coli*, whereas those received LAB around calving had a higher number of *Lactobacillus* group and *Staphylococcus* spp. compared with CTR cows. It is well known that the lactic acid – producing bacteria play an important role in maintaining the microbiobial balance and preventing infections in the reproductive tract (Reid and Bruce, 2003). On the other hand, among the contaminant bacteria, *E. coli* is one of the notorious pathogens that cause uterine infections in postpartum dairy cows (Wang et al., 2013; Williams, 2013). Either by boosting the prosperity of lactic acid bacteria or suppressing the proliferation of total bacterial and *E. coli*, the intravaginal application of LAB holds the promise to maintain a desirable microbiota in the reproductive tract in the postpartum period, which can help greatly lower the chance of uterine

infections. Furthermore, all of these results imply that intravaginal administration of LAB had both short term and long term impacts on the microbial composition of vaginal mucus of dairy cows. Therefore, the maintenance of a desirable bacterial composition can be achieved by a few times of treatment, rather than repeated administration, which makes the practice of LAB administration easier and more economical. Of note, 2 times of treatment is the minimal treatment we have tried in this study. It would be worthwhile to conduct another study to investigate whether 1 time treatment could exert the same impact on the vaginal microbiota in the future.

Moreover, health benefits included a better local humoral response as reflected by greater sIgA concentrations in the vaginal mucus of treated cows. Secretory IgA is the main immunoglobulin that protects mucosal layers from the invasion of pathogenic bacteria. One of the merits of sIgA defense is it prevents bacterial invasion and colonization by binding bacteria and forming immune aggregation, which is called immune exclusion. Such immune exclusion doesn't activate complement system and therefore is non-inflammatory (Russell et al., 1999; Boullier et al., 2009).

Apart from the local impact, intravaginal administration of LAB also affected the systemic immune response. Lipopolysaccharide-binding protein and SAA are involved in the transportation, neutralization and removal of bacterial LPS from systemic circulation. The lower concentrations of LBP and SAA in the treated cows indicate a lessened peripheral inflammatory response, suggesting that cows treated with LAB had lower translocation of bacterial toxic compounds or inflammatory

substances in the systemic circulation which might have been resulted from the lower incidence of uterine infections, compared with the control counterparts.

6.1.2 Reproduction benefits

The main objective of this study was to evaluate whether intravaginal infusion of 2 to 3 doses of LAB at 10^8-10^9 cfu/dose in the vaginal tract could improve reproductive performance of dairy cows. Indeed, cows treated with LAB before calving exhibited less days open compared to those in the control group. Lowering the number of days open is of great economic benefit to the dairy industry because this shortens the time that a cow needs to become pregnant and lowers expenses related to breeding and labor as well as the cost of hormonal treatment to bring cows to estrus. This also is related to earlier birth of the newborn calves and more milk production from the dairy cows. It has been estimated that it costs $3/cow/day for each extra day open beyond 90 days open (Smith and Becker, 1996; Keown and Kononoff, 2006). Prepartal treatment of LAB shortened the number of days open by 40 days, which implies an economical saving of $120/cow/day. Overall it can be concluded that infusion of LAB in the vaginal tract of transition dairy cows confers beneficial effects with regards to improving reproductive performance of dairy cows.

This reproductive outcome of postpartum dairy cows is influenced by uterine involution and cyclic resumption. In this study, application of LAB either before parturition (2 doses before the expected day of parturition) or around parturition (2 doses before the expected day of parturition and 1 dose on the first week after

parturition) expedited uterine involution in the treated cows. Involution rate of postpartum cows is important for their future reproductive performance. For instance, Melendez et al. (2004) demonstrated that cows with a uterine diameter lower than 5.1 cm had a conception rate at first service of 30.8%, whereas cows with uterine diameters greater than 5.1 cm had a conception rate at first service of 7.9%. In addition the same authors reported that cows with uterine diameters less than 5.1 cm were 5.5 times more likely to conceive at first service than were cows with uterine diameters greater than 5.1 cm. However, the beneficial effect of intravaginal LAB on accelerating the resumption of ovarian cyclicity has only been manifested in cows treated both before and after calving.

6.1.3 Metabolic and productive benefits

Another hypothesis of this study was intravaginal administration of LAB would confer better metabolic and productive status to the treated cows. Cows in the treated groups had lower concentrations of NEFA in the serum. The increased plasma levels of NEFA impair the migration, phagocytic and killing activity and/or the oxidative burst of neutrophils and other leucocytes, enhancing the susceptibility of the host to invading pathogens (Suriyasathaporn et al., 2000; Zerbe et al., 2000; Scalia et al., 2006).

