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Uterine immune response and microbiota composition in postpartum dairy cows

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Résumé

Les vaches laitières en transition sont sensibles aux infections utérines en raison de l'immunité compromise autour du vêlage et de la contamination bactérienne importante dans l'utérus immédiatement après le vêlage. Les vaches atteintes d'infections utérines sont plus susceptibles de développer d'autres maladies périnatales, ce qui entraîne une baisse de la production de lait et une diminution de la fertilité. L'infertilité liée aux infections utérines est devenue la principale cause d'élimination d'une vache du troupeau. Jusqu'à présent, il n'y a pas eu d'approches efficaces pour traiter les infections utérines. Environ 90 % des vaches laitières subissent une contamination bactérienne post-partum de l'utérus. La plupart des vaches sont capables d'éliminer l'infection en 8 semaines au cours du processus d'involution, mais jusqu'à 20 % des vaches développent une métrite, qui est une infection de toute la paroi utérine ; et dans certains troupeaux, 30 à 50 % des vaches développent une endométrite, qui est une infection de la paroi interne de l'utérus.

Il a été indiqué que le microbiome et les facteurs immunitaires innés de l'appareil reproducteur ont un effet sur la maladie utérine post-partum, mais le microbiome reproducteur bovin et son effet sur la fertilité suivante ne sont pas bien compris.

Les maladies sont négativement corrélées aux performances de reproduction et, combinées au taux d'incidence élevé, elles sont coûteuses pour les agriculteurs. Les études traditionnelles basées sur la culture sont biaisées en faveur des bactéries qui se développent dans un environnement de laboratoire. Dans

ce projet, la diversité bactérienne vaginale et utérine pendant la période d'attente volontaire a été étudiée par des méthodes de microbiologie moléculaire, principalement l'ARNr 16S et le séquençage de nouvelle génération.

L'objectif de cette étude est d'étudier les variations du microbiote et des facteurs immunitaires innés tels que les populations IL1, IL8 et AGP pendant la période d'attente volontaire.

L'objectif de la présente étude était de caractériser les communautés bactériennes de l'appareil reproducteur et la quantité de cytokines avant le vêlage et après le vêlage chez les vaches ayant

développé ou non une endométrite. . De plus, notre deuxième objectif de la présente étude était de décrire la réponse immunitaire innée locale cellulaire et humorale lors de la cervicite clinique dans l'utérus et les échantillons vaginaux dans les périodes pré et post-partum des vaches laitières.

Pour l'étude prospective de cohorte, un total de 61 vaches multipares ont été prélevées avec une cytobrosse dans le vagin 7 jours avant le vêlage (J-7) et dans l'utérus 7 jours (J+7), 21 jours (J+21), et 35 jours (J+35) après vêlage. Des échantillons de vaches en bonne santé (n = 11) et de vaches atteintes d'endométrite (n = 11) ont été traités pour l'extraction d'ADN et les gènes de séquençage de l'ARNr 16S.

La diversité alpha du microbiote utérin n'était pas significantment différente entre les groupes sains et malades. La diversité β n'était pas différente au niveau de l'UTO au cours de la même période d'échantillonnage entre les vaches saines et les vaches atteintes d'endométrite, mais le microbiote utérin était différent entre les groupes trois semaines après le vêlage. Les vaches malades avaient une abondance relative moindre de Firmicutes et de Bacteroidetes que les vaches en bonne santé et les vaches atteintes d'endométrite avaient une plus grande abondance relative d'Actinobacteria que les vaches en bonne santé. T. pyogenes, Peptoniphilus et Helcococcus étaient nettement plus abondants chez les vaches atteintes d'endométrite clinique.

De plus, les concentrations d'interleukine 1α (IL1), d'interleukine 8 (IL8) et de glycoprotéine acide α1 (AGP) ont été déterminées. Les cas de CC avec des signes cliniques au temps +5w tels que des pertes vaginales purulentes et une endométrite subclinique ont montré la production de cytokines la plus élevée. En conclusion, le post-partum de 3 semaines est un point critique pour évaluer les cytokines et les protéines de phase aiguë ; où la variation d'IL1 et d'IL8 a gardé une relation directe avec le nombre et la fonction des neutrophiles.
Mots-clés : les maladies après vêlage, immunité innée, vache laitière, microbiote

Abstract

Transition dairy cows are more 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 and therapeutic approaches to treat uterine infections. Currently, about 95 are of limited success. Approximately 90% of dairy cows exhibit contamination in the uterine cavity during the first 2 weeks following calving. Up to 40% of dairy cows still have contamination in the genital tract 3 weeks after calving. When infection or inflammation persists in the uterus beyond 4 weeks postpartum, the infected uterus is associated with infertility.

The microbiome and innate immune factors of the reproductive tract have been indicated to have an effect on postpartum uterine disease, however the bovine reproductive microbiome and its effect on fertility is not well understood.

Diseases are negatively correlated to reproductive performance, and in combination with the high incidence rate, they are costly for the farmers. Traditional culture based studies are biased towards bacteria that thrive in a laboratory environment. In this project, the vaginal and uterine bacterial diversity during voluntary waiting period (time between parturition and the time at which the cow os first eligible for insemination) were investigated by molecular microbiology methods, primarily 16S rRNA next generation sequencing.

The objective of the present study was to characterize the reproductive tract bacterial communities and the number of cytokines before and after calving in cows that developed or not endometritis. Also, the present study aimed to describe the innate immune response such as IL1, IL8 and AGP in the uterus and vaginal in the pre- and post-partum periods of dairy cows with clinical cervicitis.

For the cohort prospective study, a total of 61 multiparous cows were sampled with a cytobrush in the vagina 7 days before calving (D-7) and in the uterus 7 days (D+7), 21 days (D+21), and 35 days (D+35) after calving. Samples of healthy cows (n=11) and cows with endometritis (n=11) were processed for DNA extraction and sequencing of the 16S rRNA gene.

Alpha-diversity of uterine microbiota was not significantly different between healthy and endometritis groups. Microbiota composition (β diversity) was significantly different between

healthy and endometritis cows three weeks after calving. Diseased cows, but not at the other sampling times. Disease cows had lower relative abundance of *Firmicutes* and *Bacteroidetes* and greater abundance of *Actinobacteria* than healthy cows. *Trueperella spp*, *Peptoniphilus spp*. and *Helcococcus spp*. were significantly more abundant in disease dairy cows.
Additionally, in three weeks after calving the concentrations of interleukin 1α (IL1), interleukin 8 (IL8) and α1-acid glycoprotein (AGP) were determined. Cows with clinical and subclinical endometritis at time +5w (5 wks after calving) showed the highest cytokine production compared to the other sampling time (-1week, +1 week and+3 weeks).
Keywords: postpartum diseases, innate immune factors, dairy cows, microbiota

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ABBREVIATION

MCs: macrophages

NEFA: non-esterified fatty acids

PMN: polymorphonuclear neutrophils

RFM: retention of fetal membranes

URT: upper respiratory tract

VWP: voluntary waiting period

ACTH: adrenal corticotropin hormone

BCS: Body condition score

BHB: beta hydroxybutyrate

BHBA: β-hydroxybutyrate acid

bp: base pair

CC: clinical cervicitis

CE: clinical endometritis

CM: clinical metritis

CRH: corticotropin-releasing hormone

DM: dry matter

DMI: dry matter intake

E. coli: Escherichia coli

EnPEC: endometrial pathogenic *E. coli*

F.necrophorum: fusobacterium necrophorum

FSH: Follicular Stimulating Hormone

GnRH: gonadotropin-releasing hormone

IFN-t: Interferon tau

IgA: immunoglobulin A

IGF-1: insulin-like growth factor-1

IgG: immunoglobulin G

IgM: immunoglobulin M

IL: interleukin

LH: luteinizing hormone

LPS: lipopolysaccharide

MPO: myeloperoxidase

NEB: negative energy balance

NET: neutrophils extracellular traps

NF-kB: nuclear factor kB

NGS: next-generation sequencing

OTU: operational taxonomic unit

P.melaninogenica: prevotella melaninogenica

PAMP: pathogen-associated-molecular patterns

PCR: polymerase chain reaction

PGF2α: prostaglandins F2α

PM: puerperal metritis

PMN: polymorphonuclear neutrophils

PRID: progesterone-releasing intravaginal devices

PRR: pathogen-recognition-receptors

PUD: postpartum uterine disease

PVD: purulent vaginal discharge

RDP: Ribosomal Database Project

RFM: retained fetal membrane

rhIL: recombinant human interleukin

ROS: reactive oxygen species

SCE: subclinical endometritis

T.pyogenes: Trueperella pyogenes

TAI: timed artificial insemination

TLR: Toll-like receptors

Je dédie cette thèse à ma mère, qui veille sur nous de là-haut

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Literature review

1. Reproductive physiology of the transition period in dairy cows

1.1Definitions

Transition period: The time between late pregnancy and early lactation (also called the periparturient period); more precisely, it comprises the last 3 weeks before parturition and the first 3 weeks after parturition (Grummer, 1995). Most reproductive infectious diseases (e.g., retained placenta, metritis, endometritis) and metabolic disorders (e.g., milk fever, ketosis, abomasum displacement) occur during this time. Immunosuppression during the transition period leads to an increased susceptibility to mammary and uterine infection. Health problems are associated disproportionately with this relatively short period, making it a very important in terms of dairy cow management. The well-being and profitability of cows could be greatly enhanced by understanding the factors that account for the high disease incidence during the transition period.

1.2.Postpartum period: The time between the parturition and the end of uterine involution. It comprises the voluntary waiting period of 45 days, after which the pregnancy rate after insemination returns to normal.

1.3. Postpartum

1.3.1. Postpartum endocrinology

In early pregnancy, the conceptus blocks luteal regression in order to maintain progesterone production and prevent the expulsion of the fetus by the maternal immune system. Interferon tau (IFN-τ), the signal for pregnancy recognition (Bazer et al., 1997), blocks luteal regression and promotes uterine receptivity and embryo development. Signalling by the conceptus modulates local and systemic immunity to maintain the pregnancy (Vlasova et al., 2021). During the last trimester, the fetus grows very rapidly

and reaches the maximum capacity of the uterus. During that time, a reverse immunological shift (increasing T helper 1 cells) is occurring. As growth is limited, the fetal hypothalamus starts secreting corticotropin-releasing hormone (CRH), which stimulates the pituitary gland to secrete adrenal corticotropin hormone (ACTH), and further stimulates corticoid hormone secretion, including cortisol by the fetal adrenal gland. Cortisol can switch endocrine balance from progesterone into estradiol synthesis by inducing the enzyme 17α-hydroxylase and the production of prostaglandins F2α (PGF2α) due to the increase of oxytocin (Kindahl et al., 2004). After ovary-produced relaxin has ripened the cervix and pelvic ligaments, both estradiol and PGF2α 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 a neuronal reflex, which reinforces the contractions of the uterus (Kindahl et al., 2004). Therefore, the expulsion of the fetus is achieved by an increase in estradiol, PGF2α and oxytocin, as well as a decrease in progesterone. Hormonal changes during the periparturient period of dairy cows are illustrated in Figure 1-1.

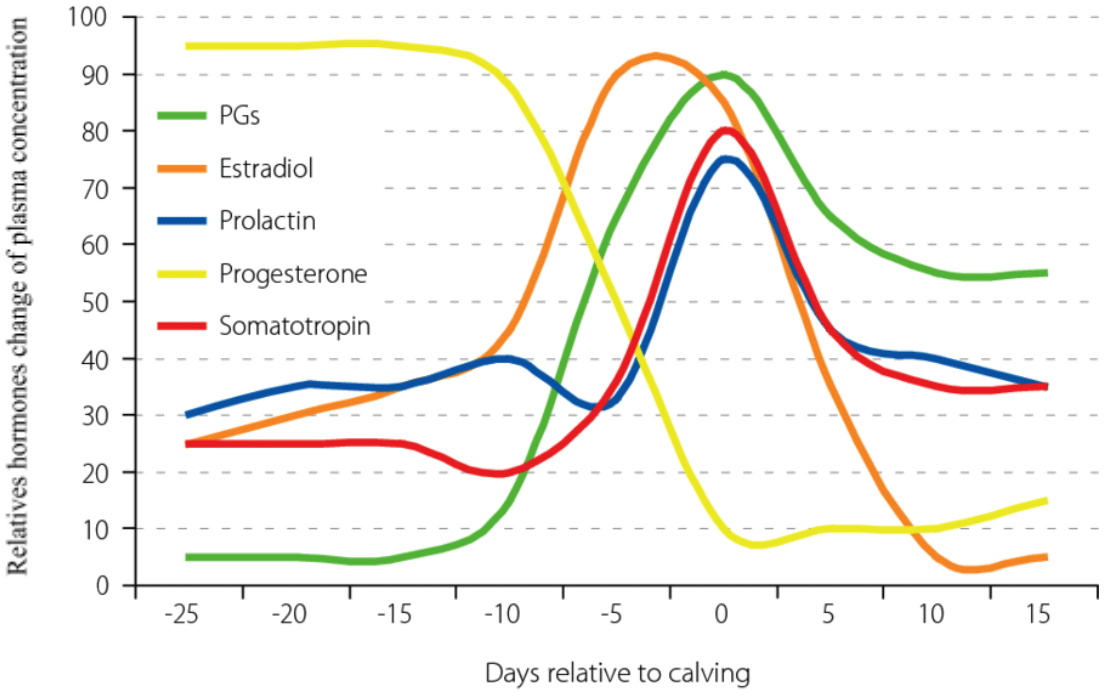

Figure 1-1. Changes in hormone concentrations around calving (Source: Ian John Lean,. 2010)

After parturition, the uterus undergoes involution, which includes shrinking 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 PGF2α, which increases sharply in the last week before calving and declines gradually to its basal level at 2 weeks after calving (Kindahl et al., 2004). PGF2α causes strong myometrial contractions, which leads to a reduction in uterine size and the expulsion of intrauterine content, thereby lowering the odds of bacterial infection in the uterus. Normally, uterine involution is completed by 5-6 weeks postpartum (Kindahl et al., 1999; Sheldon, 2004).

Besides uterine involution, the hypothalamic-hypophysis-ovarian axis needs to resume its normal cyclicity before the cow regains pregnancy capability. The anterior pituitary becomes responsive to the hypothalamus at 3-5 days postpartum and then starts releasing follicle stimulating hormone (FSH), which initiates the first follicular wave at around 7 to 10 days postpartum (Crowe, 2008). Although the average lifespan of follicles is 7-10 days (Crowe, 2008), ovulation cannot occur during the first 10-15 days postpartum due to the dominance of PGF2α (Kindahl, 1999). The occurrence of the first ovulation postpartum depends on when luteinizing hormone (LH) pulsatility returns to normal (Crowe, 2008). It has been reported that most dairy cows have a silent first ovulation after calving, meaning that the ovulation occurs without signs of heat. The first estrous cycle is short (9-11 days) due to the shorter corpus luteum lifespan (Kindahl, 1999; Crowe, 2008). The short cycle is not fertile but it is believed to prime the hypothalamus to return to a normal pulsatile state after a long period of suppression by high progesterone levels. In postpartum dairy cows, LH pulse frequency is also greatly influenced by energy status, which depends on the interactive effects of body condition score (BCS) loss, feed intake and milk production.

1.3.2. Normal uterine involution

1.3.2.1. Morphology

Normal uterine involution after calving allows the reproductive tract to return to normal function before a new pregnancy is re-established. This process is very complex and

involves morphological, endocrine, metabolic, histologic, bacteriological, biochemical and immunological changes. The process lasts about 45 days (voluntary waiting period, VWP) and during that time, the weight of the uterus decreases from 9 kg to approximately 1 kg and the uterine horns return to their normal dimensions of about 3 cm in diameter and 30 cm in length (Gier et al., 1968; Sheldon et al., 2013). At the end of the first week postpartum, the caruncles have necrotized and the stratum compactum is infiltrated by neutrophils (Weber et al., 2001). In the second week, the necrotic tissues detach and mix with blood, endometrial exudates and cellular debris in the uterine cavity to make the lochia, which is then gradually expelled during the first rise in estrogen during the first follicular wave (Moller, 1970; Sheldon et al., 2008). Even after the first ovulation, the process of sloughing and regeneration of the endometrium will not allow for implantation of a potential embryo if the cow were to be inseminated (Kiracofe, 1980; Short et al., 1990). About 20 days after calving, tissue sloughing and hemorrhaging have stopped. At this stage, uterine size has decreased by more than 80%, but full restoration of all layers of the uterine wall requires more time (about 45 days) (Sheldon et al., 2008). The epithelialization of the endometrium then progresses to completion (Deng et al., 2014) by the end of the VWP, when the uterine environment is normal and ready to establish a new pregnancy. To prevent monetary losses for the producer due to increased days open after parturition, it is crucial that uterine involution occurs completely, in a very orderly fashion and within a short period (45 days). However, complications can occur, leading to postpartum uterine disease and subfertility.

1.3.2.2. Endocrine system: Resumption of ovarian cyclicity

Follicular waves in the cow continue through the first two trimesters of pregnancy and until the final month of gestation, when they are impaired by rising estradiol levels caused by the placenta suppressing the Follicular Stimulating Hormone (FSH) pulses (Ginther et al., 1996; Crowe et al., 1998). A rise in FSH occurs between 1 and 5 days postpartum (Beam and Butler, 1997) and a follicular wave occurs in the second week postpartum. By 10 days postpartum, a dominant follicle can be found in most cows with an increasing circulating estradiol concentration and an eventual surge of luteinizing hormone (LH), and ovulation in 40% of cows (Beam and Butler, 1997; Sakaguchi, 2011).

Time to first ovulation is highly correlated with the nadir of negative energy balance (NEB) (Canfield and Butler, 1990). The early resumption of ovarian cyclicity in postpartum is associated with better reproductive performance (Savio et al., 1990; McDougall et al., 1995; Galvao et al., 2009). Cows that are cycling by 21 days postpartum have better conception rates at the first insemination postpartum than do cows that are cycling by 49 days postpartum, and the latter have higher first-service conception rates than cows that are not cycling at 49 days postpartum (Galvao et al., 2009).

Most cows will develop a dominant follicle during the first follicular wave. The difference between follicles that go on to ovulate and those that do not lies in the magnitude of the rise in circulating estradiol concentrations produced by those follicles (Beam and Butler, 1997). Non-ovulatory follicles appear normal on ultrasound examination; however, most of them do not produce estradiol that is detectable in circulation (impairment of estradiol by granulosa cells). However, non-ovulatory follicles that do show a rise in circulating estradiol mostly develop into cystic follicles. In ovulatory cows, the high-circulating estradiol concentration is detected by the hypothalamus, which releases a surge of gonadotropin-releasing hormone (GnRH), which in turn stimulates the pituitary to release the LH surge (Crowe, 2008).

1.3.2.3. Metabolic changes

The metabolic state of postpartum dairy cows is strongly associated with an early return to ovarian cyclicity (Beam and Butler, 1997; Opsomer et al., 2000; Wathes et al., 2007). Dairy cows enter a state of NEB during the early postpartum period, which reduces LH pulsatility (Canfield and Butler, 1990) and increases negative feedback on the hypothalamus by estradiol (Beam and Butler, 1997). High energy demands from late pregnancy and early lactation (transition period), combined with reduced energy intake, result in a rapid mobilization of energy from lipid storage. These increases in the concentrations of β-hydroxybutyrate acid (BHBA) and non-esterified fatty acids (NEFA) have been associated with impaired follicle function (Jorritsma et al., 2004; Vanholder et al., 2005). In addition, circulating insulin and insulin-like growth factor-1 (IGF-1) levels are lower in non-ovulatory cows (Butler et al., 2004). Insulin resistance occurs in late

gestation and early lactation in high-producing dairy cows and is associated with impaired ovarian function (Butler et al., 2003; Leury et al., 2003; Webb et al., 2004). Insulin resistance prevents lipolysis (Bell, 1995), thereby increasing BHBA and NEFA concentrations. Insulin also directly affects follicle function: both granulosa and theca cells require insulin in order to be able to respond to gonadotropin stimulation (Poretsky and Kalin, 1987; Stewart et al., 1995; Silva and Price, 2002).

1.3.2.4. Bacteriological effects

The development of uterine health disorders and the delayed resumption of ovarian cyclicity share some common risk factors, including decreased dry matter intake, low BCS and NEB (Beam and Butler, 1997; Butler et al., 2006; Hammon et al., 2006; Huzzey et al., 2007; Patton et al., 2007; Crowe, 2008; Sheldon et al., 2008; Galvao et al., 2009; Galvao et al., 2010). The uterus influences ovarian function in several ways. The first dominant follicle in postpartum emerges from the ovary contralateral to the previous pregnant uterine horn (Kamimura et al., 1993). The close association between venous circulation from the uterus and arterial circulation to the ovary in the cow enables prostaglandin F2α (PGF2α) produced by the uterus to be transported to the ovary for luteolysis. This transport mechanism may also be used for other molecules, such as inflammatory mediators and endotoxins produced by the uterus and bacteria, respectively.

Bacterial contamination of the uterus postpartum is associated with uterine disease and also with impaired ovarian function (Opsomer et al., 2000; Sheldon and Dobson, 2004; Williams et al., 2007). Cows with higher uterine bacterial contamination have slower dominant follicle growth and so produce less estradiol (Sheldon et al., 2002; Williams et al., 2007). If ovulation occurs before clearance of bacterial contamination in the postpartum cow, the progesterone exposure may impair the immune system and exacerbate the infection, resulting in pyometra (Olson et al., 1984). Administration of exogenous GnRH to early postpartum cows may also result in pyometra (Etherington et al., 1984). Therefore, ovulation before the completion of uterine involution is potentially detrimental to uterine health. Cows with uterine disorders may also present a prolonged luteal phase (Opsomer et al., 2000; Ranasinghe et al., 2011). Uterine infection results in a

shift from the production of prostaglandin F2α (luteolytic effect) to prostaglandin E2 (luteotropic effect) (Herath et al., 2009). Uterine health and the resumption of ovarian cyclicity are intertwined and both are critical to a timely return to fertility in postpartum cows.