Moreover cows treated with intravaginal LAB produced more milk compared with their control counterparts. This is the first time that a green technology as probiotics can cause increased milk production in transition dairy cows without

residual in the milk. The most successful previous technology is utilization of recombinant bovine somatotropin (r-bST) hormone (i.e., growth hormone) to increase milk production. This technology has been the center of public criticism because r-bST is transported into the milk and has been associated with negative health effects on the cows and consumers, and its utilization by dairy industry has been banned in Canada (Health Canada, 1999). An European Union report and two metaanalytical studies on effects of r-bST on dairy cow welfare and health status indicates that its usage results in severe and unnecessary pain, suffering, and distress for the cows and is associated with increased incidence of mastitis, foot disorders, and reproductive problems (Dohoo et al., 2003a, 2003b). In addition various investigations have suggested that high amounts of insulin growth factor-1 (IGF-1) in the milk of cows treated with r-bST poses a great risk for development of various cancers in human subjects (Baserga et al., 2005). Other health benefits of intravaginal LAB were greater IgG in the milk of the treated cows. This result is of utmost importance for the newborn calves that might be provided with greater immunoglobulins during the first weeks of birth when they are more vulnerable to gastrointestinal and pulmonary infections. It is a well-known fact that calves receiving more immunoglobulins from the colostrum during the first 12 h postpartum are better protected from infection by enteropathogenic bacteria and lower incidence of pneumonia (Roy, 1980).

6.2 Future implications of LAB administration

Several issues need to be better understood in order for this exciting technology to be used widely by the dairy industry to improve the incidence rate of uterine infections, reproductive performance and productivity of dairy cows. One of the issues that deserve to be studied in the future is the frequency of administration of probiotics into the vaginal tract. Two separate studies conducted by our team using 6, 3 and 2 treatments per week around calving have given very encouraging results. Data from the present study indicated that LAB survived in the vaginal mucus for several weeks and triggered alterations in the composition of bacterial composition of vaginal mucus. Should we utilize 2, 3, 6 or more administrations of LAB? This warrants further investigation.

Another issue that needs to be further studied is the time of administration of probiotics around calving. We arbitrarily chose to start administration of LAB at 2 wk before the expected day of parturition; however, starting earlier than 2 wk might be interesting to be studied. This suggestion comes from the fact that LAB survived for several weeks in the vaginal mucus of the treated cows.

Another important issue that deserves to be studied further is the dose of LAB utilized. We used two different doses of LAB in our two separate studies. In the first LAB study we used a greater dose of LAB at 10^{10} to 10^{12} cfu/dose; whereas at the present study we used 10^{8} to 10^{9} cfu/dose. Both doses gave similar effects on reproduction outcomes and production benefits. Therefore these data argue for a dose study in the near future.

In an accidental experiment we noticed that LAB used in around 15 cows per group were not efficient with regards to reproductive benefits we observed in our first probiotic experiment. These LAB strains were stored at -80 for almost a year. Culture analyses of these LAB strains indicated that the number of cfu in the 1-year old probiotics had decreased to 10^7 cfu/dose from 10^{10} to 10^{12} cfu/dose from previous year. It will be interesting to study the shelf life of probiotics and better preservatives to prolong their shelf life.

There are two last issues that need to be addressed with regards to application of probiotics for reproductive performance of transition dairy cows. The first is to increase the number of cows used in the experiment. In the two studies conducted by our team we have used 30-40 cows per group. This limitation is related to the low number of milking cows at the Dairy Research and Technology Centre at the University of Alberta, which has a total of 150 cows. These cows are used by various investigators during the year and do not allow to increase the number more than 150. Another limitation is the 1-2 years of research grants supported by funding agencies. In these occasions extended research grants to 3-4 years would be beneficial for using a greater cohort of animals in the experiment. Application of LAB in two conventional dairy farms in Albert or Canada would be of great interest to see if the strains of LAB selected from the cows at University of Alberta dairy farm would work in cows to other farms. Determination of vaginal bacterial composition in various dairy cows in conventional farms in Alberta and beyond would be of interest

to identify and isolate other potential probiotic bacteria to be used in the future in different dairy farms.

6.3 Conclusions

Taken together, the data obtained from this project demonstrated that intravaginal infusion of LAB around calving lowered uterine infections of postpartum dairy cows through modulating local microbiota and enhancing local sIgA production. Improved reproductive health was associated with other benefits including less activation of innate immunity, better metabolic status and hormonal production, which promoted reproductive and milk production performance. These encouraging results suggest importance of reproductive tract microbiota in cow health and productivity. However, further investigation is warranted to determine the optimal treatment time, the frequency of probiotic applications, and optimal dose of probiotics. With regards to dairy farm application of this new technology, testing probiotics on other locations is desirable at present.

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