1.3.2.5. Immune response of the postpartum uterus

The immune system plays a major role in uterine defence mechanisms; however, our understanding of its role is still limited because of scarce information on the mediators between the immune defence, the metabolic status, and the microbiota. During the transition period (3 weeks prior to and 3 weeks after calving), dairy cows experience immune and metabolic dysregulation, which makes them vulnerable to various infectious and non-infectious diseases. Even with treatment (antibiotics and other molecules), diseases still persist and cause infertility. Therefore, further research is needed to know the molecules that link the three systems and to understand their regulation mechanisms. .

In general terms, the first line of defence for the reproductive tract is physical barriers, including mucosal membranes. Epithelial cells secrete several antimicrobial factors, including antimicrobial peptides and defensins, which play an important role in the innate immune response. Other important cells are polymorphonuclear neutrophils (PMNs), macrophages (MCs), natural killer cells, dendritic cells, gamma delta T cells, mucosa-associated invariant T cells and granulocytes. Although most of the information on the innate immune response comes from human research, much is known about the process specific to cows (Vlasova and Saif, 2021). Most of the above-mentioned cells are powered by pathogen-recognition-receptors (PRRs), which interact with pathogen-associated-molecular patterns (PAMPs) to initiate the immune response cascade. Upon activation, inflammatory cytokines and chemokines are produced and induce migration of neutrophils and monocytes into the affected area. As the process continues, signals are sent to the dendritic, natural killer, B and T cells (Dannermann et al., 2009) to promote resolution of the inflammation and control the immune response.

Cellular defense against bacterial contaminants is provided by uterine leukocytes (Stossel, 1975), with PMNs being the main leukocyte involved in bacterial clearance after uterine infection (Hussain 1989; Gilbert et al., 2007). The PMNs recruited from circulation to the infected uterus by chemotactic factors enhance phagocytosis of the bacterial particles. It has been shown that the increase in leucocytes is followed by a reduction in total leucocyte counts one week postpartum and a return to normal levels over the following three weeks (Hussain et al., 1992; Cai et al., 1994; Mateus et al., 2002; Kim et al., 2005; Singh et al., 2008). This phenomenon is not always observed (Bazzazan et al., 2022). A rise in cortisol levels during calving is the main cause of leukocytosis (Preisler et al., 2000), while a decline in leukocyte numbers has been associated with their migration into the uterine lumen and mammary glands (Singh et al., 2008). Bacterial contamination of the uterus in postpartum dairy cows is ubiquitous, and the endometrium is the first line of defense against bacterial invaders (Galvão et al 2011; Sholden et al., 2004a). Receptors on endometrial cells and macrophages, called Toll-like receptors (TLRs), recognize highly conserved molecular patterns present on bacteria (PAMPs). TLR binding of these PAMPs stimulates cells to produce and release pro-inflammatory cytokines and chemokines, including TNF-α, interleukin-6 (IL-6) and interleukin-8 (IL-8) (Tzianabos, 2000; Beutler et al., 2003). IL-6 and TNF-α stimulate the production of antimicrobial peptides, which assist in the elimination of pathogenic bacteria from tissues. Fischer et al. (2010) showed that pro-inflammatory cytokines like IL-6, IL-8 and TNF-α may accelerate PMN infiltration into the endometrium of cows following infection. The role of TNF-α is to stimulate the expression of IL-8 and adhesion molecules on vascular endothelial cells. IL-8 plays an important role in PMN and monocyte chemo-attraction and activation. These responses ultimately increase phagocytosis and bacterial killing (Roach et al., 2002). IL-6 is a pro-inflammatory cytokine that has various roles during the early stages of the inflammatory process. It promotes PMN maturation, differentiation of monocytes into mature macrophages, and the production of natural killer cells (Ishikawa et al., 2004). It is believed to increase the expression of oxytocin receptors on myometrial cells. Ishikawa et al. (2004) showed that IL-6 is present in the bovine uterus in high concentrations before parturition and decreases to baseline values by 8 days postpartum. Zerbe et al. (2003) showed that

infusing recombinant human IL-8 (rhIL-8) into the uterus of cows attracted PMNs into the uterus, while anti-IL-8 monoclonal antibody treatment prevented PMN-dependent tissue damage as well as PMN infiltration. These observations confirm that IL-8 is an important chemoattractant for PMNs. Although the presence of recombinant IL-8 in the uterus has been shown to increase the influx of PMNs and other leucocytes into the bovine uterus, the question of whether IL-8 is generated in the uterus of infected cows is not clear.

Galvão et al. (2011) compared uterine endometrial cytokine gene expression between cows diagnosed with endometritis (>10% PMNs in low-volume uterine lavage) at 35 days postpartum and cows without endometritis from calving to 7 weeks postpartum in order to evaluate differences in the pro-inflammatory cytokine production in cows with and without disease. Tumour necrosis factor-α gene expression during the first week postpartum was lower in cows with endometritis than in control (healthy) cows. These researchers postulated that this lower TNF-α response may compromise the PMN and monocyte response to bacterial contamination of the uterus, decreasing bacterial clearance and contributing to the development of uterine disease. In contrast, IL-6 and IL-8 gene expression were elevated at 5 and 7 weeks after calving in cows with endometritis. Chapwanya et al. (2009) also reported that in uterine biopsies collected at 2 weeks postpartum, TNF-α expression was decreased in cows diagnosed with endometritis later in the postpartum period. However, other work examining endometrial cytobrush cytology samples collected between 3 and 4 weeks postpartum showed that both TNF-α, and IL-6 were usually increased in cows with endometritis (Gabler et al., 2009; Fischer et al., 2010). Therefore, the pro-inflammatory response to bacterial infection appears to change throughout the postpartum period.

Cows with endometritis may have a compromised ability to respond to bacterial contamination because pro-inflammatory cytokine gene expression is up-regulated later after calving than in their non-diseased counterparts. Decreased pro-inflammatory cytokine gene expression could lead to poor chemotaxis and activation of PMNs, which would result in delayed bacterial clearance and the development of endometritis (Galvão

et al., 2011). In other words, healthy cows may have a greater and quicker pro-inflammatory response to bacteria invading the uterus after calving and are therefore able to eliminate infection successfully and more quickly (Galvão et al., 2011). Nino-Soto et al. (2008) and Hammon et al. (2006) postulated that lower expression of TNF-α and IL-6 in postpartum cows that developed endometritis could be due to an intrinsic defect in endometrial cell function or extrinsic mechanisms affecting endometrial cell activity, such as a negative energy balance. Beam et al. (1998) reported differences in cytokine gene expression between cows that developed endometritis and healthy cows when a negative energy balance was more pronounced. It was postulated that the ability of cows with endometritis to up-regulate the expression of pro-inflammatory cytokine genes was affected. Cows that develop endometritis due to a severe negative energy balance may have an impaired ability to up-regulate pro-inflammatory cytokines in the first week after calving, but are able to mount an inflammatory response as soon as their nutritional status improves. Therefore, maintaining a good energy balance during the transition period could potentially reduce the risk of uterine diseases. The delayed uterine immune response and impaired leukocyte function that is related to an energy deficit may be complicated by the increases in estradiol and cortisol that occur after calving (Goff et al., 1997; Wathes et al., 2009; Galvão et al., 2011).

The immunoglobulins derived locally from the uterus (IgA, IgG1, IgM) and from serum (IgG2) also contribute to uterine defense through enhanced bacterial opsonization and antigen presentation (Dhaliwal et al., 2001). Immunoglobulin G and IgM concentrations in lochia from healthy cows decrease after calving, but in cows with an abnormal puerperium, IgA and IgG concentrations in uterine fluids increase rapidly as endometritis develops (Aknazarov, 1998; Bondurant, 1999). These humoral immune mechanisms are also influenced by increasing steroid hormone levels, which are reportedly due to the influence of elevated estrogen concentrations (Cobb et al., 1995). In the early postpartum period, the low-estrogen-concentration environment that occurs prior to the resumption of cyclicity predisposes the uterus to bacterial colonization through the disarming of specific immune responses, leaving only non-specific responses such as neutrophil infiltration.

However, the role of humoral immunity in bovine uterine disease is not fully understood (Lewis, 1997; Dhaliwal et al., 2001).

1.3.2.5.1. Neutrophil physiology

The development of neutrophils takes approximately 14 days (Bainton et al., 1971; da Silva et al., 1994) and is under the influence of granulocyte colony-stimulating factor (G-CSF). Neutrophils start as pluripotent stem cells and then become, in succession, myeloblasts, promyelocytes, neutrophilic myelocytes, neutrophilic metamyelocytes, band cells and segmented PMN mature neutrophils (Bainton et al., 1971; Jain, 1976; Mehta et al., 2015). The formation of azurophilic (primary) granules containing myeloperoxidase (MPO), serine proteases and antibiotic proteins occurs during the differentiation of promyelocytes (Faurschou et al., 2003). Specific (secondary) granules that contain high levels of lactoferrin are formed during the differentiation of myelocytes and metamyelocytes (Faurschou et al., 2003). During the differentiation of band cells and segmented neutrophils, gelatinase (tertiary) granules are formed, followed by the secretory vesicles found in segmented and mature cells (Faurschou et al., 2003). Bone marrow contains three functional pools of developing neutrophils; myeloblasts, promyelocytes and myelocytes (mitotic pool) (Jain, 1976), where cells reside for an average of 7.5 days in mammals (Bainton et al., 1971; da Silva et al., 1994). Cells then spend an average of 6.5 days (Bainton et al., 1971) in a maturating pool containing metamyelocytes and band cells (Jain, 1976). Band cells and segmented neutrophils reside in storage pools for an average of 7 days before being released into circulation (Jain, 1976).

A mature lactating dairy cow has an average of 2×10^{11} segmented neutrophils in marrow and in circulation (da Silva et al., 1994). The production of neutrophils can increase tenfold during acute inflammation or the administration of granulocyte colony stimulating factor (Van Schyndel et al., 2018). Although neutrophil kinetics are not well documented in dairy cattle, research suggests that mature neutrophils travel in circulation and have an average half-life of 8.9 hours in healthy cows (Paape et al., 2002) and function in tissues for up to 2.5 days (da Silva et al., 1994). Cells in the marrow storage

reserves can be quickly mobilized and directed by chemoattractants to sites of infection (Paape et al., 2002). Under stimulus from infection, the time required for a myelocyte to mature and appear in circulation can be decreased to as little as 48 hours, in comparison to the 96 to 144 hours in a normal situation (da Silva et al., 1994).

At the first signs of bacterial contamination, local epithelial cells and macrophages initiate the inflammatory response by releasing chemoattractants (Paape et al., 2002). Neutrophil recruitment appears to have tissue-specific features (Margraf et al., 2019). Mature neutrophils are recruited from the bloodstream, migrating from circulation through a process called diapedesis. This involves the following processes: tethering, a transient adhesion to the endothelium surface in response to various ligands; rolling, which is mediated by selectins expressed on inflamed endothelial cells; firm adhesion of neutrophils to endothelial walls via selectins and integrins; and, finally, transmigration through the endothelial junction (Witko-Sarsat et al., 2000; Kobayashi et al., 2003). Neutrophils then follow the chemotactic gradient toward the site of infection, where bacteria are bound to membrane receptors and phagocytized (Wright et al., 2010). Once in the tissues, neutrophils interact with damaged cells or bacteria to remove them through a variety of mechanisms: 1) phagocytosis and intracellular digestion by oxidation (oxidative burst in lysosomes), 2) extracellular release of oxidants from neutrophil granules (e.g., myeloperoxidase), or 3) casting neutrophils extracellular traps (NETs) of DNA (Nauseef and Borregaard 2014, Liew and Kubes 2019). Following ingestion, neutrophil effector systems are activated, including both oxidative and non-oxygen-dependent microbicidal pathways (Witko-Sarsat et al., 2000). Oxidative burst involves the activation of the NADPH-oxidase complex and MPO pathway, resulting in the production of reactive oxygen species (ROS), such as hydrogen peroxide, superoxide anion, hypochlorous acid and other hydroxyl radicals (Faurschou et al., 2003; Kobayashi et al., 2003). The non-oxygen-dependent pathway involves the release of serine proteases, antibiotic and antimicrobial proteins, and metalloproteinases from granules into the phagolysosome (Witko-Sarsat et al., 2000). Neutrophils can also kill pathogens extracellularly using NETs to supplement phagocytic killing and control infection. NETs are induced as a response to different inflammatory mediators and

provide a fibrous chromatin network to capture pathogens (Parker et al., 2012). There is also a substantial amount of NET-associated active MPO, which is involved in the microbicidal capabilities of NETs (Parker et al., 2012).

Neutrophils have to respond promptly to stimuli and although they are capable of protein synthesis and may tailor cytokine production in situ, it is probable they must have already synthesised all effector molecules that they may need for their functioning (Kubes 2018). Neutrophils may die by apoptosis or necrosis. Apoptosis is programmed cell death with controlled clearance of the potentially damaging content by macrophages. Necrosis involves cell rupture, contributing to additional damage in nearby cells and inflammation (Paape et al., 2003). It is believed that apoptotic neutrophils return from the inflammatory sites to be cleared from the bone marrow or from resident macrophages in the liver or spleen in order to further modulate the neutrophil response, resolve the inflammation and trigger the healing process (Kolaczkowska and Kubes, 2013).

Neutrophils are activated by ligand 8 (CXCL8 or IL-8), platelet-activating factor, TNFα, and complement component C5a (Bassel and Caswell, 2018). They can be directly activated by PAMPs through TLRs, of which TLR1, 2, 4, 6, 7 and 10 are known to be present in bovine neutrophils. TLR4, which responds to LPS, is especially relevant in the postpartum dairy cows. Once stimulated by CXCL8, by TNFα released from macrophages resident in damage tissue, or by epithelial cells reacting with PAMPs, neutrophils begin migration (Sheldon et al., 2019).

1.3.2.5.2. The effect of parturition on neutrophil function

Parturition and the onset of lactation cause changes in feed intake, nutrient partitioning and metabolic demand (Crookenden et al., 2016, Rinaldi et al., 2008). These changes often cause dysfunction of the innate immune system and have a particularly negative effect on neutrophils (Hammon et al., 2006). There is an important link between metabolism (adipose metabolism and insulin resistance) and immune function and inflammation in humans and animals (Hotamisligil and Erbat, 2008; Osborn and Olefsky 2012; Bradford et al., 2015). Both obese people and periparturient dairy cows are

characterized by elevated circulating NEFA, insulin resistance, hepatic accumulation and systemic inflammation. NEFA, and particularly saturated fatty acids which dominate in transition dairy cows, may impair neutrophil function (Ingvartsen and Moyes, 2013). Minimizing the decrease in dry matter intake (DMI) and the degree of negative energy balance (NEB) during the periparturient period has been shown to improve neutrophil function (Hoeben et al., 1997; Hammon et al., 2006). Cows with plasma NEFA $\geq$ 0.400 mEq/L within 2 weeks before calving had significantly lower MPO activity than those with lower NEFA concentrations (Hammon et al., 2006). An in vitro study by Scalia et al. (2006) found no differences in the phagocytic activity of neutrophils in mid-lactation cows incubated with varying levels of NEFA, but a significant reduction in cell viability. Certain NEFA activate TLR4, a main receptor for lipopolysaccharide (LPS), which activates nuclear factor κB (NF-κB) and leads to secretion of tumour necrosis factor α (TNFα), interleukin-1 (IL-1) and IL-8. TNFα and IL-1 act on intracellular messengers to upregulate inflammation and increase insulin resistance.

Neutrophils may be involved in chronic, systemic and sterile inflammation, including lipolysis, ketosis and a lack of supply substrates for immune cells, conditions that are common in dairy cows (Buck et al. 2017). Hoeben et al. (1997) demonstrated that cows with ketosis also experience impaired neutrophil function. Inducing elevated BHB levels with 1 to 2.5 mmol/L of butyric acid in vitro inhibited respiratory burst activity in bovine neutrophils isolated from the peripheral blood of 7 clinically healthy periparturient cows (Hoeben et al., 1997). These results show that high-producing cows with elevated levels of BHB or severe NEB may be at greater risk of local and systemic infections during the postpartum period (Hoeben et al., 1997; Hammon et al., 2006).

In a trial investigating the effect of different dietary energy levels on neutrophil function during the transition period, 10 control cows were fed a diet of 100% NEL with an energy content of 1.34 Mcal/kg dry matter (DM) and 9 overfed cows were fed 150% NEL at 1.62 Mcal/kg DM throughout the 45-day dry period (Graugnard et al., 2012). Both groups experienced NEB during the first week postpartum but overfed cows showed a much larger decrease in energy balance at parturition. Concentrations of NEFA and BHB

did not differ between the groups, which is interesting since overfed cows had a more dramatic decrease in energy balance between the prepartum and postpartum periods. However, concentrations of both glucose and insulin were much greater in the overfed group at -14 days and 7 days compared to the measured pre-feeding. The overfed group had a lower average phagocytic capacity of 32.7% at 14 days prepartum, compared to the control group at 46.5%. Seven days after calving, phagocytosis in the control group was constant, whereas there was an increase in the overfed group to similar levels. This increase in the overfed group is probably related to the greater concentration of glucose (3.5 and 3.3 mmol/L in overfed and control groups, respectively), the preferred energy substrate for immune cells (Graugnard et al., 2012). However, at 7 days, compared to control cows, overfed cows had higher glucose and insulin concentrations and significantly lower neutrophil phagocytic ability. Although it is not sufficiently understood, the authors hypothesized that elevated levels of insulin are responsible for impaired neutrophil function at calving (Graugnard et al., 2012).

Calving also causes changes in gene expression associated with immune function. In samples collected from cows at -1, 0, 1, 2 and 4 weeks relative to calving, Crookenden et al. (2016) found reduced expression of genes for pro-inflammatory cytokines and increased expression for anti-inflammatory cytokines on the day of calving compared to all other time points. There was also variation in gene expression associated with neutrophil adhesion, maturation and apoptosis throughout the transition period. During and immediately following parturition, pro-inflammatory cytokines are needed to stimulate an inflammatory response and initiate uterine involution. The down-regulation of components involved in the inflammatory cascade can result in a predisposition to infectious disease (Crookenden et al., 2016).

MPO activity, cytochrome c reduction activity, ROS production, and oxidative burst are indicative of neutrophil function and efficiency. Kehrli et al. (1989) demonstrated maximum neutrophil function 2 weeks before parturition and minimum function during the first week of lactation based on stimulated native chemiluminescence, bacterial ingestion assays and random migration under agarose in blood samples from 8 heifers

during the periparturient period. This study also found an impairment in neutrophil random migration and phagocytosis of labelled *Staphylococcus aureus* during the first week after calving (Kehrli et al., 1989). Gilbert et al. (1993) demonstrated that superoxide anion production was lower in the first week postpartum compared to the subsequent weeks of lactation. They discovered that cows in their fourth or greater lactation had more profound impairment of superoxide anion production compared to cows with fewer than four lactations.

To distinguish between the effects of parturition and lactation, Kimura et al. (1999) compared expression of neutrophil adhesion molecules and MPO activity in 10 mastectomized and 8 intact cows. Cows from each group were sampled twice weekly from 3 weeks prior to calving to 3 weeks after calving, and 4 non-pregnant heifers were used as a control. Neutrophil function declined substantially and equally in both groups at the onset of parturition, suggesting a direct effect of calving itself. However, MPO activity recovered to prepartum values within 7 days in the mastectomized cows but took as long as 20 days in the intact cows. This suggests that although parturition causes an initial immunosuppression, the demands of lactogenesis contribute to the prolonged suppression of neutrophil function in the postpartum period (Kimura et al., 1999).

1.3.2.6. Bacteriology in postpartum uterine infections: culture-dependent vs culture-independent perspective

Approximately 95% of cows after calving have their uterus contaminated with bacteria, regardless the presence of signs of disease (Foldi et al., 2006; Paisley et al., 1986; Sheldon and Dobson, 2004). In addition to the loss in anatomical barriers, the negative pressure created by the phenomenon of uterine contraction and relaxation enhances the entry of bacteria, allowing for rapid colonization of the uterus. Gram-negative bacteria predominate in the uterus during the first week after calving, but are gradually replaced by Gram-positive bacteria. By day 15 postpartum, 78% of cows still have bacteria in the uterus; by day 60, only 9% do (Wira et al., 2005). During normal uterine involution, the uterus clears bacteria by week 6 after calving (Sheldon et al., 2002). However, 40% of

dairy cows are not able to completely eliminate the bacterial infection (Sheldon et al., 2008).

Based on culture-dependent studies, the most common bacteria involved in postpartum uterine disease (PUD) are *T. pyogenes*, *E. coli*, *F. necrophorum*, *Prevotella melaninogenica* and *Bacteroides* species (Griffin et al., 1974; Studer and Morrow, 1978; Ruder et al., 1981; Olson et al., 1984; Bonnett et al., 1993; Dohmen et al., 1995; Huszenicza et al., 1999; Dohmen et al., 2000; Williams et al., 2005; Sheldon et al., 2009b). Pathogenic bacteria have specific characteristics, such as a capacity to adhere to mucosa, to colonize and penetrate the epithelium, and to release bacterial toxins that lead to the establishment of PUD (Sheldon et al., 2009). For example, *E. coli* is the predominant uterine pathogen during the first week postpartum and is associated with metritis because of virulent factors that enable bacteria to attach to the endometrium, as is the case for endometrial pathogenic *E. coli* (EnPEC) (Esposito et al., 2014; Sheldon et al., 2010). Although an association between *E. coli* and uterine infection is not consistently found, *E. coli* is often considered a colonizer that changes the uterine environment predisposing the establishment of other pathogens (Robert and Santos, 2016). One of these is *T. pyogenes*, which causes inflammation and lesions in the endometrium, and significantly slows the process of uterine involution by producing the toxins pyolysin and cytolysin (Newsholme et al., 1987). *Trueperella pyogenes* predominates during the three weeks postpartum, becoming the main bacteria associated with clinical endometritis. *Trueperella pyogenes* and *E. coli* isolates contain antibiotic resistance genes associated with biofilm formation (Kasimanickam et al., 2016). *Fusobacterium necrophorum* is another later developing pathogen that acts synergistically with *T. pyogenes* and is capable of inhibiting phagocytosis using a leucocidal toxin (Roberts, 1967; Sheldon et al., 2004; Singh et al., 2008).

Culture-dependent studies have revealed critical information about the pathogens involved in PUD; however, only bacteria with a well-established culture system and identification methods can be detected. In fact, less than 1% of bacteria can be cultured in the laboratory (Kaeberlein et al., 2002). Interactions between bacteria are not completely

understood, but it is possible that synergic relationships may facilitate the establishment of PUD. A major consequence of the presence of pathogenic bacteria causing PUD is delayed uterine involution, which delays the return to cyclicity and increases services to conception (Bonnett et al., 1993; LeBlanc et al., 2002; Ribeiro et al., 2013). Conversely, some studies have shown that Group A hemolytic streptococci are associated with uterine health (Bonnett et al., 1991; Dohmen et al., 1995; Gilbert and Santo, 2016). Immediately after calving (20 minutes), the uterine microbiota is already well established. It then proceeds to change over 2 days, either deviating toward PUD (dysbiosis) or toward a healthy postpartum (Jeon et al., 2015). Even though diseased (metritic) and healthy cows present the same phylum abundance (*Firmicutes, Bacteroides, Proteobacteria* and *Fusobacteria*), the uterine microbiota of diseased cows has reduced richness and diversity compared to healthy cows (Bicalho et al., 2017; Machado et al., 2012; Peng et al., 2013; Knudsen et al., 2015; Wagener et al., 2015). With next-generation sequencing (NGS), pathogens have been detected that were previously unknown based on culture-dependent methods, such as certain species of *Bacteroides* and *Helcococcus,* while others have been found to be more abundant in healthy postpartum cows compared to cows with metritis, such as *E. coli*, *Mycoplasma* and *Ureaplasma* (Galväo et al., 2019). Cows with clinical endometritis have a uterine microbiome very similar to cows with metritis, with the exception of the relative abundances of Tenericutes, Bacteroidetes and *Fusobacterium*, which are higher compared to normal cows (Miranda-Caso Luengo et al., 2019; Wang et al. 2018).

Because of the high prevalence of PUD in cows, most studies have focused on the early postpartum period (less than 5 weeks) before the completion of uterine involution and the reestablishment of a complete partitioning of the reproductive tract, assuming that vaginal findings during this period are linked to the uterus. In addition, clinical and visual signs of infection or inflammation of the vaginal vault are rare in the postpartum period in cows. For these reasons, studies on the vagina in postpartum cows are considered less relevant, which explains the scarcity of scientific information on this subject. The first study to evaluate the microbiome of the bovine reproductive tract using 16s rRNA sequencing was Swartz et al. (2014), which examined the vaginal microbiome of cows

and ewes. It found high bacterial species diversity and its results were very different from human studies. Interestingly, *Lactobacillus* was common among samples but had an average relative abundance of less than 1%, resulting in a more neutral pH of the vagina. Pregnant cows and heifers had significantly lower bacterial species diversity than non-pregnant animals (Pascottini et al., 2020). However, non-pregnant animals were not controlled for estrous cycle stage, which could possibly have affected the results due to physiological differences in hormone concentrations. Laguardia-Nascimento et al. (2015) and multiple other studies agree that the reproductive tract of cows is dominated by the phyla Firmicutes, Bacteroidetes and Proteobacteria, but low levels of *Lactobacillus* species are also present (Moore et al., 2017). In contrast to the highly diverse microbiome of cows, the vagina in women has low species diversity, consisting predominately of *Lactobacillus* in healthy nonpregnant and pregnant women (The Human Microbiome Project, 2012; Walther-Antonio et al., 2014).

1.4. The microbiota of the human reproductive tract

The microbiota of the vaginal tract was characterized by Ravel et al. (2011). In this study, the vaginal bacterial communities of reproductive-age women (n=396) were analyzed using the 16S rRNA gene. Subjects were of different ethnic and racial backgrounds (Caucasian, African American, Hispanic and Asian) and all were currently living in North America. Results showed that the majority of bacteria within the subjects' vaginal tracts had the common physiological ability to produce lactic acid. However, Caucasian and Asian women (pH=4.2) had more *Lactobacillus* species than African American and Hispanic women (pH=5.0). *Lactobacillus* species in the woman's vaginal microbiota is crucial for vaginal health (Redondo-Lopez et al., 1990).

Lactic acid facilitates the maintenance of an acidic vaginal environment that can prevent the growth and attachment of potential pathogens (Osset et al., 2001). Ronnqvist et al. (2006) evaluated the role of *Lactobacillus* species in the vaginal tract and their relationship to other genital microbes and vaginal pH. In the 191 women evaluated, the overall conclusion was that *Lactobacillus* species contribute to maintaining a low vaginal pH, which has a negative impact on the growth of Group B Streptococci. The latter is

associated with preterm birth, fetal injury, neonatal mortality in newborns and fetal infection in utero (Whidbey et al., 2013). Therefore, it can be inferred that an acidic vaginal environment is beneficial in women for preventing the growth of pathogenic bacteria, but as mentioned in Ravel et al. (2011), what constitutes a physiologically normal pH and the specific bacteria that induce the acidic environment varies among ethnic groups. The significance of the vaginal microbiota and its importance on fertility and pregnancy maintenance is still under debate (Baker et al., 2018).

Within the normal uterus, the most abundant phyla reported include Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria (Chen et al., 2017; Walther-Antonio et al., 2016). However, the Firmicutes phylum is the most dominant of this group (Mitchell et al., 2015; Miles et al., 2016; Tao et al., 2017). Fang et al. (2016) reported that women with endometrial polyps and chronic endometriosis had a higher relative abundance of *Lactobacillus* species in their uteruses than healthy women. By contrast, Moreno et al. (2016a) reported that high levels of *Lactobacillus* species in the uterus increased success rates in women undergoing in vitro fertility treatments and embryo implantation. These contradictory results may be explained by some studies having a sampling pool of patients with both healthy and unhealthy reproductive tracts, and the risk of vaginal contamination.

1.5. Microbiota of the bovine reproductive tract

Studies of the microbiota in the reproductive tract (vagina and uterus) have tried to understand its impact on reproductive performance, as well as animal health and well-being. While these studies have a number of limitations, such as no contamination check, small number of animals and a lack of in-depth analysis, they have yielded some interesting results.

Compared to humans, the bovine vaginal tract has low levels of *Lactobacilli* and a neutral pH. The three most abundant phyla are Bacteroidetes, Fusobacterium and Proteobacteria (Swartz et al., 2014). Comparing the vaginal microbiota in four subsets of Nellore cattle (pregnant cows (n=5), nonpregnant cows (n=5), pregnant heifers (n=5), and nonpregnant heifers (n=5)), Nascimento et al., (2014) showed results similar to those of Swarz et al. (2014), with the most abundant phyla in all cows being Firmicutes, Bacteroidetes and

Proteobacteria. Moreno et al. (2016b) investigated the impact of progesterone-releasing intravaginal devices (PRIDs) on the vaginal microbiota. After PRID removal, cows were checked for vaginal discharge (present/absent) and for levels of Proteobacteria and opportunistic pathogens. However, synchronization protocol with a progesterone device (PRID) in cows with or without vaginal discharge has been shown to be equally effective in terms of pregnancy rate. Ault et al. (2019) investigated the effect of progesterone on the vaginal and uterine microbiota prior to timed artificial insemination (TAI). Samples were collected from pregnant (n=10) and nonpregnant (n=10) cows at three time points (d-21, d-9, and d-2 relative to TAI) from the vagina and uterus via lavage. Serum was also collected at these time points to measure progesterone concentration. There were no differences in the alpha diversity of vaginal microbiota species abundance at any sample date prior to TAI. However, operational taxonomic unit (OTU) analysis showed that diversity significantly decreased in the uterus over time (Ault et al., 2019). The principal coordinate analysis to visualize beta diversity within the samples showed a significant clustering at d-2 in the uterus (P=0.005), and at d-21 (P=0.002) in the vagina between pregnant and nonpregnant cows. However, no direct relationship between progesterone concentration and vaginal or uterine microbiota was found. Moore et al. (2017) sampled the entire reproductive tract of virgin heifers and pregnant cows to establish whether a resident microbiota exists. Researchers collected endometrial biopsies of 10 virgin dairy heifers, and collected amniotic fluid, placentomes, intercotyledonary placenta, cervical mucus and vaginal tissue from 5 pregnant dairy cows after slaughter. All samples showed a similar microbiota, leading researchers to conclude that both pregnant and virgin uteri contain a resident microbiota with Firmicutes, Bacteroidetes and Proteobacteria being the most abundant phyla in both the pregnant and virgin uteri.

In addition to maternal health and fertility, the vaginal microbiota may affect the cow's offspring as well. Lima et al. (2018) sampled the vaginal and fecal microbiota of Holstein cows (n=81) and compared it to the fecal and upper respiratory tract (URT) microbiota of their calves (n=81) at 3, 14 and 35 days of age to evaluate if maternal microbiota can influence calf health. They found that the URT microbiota of calves and their mothers'

vaginal microbiota were quite similar (63% overlap with 253 shared OTUs), regardless of calf age. *Mannheimia* and *Moraxella*, two bacteria involved in bovine respiratory disease in calves, were some of the most prevalent bacteria shared between mother and calf, suggesting that the health of a calf could be affected by mother-to-offspring transmission during parturition.

A study on uterine bacterial communities by Wang et al. (2018) focused on characterizing the uterine microbiota of postpartum dairy cows with clinical endometritis (CE) and subclinical endometritis (SE). Researchers collected uterine samples with small volume lavage from 13 healthy, 5 SE, and 9 CE cows at 30 days postpartum. All cows had a similar microbiota with 293 of 445 shared OTUs. Clinical endometritis was characterized by an increased abundance of *Fusobacterium*, *Trueperella* and *Peptoniphilus*. Subclinical cows had no uterine pathogens, but an increased abundance of *Lactococcus* and *Acinetobacter*. Therefore, healthy, subclinical and clinically infected cows seem to share a similar microbiota; however, the competitive and cooperative interactions within the microbiota still need to be established to determine how uterine pathogens interact and promote a healthy uterus. Santos et al. (2011) also found that the phylum *Fusobacteria* was associated with metritic postpartum cows, but no differences were found between healthy and metritic cows.

Interestingly, Jeon et al. (2017) evaluated the possibility of pathogens being introduced into the uterine postpartum via the blood. They compared bacterial communities from blood, feces and uterine samples at day 0 and day 2 postpartum, and a vaginal sample taken 7 days prior calving. Results of the principal coordinate analysis showed distinct clustering of blood and fecal bacterial communities, but more scattered clustering of uterine and vaginal communities (Jeon et al., 2017). Additionally, major uterine pathogens were detected in blood samples, and there was a strong and significant interaction of uterine pathogens and the blood microbiota.

1.6. POSTPARTUM UTERINE DISEASE

Postpartum uterine disease (PUD) is the leading cause of reproductive inefficiency in dairy cattle (Barlund et al., 2008). Almost all dairy cows (80 to 100%) experience bacterial contamination of the uterus immediately after calving (Sheldon et al., 2008, 2009; Herath et al., 2004). Because of this bacterial contamination and the substantial repair of the endometrium following parturition, uterine inflammation is an unavoidable and necessary process of postpartum uterine involution (LeBlanc, 2014). However, in a high proportion of postpartum cows, the prolonged hyperinflammation state leads to uterine disease (Chapwanya et al., 2010). The PUD complex includes retained fetal membranes (RFM), clinical metritis (CM), clinical endometritis (CE) and/or purulent vaginal discharge (PVD), pyometra and subclinical endometritis (SCE) (Table 1).

Postpartum uterine disease	Definition	Days after calving and reported incidence	Treatment
Retained fetal membranes	Failure to expulse the placenta 24 hours after parturition	24 hours after parturition Incidence: 4-12%	Manuel's removal is discouraged No treatment with no systemic signs
Metritis (puerperal and toxic)	-Enlarged, atonic uterus -Fetid, watery red-brown discharge -Signs of systemic illness (fever >39.5°C, decreased milk yield, toxemia)	Within 21 days after calving Incidence: 5-15%	When cows become febrile, systemic antibiotics for at least 3 consecutive days Supportive therapy if required
Clinical endometritis and/or purulent vaginal discharge	-Local inflammation of the endometrium -Presence of purulent or mucopurulent material in the vagina (clinical endometritis) -Absence of systemic symptoms (fever)	≥21 days after parturition Incidence: 20-30%	Intrauterine antibiotics (cephapirin) ≥26 days after parturition
Pyometra	-Presence of purulent material in the uterine lumen -Persistence of the corpus luteum Cervix often closed	Depending on the days post calving after first ovulation Incidence: 1-2%	Two doses of PGF2α with an interval of 11-14 days between applications
Cytological endometritis	-Abnormal presence of PMNs in endometrial cytology samples -Absence of any clinical signs	-Usually, ≥21 days after calving Incidence: 25-35%	No treatment

Table 1. Definition, incidence and treatment of the different PUDs.

1.6.1. Retained fetal membranes

Retained fetal membranes (RFM) is the failure to expulse the placenta between 12 to 24 hours after parturition (Drillich et al., 2003; Fourichon et al., 2000; Paisley, 1986). In RFM, the membranes are retained in the uterine lumen for an average of 7 days (Eiler et al., 1997), leading to increased bacterial contamination and delayed uterine involution (Laven and Peters, 1996). The incidence of RFM ranges from 4 to 12% (Drillich et al., 2003), with a median incidence rate of 8.6% (Kelton et al., 1998). Predisposing factors are twins, dystocia, stillborn calf, abnormal length of gestation, induced parturition, abortion, nutritional imbalances, fetotomy, CS (non-elective), advanced age and seasonal effects (Laven and Peters, 1996; Beagley et al., 2010). RFM has no direct impact on milk production, reproduction or culling if the condition does not evolve to CM, CE or SCE (Dubuc, 2011). The increased risk for PUDs is the main reason for RFM's economic importance. While a variety of methods have been used to treat RFM, the optimal treatment is under debate. Manual removal, local or systemic antibiotics and ecbolic drugs (e.g., oxytocin) are commonly used treatments, although current evidence does not support their use (Stevens et al., 1997; Gilbert, 2016). Intrauterine infusion of oxytetracycline is a common treatment among practitioners. Immunity dysfunction may be associated with RFM. Because of the metabolic stress associated with hormonal and metabolic changes, such as negative energy balance and a shortage of proteins, minerals and vitamins, the hypothalamic-pituitary-adrenocortical axis is activated. This results in a several fold increase in cortisol relative to the situation of controlled stress. As cortisol is a powerful immune suppressive agent, leukocyte proliferation and function are depressed, thereby compromising the rejection of the fetal membranes. In addition, metabolic periparturient stress triggers the production of catecholamines and more specially adrenalin, causing hypotony of the uterus. Therefore, immunosuppression and hypomotility of the uterus in high-producing dairy cows are very significant and compromise expulsion of the placenta (Mordak and Stewart, 2015).

1.6.2. Puerperal metritis

Clinical metritis is characterized by an enlarged uterus and a watery red-brown fluid to viscous off-white purulent uterine discharge, which often has a fetid odor, and occurs within 21 days postpartum (Sheldon et al., 2006). The diagnosis of puerperal metritis (PM) is made on the basis of clinical signs of fetid uterine discharge and/or systemic illness. The severity of the disease is graded as 1, 2 or 3 based on symptoms (Sheldon et al., 2009). In metritis, grade 1 is characterized by an abnormally enlarged uterus and uterine discharge without systemic signs of illness (Sheldon et al., 2006). Grade 2 metritis (or puerperal metritis) is characterized by additional signs of systemic illness, such decreased milk production, dullness and fever >39.5°C (Sheldon et al., 2006). Cows with grade 3 metritis (or toxic metritis) exhibit clinical signs of toxemia, such as inappetence, cold extremities, depression and/or collapse (Sheldon et al., 2009). Risk factors for any degree of metritis are often associated with RFM, dystocia, stillbirth or twins, with the metritis usually appearing within 10 days after calving (Drillich et al., 2001; Markusfeld, 1984). The impact of metritis on milk production and reproductive efficiency is under debate (Dubuc, 2011). When reported as detrimental, the impact on milk production is between 2 and 13 kg of milk lost per day over a period of 2 to 20 weeks (Overton and Fetrow, 2008; Wittrock et al., 2009; Giuliodori et al., 2013). Giuliodori et al. (2013) suggested that PM is associated with impaired early pregnancy rates and extended calving to conception intervals, but other studies have not found a link between PM and reproductive capacity (Dubuc, 2011). A common treatment for PM (puerperal/toxic) is the intrauterine infusion of antibiotics. However, the efficacy of local antibiotic treatment is a controversial issue (Drillich et al., 2001). Nowadays, the recommendation is to use systemic antibiotics (ceftiofur for 3 consecutive days) in cows with fetid vaginal discharge at days 4 to 6 after calving and a rectal temperature ≥39.5°C (Drillich et al., 2001; Zhou et al., 2001). A recent randomized clinical trial studied the efficacy of the initial use of ketoprofen (anti-inflammatory drug) versus ceftiofur (antibiotic) in cows with PM to reduce the use of antibiotic drugs (Pohl et al., 2016). However, no beneficial effects were found when cows were only initially treated with ketoprofen and more doses of medical applications eventually had to be used (Pohl et al., 2016).

1.6.3. Clinical endometritis and/or purulent vaginal discharge

Clinical endometritis (CE) refers to a local inflammation of the endometrium that is characterized by the presence of purulent or mucopurulent (>50% pus) material in the vagina ≥21 days postpartum that originated from the uterus and is not accompanied by systemic illness (Sheldon et al., 2006; Dubuc et al., 2010). It affects around 20% of dairy cows between 21 to 40 days postpartum (LeBlanc et al., 2002). Usually, CE is diagnosed by means of vaginoscopy, a gloved hand exam or Metri-checking (Pleticha et al., 2009; McDougall et al., 2007). However, the presence of abnormal vaginal exudates may not always be related to endometrial inflammation. Inflammation of the endometrium requires endometrial cytology or biopsy (or ultrasound examination) (Gilbert, 2016). The presence of vaginal exudate nowadays is referred to as "purulent vaginal discharge" (PVD). It is generally assumed that PVD is the result of endometritis, cervicitis, vaginitis or the combination of the former (Deguillaume et al., 2012). Deguuillaume et al. (2012) reported that 13% of cows had endometritis, 11% had cervicitis and 32% had both of these conditions. The detrimental effects of endometritis, cervicitis and vaginitis on reproductive performance are additive (Dubuc et al., 2010). In general, cows affected by PVD need on average 30 more days to become pregnant compared to unaffected cows (Tison et al, 2015; LeBlanc, 2008). The current literature on the efficiency of treatment protocols for CE/PVD is contradictory. However, two main approaches are commonly used: parenteral injections of prostaglandins (PGF2α) and intrauterine antibiotics. Prostaglandins are reported to be slightly beneficial (Kasimanickam et al., 2006) or inefficient (Lefebvre and Stock, 2012). The administration of intrauterine cephapirin after 26 days postpartum has been proven to be useful in treating PVD (Tison et al., 2017; McDougall, 2003; Runciman et al., 2008).

1.6.4. Pyometra

Pyometra is defined as the accumulation of purulent or mucopurulent material in the uterine lumen causing distension of the uterus, accompanied by the presence of an active corpus luteum (Sheldon et al., 2006). In pyometra, the cervix is often closed, although its

lumen is not always completely occluded and some purulent material may discharge through the cervix, vagina or vulva (Sheldon et al., 2006). *T. pyogenes* is the major etiological agent: it destroys the endometrium and eliminates the production of prostaglandins Fα, thereby allowing the persistence of the corpus luteum. A large field study showed that pyometra affected approximately 1.2% of cows (Busch et al., 2009) and another study showed that in most cases the disorder was related to problems during the postpartum period (Opsomer et al., 2000). In general, pyometra delays ovulation (Sheldon et al., 2002; Gilbert, 2016). Its diagnosis can be made by rectal palpation and/or ultrasound, the latter being the preferred and most accurate method. A fairly high rate of treatment success is obtained with two doses of PGF2α at an interval of 11 to 14 days between injections (Gustafsson et al., 1976; Olson et al., 1986; de Kruif et al., 1977). The prognosis after PGF2α treatment is generally favorable, with a first service conception rate of approximately 30% and an expected pregnancy rate of 80% after three or four inseminations (Fazeli et al., 1980).

1.6.5. Subclinical endometritis

Subclinical endometritis (SCE) is defined as an increased proportion of PMNs in the superficial endometrium that is associated with impaired reproductive performance and the absence of abnormal vaginal discharge and systemic signs (Sheldon et al., 2009). It can be diagnosed in different ways, including by endometrial biopsy or endometrial cytology via cytobrush or low-volume lavage (Barlund et al., 2008; Melcher et al., 2014). Biopsy involves the histopathological evaluation of an endometrial sample containing the surface epithelium, lamina propria and endometrial glands (Meira et al., 2012). This method provides the most accurate characterization of endometrial stratum compactum (Meira et al., 2012; Pascottini et al., 2016), but it is an invasive technique and some suggest that the biopsy itself may have negative effects on pregnancy (Bonnet et al., 1991). Endometrial cytology is a reliable diagnostic technique for SCE, regardless of whether cytobrush (Madoz et al., 2012) or low-volume lavage is used (Kasimanickam et al., 2005; Cheong et al., 2012) is used.

As there is no true gold standard diagnostic method for the diagnosis of SCE, the diagnostic threshold value for % PMNs in dairy cattle ultimately depends on DIM at diagnosis and the diagnostic method. The literature shows that the diagnostic threshold for % PMNs varies considerably, as does the threshold validation method used (de Boer et al., 2014; Melcher et al., 2014). For example, one study showed that at between 35 and 40 days postpartum, reproductive outcomes were generally impaired when the uterine cytology sample showed >5% PMNs (de Boer et al., 2014). Other groups have used a PMN threshold of >18% collected 21-33 days postpartum, and >10% at 34-47 days for the diagnosis of SCE (Kasimanickam et al., 2004; Sheldon et al., 2006; Sens and Heuwieser, 2013). In reproductive analyses using cytology, Barlund et al. (2014) used a threshold of >8% PMNs, Denis-Robichaud et al. (2015) used ≥6%, and Pascottini et al. (2016) used ≥3%, to name a few. Madoz et al. (2013) used optimal sensitivities and specificities to define critical thresholds for the diagnosis of SCE in grazing cows. Their trial resulted in thresholds of 8% PMNs for 21-33 days postpartum, 6% PMNs for 34-47 days, 4% PMNs for 48-62 days, and 5% PMNs for the full period of 21-62 days postpartum. Efficacy of treatment was not reported.

1.7. ISOLATION OF BACTERIA: TECHNICAL INFORMATION

1.7.1. Culture-based methods

Years of experimentation were required for scientists to establish a reliable culturing method for bacteria. The first culture media used were heart and brain cells. These were nonselective, thus permitting the excessive growth of microorganisms. The advent of the Petri dish, with its transparent lid that allowed researchers to observe the formation of colonies while limiting contamination, was revolutionary. Then, solid culture media composed of gelatin and agar allowed researchers to observe the growth of pure cultures (Guthertz, 2017). Next, scientists created the enriched growth medium through the addition of animal blood, which encouraged the growth of varied so-called fastidious microorganisms (Russell et al., 2006). Finally, researchers determined that select cultures could be developed by regulating four primary environmental parameters: nutrients, atmosphere and humidity, optimal temperature and incubation time (Bonnet et al., 2019).

This led to the creation of selective or differential media that could encourage the growth of certain microorganisms while inhibiting the growth of others. Such media are created through the addition of substances like organic and inorganic components and minerals. For example, crystal violet is a dye that helps classify bacteria since it inhibits the growth of Gram-positive bacteria. Antibiotics and antiseptics are also used to prevent or correct sample contamination and decrease the rapid overgrowth of commensal bacteria (Bonnet et al., 2019). Other forms of control in selective media are just as important; for example, regulating environmental parameters like temperature and atmospheric conditions provides aerophilic or anaerobic conditions based on varying oxygen levels (Stieglmeier et al., 2009). Meanwhile, temperature is key when developing cell cultures to grow and isolate intracellular bacteria: mammalian cells must be kept at 36.0°C, while other cell lines (e.g., amphibian, tick, mosquito and fish) prefer 28.0°C (Stewart, 2012; Penzo-Mendez, et al., 2012). A third method of control is to regulate incubation time since most pathogens grow over 24 to 48 hours, while pathogen require up to five days (Dunn et al., 1997). All of these factors must be considered in order to provide optimal growth conditions for the desired microorganism being cultured.

Culture-dependent methods have an inherent limitation of only being able to detect bacteria for which a culturing and identification method has been established. Indeed, it has been estimated that less than 1% of bacteria can be cultured in the laboratory (Kaeberlein et al., 2002). To overcome this limitation, culture-independent techniques such as PCR, 16S rRNA metagenomic sequencing, and whole-metagenome sequencing have been used to elucidate the role of the main uterine pathogens identified in culture-dependent studies. Nonetheless, these techniques are not without drawbacks. Limitations include the following: (1) bacterial viability cannot be assessed (if transcriptomics is not used), evaluated; (2) sequencing depth and coverage are low when using clone library sequencing; (3) operational taxonomic unit classification may change depending on the variable region of the 16S rRNA gene that is targeted for sequencing; (4) classification based on 16S rRNA gene sequencing is not accurate at the species level may not be reliable,; and (5) cost, which tends to be higher, particularly for whole metagenome sequencing (Galväo et al., 2019).

1.7.2. Metagenomic approaches

Given the important role of the vaginal microbiome in human reproductive health, it is important to understand which microorganisms are present in the vaginal microbiome, how many of which microbes are present and what the microbes are doing. Currently, there are two popular culture-independent methods to analyze microbiomes without the need for culturing: amplicon sequencing and whole genome shotgun (WGS) sequencing (Kuczynski et al., 2011; Sharpton, 2014; Shah et al., 2011). Amplicon sequencing uses the polymerase chain reaction (PCR) to amplify marker genes, such as the 16S ribosomal RNA (rRNA), from genomic DNA and map marker genes onto known sequence databases. This method can answer the questions of what microorganisms are present in the vaginal microbiome. WGS sequencing is the sequencing of genomic DNA directly from any environment (without PCR) to reveal the genes present. This method can answer the questions of which microorganisms are present in the vaginal microbiome, how many of which microbes are present and what functions can be performed by the microbes present.

1.7.3. 16S rRNA gene sequencing

The 16S rRNA gene is the most commonly used marker for bacterial identification (Woese et al., 1999). The 16S rRNA genes exists in all bacteria and contain hypervariable regions that are species-specific and can be used to identify bacteria, as well as strongly conserved regions that can be used to design primers to amplify hypervariable regions (Hugenholtz et al., 1996). 16S rRNA genes contain nine hypervariable regions (V1-V9) (Shah et al., 2011), that can be used to identify bacteria. Although 16S rRNA gene sequencing is a very powerful tool for identifying bacteria, it has three drawbacks. First, PCR amplification potentially introduces bias. Known sources of bias are the design of the universal primers for 16S rRNA genes and the PCR conditions used for the analysis of 16S rRNA genes (Hongoh et al., 2003; Grice et al., 2012). Second, 16S rRNA genes are not single-copy genes: the copy number varies from 1 to 15 (Klappenbach et al., 2001; Vetrovsky et al., 2013). This leads to the under or overestimation of bacterial

community composition. Lastly, an analysis of 16S rRNA genes does not provide functional information. Although Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) can be used to predict function from 16S sequencing data, this method predicts function from phylogeny based on shared gene content and does not consider variation among species (Langille et al., 2013). Moreover, the prediction accuracy of PICRUSt depends on the closeness between reference genomes and query microbiome (Langille et al., 2013). Therefore, 16S rRNA gene sequencing is not sufficient to elucidate the relationship between the vaginal microbiome and reproductive health.

The general workflow for 16S rRNA genes hypervariable tag sequencing technologies is as follows: 1) extraction of genomic DNA, 2) PCR amplification of certain regions of the 16S rRNA gene, 3) amplicon sequencing, and 4) bioinformatics analysis, including alignment and classification based on reference databases (Yang et al., 2013).

1.7.4. Next-generation sequencing

Recent years have seen an enormous reduction in the cost of high-throughput sequencing technologies, also known as next-generation sequencing (NGS). These technologies have the capacity to produce large genomic sequence datasets over a relatively short period of time at a reasonable cost. The advancement of NGS technology has enabled a 1,000-fold reduction in sequencing costs, from $500 per megabase using Sanger sequencing to <$0.5 per megabase using an Illumina platform (Kircher et al., 2010; Kircher et al., 2012). It is now possible to sequence the complete human genome within days for <$1,000; this represents a massive advance, considering that the first human genome sequence was completed just over a decade ago after 13 years of work and at a total cost of ~$3 billion (Kircher et al., 2009; Kircher, 2014).

The cost of NGS has reduced to the point where it is now feasible to use it in the clinical diagnosis of infection. Previous studies have shown that NGS can reliably identify microorganisms, including viruses, at levels beneath the detection sensitivity of

conventional microscopy and/or serological tools (Kircher et al., 2005). The technology also facilitates the discovery of genes that serve as biomarkers in various pathological conditions. In fact, the application of NGS in the area of genetic diseases and cancer has facilitated the identification of genes that serve as biomarkers for diagnosing genetic abnormalities and certain types of cancers, as well as for monitoring disease progression (Kircher et al., 2008). In the field of microbiology, NGS provides huge amounts of sequence data for genetic profiling, the study of biodiversity and pathogen discovery.

16S rRNA sequencing:

The 16S rRNA gene has been used to identify bacteria based on their DNA sequence since the invention of the PCR reaction (Wang and Qian 2009; Janda and Abbott, 2007). The 16S rRNA gene has several features which make it good for identifying bacteria. The gene codes for ribosomes, the small subunit that translates mRNA into proteins (Moore and Steitz, 2002). This function is essential for bacteria; thus, the gene is highly conserved (Wang and Qian, 2009). the widespread use of the 16S rRNA gene to identify bacteria has given rise to large databases with reference sequences. For example, the Ribosomal Database Project (RDP) contained 643,915 16S rRNA entries in September 2008 (Cole et al., 2009) and 3,019,928 16S rRNA entries in September 2014. However, sequencing a small part of the 16S rRNA gene, such as one or two hypervariable regions, may not provide sufficient resolution to distinguish between closely related bacteria with very similar 16S rRNA gene sequences (Amir et al., 2013). Furthermore, the copy number of the 16S rRNA gene can vary between bacterial species, so a direct quantification of the number of bacteria based on the 16S rRNA observed may be inaccurate (Zoetendal et al., 2004).

Conclusions:

The microbiome of uterus uterine microbiota was generally same bacterial diversity during one week to five weeks after calving in healthy cows. Disease cows with had a different microbiome, with the increased relative abundance of *Bacteroidetes* and *Fusobacteria* (phylum level), and *Fusobacterium* and *Trueperella* (genera level) than healthy cows. Bacteria that grew in culture were often present within the most abundant bacteria in the16S rRNA gene sequencing in 21

days after calving. Bacterial composition was not significantly different between healthy and disease cows during postpartum. This study supports the hypothesis that bacterial pathogens in the uterus are associated with postpartum uterine diseases, which points to the alternative hypothesis that regulation of uterine inflammation is worthy of pursuit for the prevention and treatment of postpartum uterine diseases. In addition, the 3-week postpartum was found a critical point showing a significant increase of the concentration for all molecules studied. Variations of IL1 and IL8 during the time course keep a relation with the neutrophils function as a normal homeostatic process. During all the time-course analyses, these cytokines had a major concentration in cows with clinical cervicitis than in healthy cows. With a proinflammatory protective effect, IL1, IL8 and AGP can modulate locally the uterine innate immune response involving neutrophils in normal uterine involution, cervicitis or endometritis, without the presence of a systemic inflammatory process.

Suggestion:

There is still a gap in knowledge in the linking between immune defence and the microbiota of the reproductive tract in diseases dairy cows. The first problem is the compartmentalization of the reproductive tract (vagina, cervix, and uterus). Practically no information is available on cervicitis and vaginitis in postpartum diseased cows. Therefore, the first objective was to assess the role of cervicitis and vaginitis in PUDs in dairy cows. The objective was to measure the association between cervicitis/vaginitis with the most important uterine diseases like metritis, and endometritis. Once the relative importance of cervicitis was measured, information about the immune response associated with cervicitis was very important to assess. Therefore, Interleukin 1-alpha, IL-8 and alpha-1 acid glycoprotein were measured in the case of cervicitis and vaginitis. Finally, there areare controversial results of the microbiota of the reproductive tract in postpartum dairy cows. With the present definition of postpartum endometritis, we have to determine the microbiota pre and postpartum in dairy cows.

1.8. OBJECTIVES AND RATIONALE

This series of experiments had two objectives. The first was to define the relationship between the different postpartum diseases (vaginitis, cervicitis and endometritis) that affect the genital tract of high-producing dairy cows. The second was to characterize the immune response and changes in the microbiota during the transition period, specifically in the case of cervicitis and clinical endometritis. The hypothesis is that a cow's immune response and microbiota change during the transition period according to its uterine health status.

In the first set of experiments, I hypothesize that cervicitis is associated with an immune response that is similar to that in the uterus in the postpartum period. The objective is to describe the cellular and humoral local innate immune response during clinical cervicitis (CC) in the uterus and vaginal fornix during the pre-and postpartum periods.

In the second set of experiments, it was hypothesized that clinical cervicitis and vaginitis are components of reproductive tract inflammatory disorders in postpartum dairy cows and that they have a precise association with the other postpartum uterine diseases. The objective is to investigate whether cervicitis and vaginitis are potentially significant problems in high-producing dairy cow's involution process and post-partum diseases.

In the third set of experiments, I hypothesize that dynamic changes in the vaginal prepartum and postpartum uterine microbiota differ between healthy and diseased dairy cows. The goal is to characterize bacterial communities in the vagina before calving and in the uterus after calving using NGS in healthy and endometritis in dairy cows. I also compare the uterine bacteria recovered in culture with the metagenomic profile of samples collected at 21 days after calving.

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CHAPTER 2: ARTICLE 1

Pre- and Post-partum Concentrations of Interleukin 1α, Interleukin 8, and α1-Acid Glycoprotein in Vaginal Fornix and Endometrium of Dairy Cows With Clinical Cervicitis

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Abstract

Innate immunity is the principal sensor responsible of the local immune response to control mucosal bacterial contamination of the uterus after parturition, triggering a pro-inflammatory process in tissues other than the endometrium like the cervix uteri and vagina. However, knowledge about the inflammation process and outcome of the cervix in dairy cows is scarce. The objective of the present study was to describe the cellular and humoral local innate immune response during clinical cervicitis (CC) in the uterus and vaginal fornix in pre- and post-partum periods of dairy cows. A retrospective descriptive study was performed involving 26 animals, characterized as clinical cervicitis cows (n=19) and healthy cows (n=7). Eighty-five pre- and post-partum period dairy cows, blood and mucus samples were selected for this research; including records of cow´s clinical exam performed four times: -1w (day -7±2, prepartum), +1w (day +7±4), +3w (day +21±4) and +5w (day +35±4) postpartum. For animal selection, cases were defined at the five-week examination. Clinical cervicitis was defined as cows exhibit cervix grade–2 and healthy cows were defined as a cow clinically normal with a grade-0 cervix. The concentrations of interleukin 1α (IL1), interleukin 8 (IL8) and α1-acid glycoprotein (AGP) were determined. Cases of CC with clinical signs at time +5w such as purulent vaginal discharge and subclinical endometritis shown the highest cytokine production. In conclusion, the 3-week postpartum is a critical point to evaluate cytokines and acute phase proteins; where IL1 and IL8 variation kept a direct relation with neutrophils numbers and function. During all the time-course analysis, these cytokines had major concentration in cows with clinical cervicitis than healthy cows. The presence of AGP in the endometrium infer a homeostatic proinflammatory protective balance effect, modulating the local uterine innate immune response during peripartum.

Key words: *uterine disease, cervix, innate immunity, Cytokines*

1. Introduction

Most parturient cows experience bacterial contamination of the uterine cavity and endometrial damages that trigger an active inflammatory response to clear the infection and repair the tissues respectively. However, the post-partum physiological complex, and the bacterial load, does not allow the innate immune response to control uterine bacterial imbalances; therefore facilitating prolonged genital tract inflammation stage, and a delay uterine involution indulging a postpartum uterine disease (Carneiro et al., 2016; Herath et al., 2009; Herath et al., 2007; Williams, 2013). The incidence of postpartum uterine disease (PUD) is high in dairy cows worldwide and includes clinical (metritis, endometritis) and non-clinically conditions (subclinical endometritis) (Sheldon et al., 2009).

The diagnosis of PUD is limited by the lack of clarity regarding the case criteria, difficulties associated with the diagnosis of subclinical conditions, and variable sensitivity and specificity of the different diagnostic tests in relation to the absence of direct visual assessment of the endometrium (de Boer et al., 2014; Wagener et al., 2017). The discrepancies between clinical findings and the diagnostic test could be explained by the presence of an inflammatory process in tissues other than the endometrium like the cervix uteri and vagina (Hartmann et al., 2016). The cytological evidence of cervical inflammation was reported in 19% of sub-fertile cows (Sylte et al., 2010). Hartmann et al. (2016) and Deguillaume et al. (2012) reported that 60.8% and 45% of dairy cows had clinical cervicitis and cytological cervicitis respectively between 42 and 50 days postpartum, affecting. However, knowledge of inflammation or bacterial contamination of the cervix and the subsequent influence on reproduction in dairy cows is limited.

Bacterial contamination of the uterine lumen is an event that occurs in more than 90% of cows during the first postpartum days; the capacity of the uterus to resolve an infection depends on its ability to detect and respond to microbial ligands (Chapwanya et al., 2009; Sheldon and Dobson, 2004). Innate immunity is the principal sensor responsible of immune response to control bacterial contamination of the uterus after parturition. The pathogen-associated molecular patterns (PAMPs) present in the bacterial are quickly detected by the pattern recognition receptors (PRRs) in the host cells; the engage between PAMPs and PRRs such as Toll-Like Receptor (TLRs) initiate a signaling cascade resulting in the synthesis and production of pro-inflammatory cytokines such as TNF-α, IL-1-α, IL-6 and chemokines (IL-8) (Adnane et al., 2017; Singh et al., 2008). For example the chemokine IL8 is produced mainly by activated macrophages in response to bacterial PAMPs directing the upregulation of adhesion molecules to enhance neutrophil recruitment to the inflammatory area (Chapwanya et al., 2012; Fischer et al., 2010). As a result, uteri inflammation environment is characterized by the significant presence polymorphonuclear leukocyte infiltration (Neutrophils) as well (Adnane et al., 2017).

The lack of a complete genital examination in postpartum dairy cows without appreciation of the molecular immunological mechanisms underlying changes at the cellular or systemic level, could explain the limited understanding of the general process involved in PUD (Chapwanya et al., 2009). Therefore, the aim of the present study was to describe the innate immune response in the uterus and vaginal fornix in the pre- and post-partum periods of dairy cows with clinical cervicitis.

2. Materials and methods

2.1 Ethical Statement

This research was performed in compliance with the experimental practices and standards approved by the animal care committee of the University of Montreal.

2.2 Study population and sampling.

A retrospective descriptive study (Figure 1) was performed involving 26 animals defined as, clinical cervicitis cows (n=19) and healthy cows (n=7). Cows were selected from a database of a previous study in which 85 dairy cows (purposively selected) were following-up and sampling (blood and cervix / uterine mucus) during the pre- and postpartum period. For the present study, clinical data of the database and the stored blood and mucus samples were used.

The cows came from three commercial dairy herds in Quebec (Canada) that were housed in a tie-stall barn and milked twice daily. The rolling herd average for milk production was 9000 kg. Cows were fed a total mixed ration of corn and hay silage to meet the nutrient requirements of dairy cows recommended by the National Research Council (NRC, 2011). Farms were visited weekly by the same veterinarians and all cows were vaccinated 2 times against E. coli (J-VAC®, Boehringer Ingelheim, Burlington, Ontario, Canada, L7L 5H4) before calving (2 ml IM, D-40 and D-26 before parturition) and once against IBR, BVD type 1 and 2, PI3, and BRSV (Bovi-shield GOLD5® FPTM 5 L5, Zoetis, Kirkland, Québec, Canada, H9H 4M7) after calving (2 ml IM, between D-10 and D-30 postpartum). In addition, all pregnant cows were injected with Se (5.0 ml, D-60 before calving, MU-SE, Intervet, Merk, Kirkland, Québec, Canada, H9H 4M7).

The database includes records of cow´s clinical exam performed four times: -1w (day -7±2, prepartum), +1w (day +7±4), +3w (day +21±4) and +5w (day +35±4) postpartum (Figure 1). Data collection included clinical data associated with reproductive assessment (vaginoscopy,

transrectal exams, purulent vaginal discharge, cervix characteristics, uterine horn symmetry, and ovarian structures), cytological exams (neutrophil count in the fornix of the vagina and in the uterine) and blood samples (white blood cell count). Samples were stored frozen at -80°C and used in the present study to determine the immune response.

2.3 Case definition and animal selection.

For the animal selection, postpartum disorders were defined. Clinical metritis was characterized as abnormally enlarged uterus and purulent uterine discharge within the first 21 days postpartum without systemic clinical sings (Giuliodori et al., 2013). At 5 weeks postpartum, clinical endometritis (CE) was defined as a cow exhibiting purulent vaginal discharge – PVD (Sheldon et al., 2008) or subclinical endometritis (SE) in cows with a proportion of neutrophils exceeding 5% on endometrial cytology in absence of PVD and anomalies on transrectal examination (Wagener et al., 2017). On vaginoscopy, cervix was classified as GRADE 0 (Normal) without abnormality; GRADE 1 (normal) with the second cervical fold swollen without redness and prolapsing through the first ring, and GRADE 2 (Clinical Cervicitis, CC) with the second fold swollen and red prolapsing through the first ring in absence of PVD (Hartmann et al., 2016).

Based on reproductive assessment records, as exclusion criterion, animals were rejected of the database because of: 1) systemic illness or clinical conditions other of the reproductive tract; 2) calving disorders (dystocia, twins, fetal membranes retention) and; 3) animals that in the final reproductive exam (+5w), exhibit endometritis (clinical or subclinical) without clinical evidence of clinical cervicitis. Clinical cervicitis cows (CC) were defined as a cow, which, posterior to applying the exclusion criterion, exhibited cervix grade–2 at the five-week examination. Healthy cows (CH) were defined as a cow clinically normal with a grade-0 cervix

at the 5w plus any evidence of postpartum disorders during all the follow-up period (-1w +1w; +3w; +5w postpartum).

From the database (n=85), animals were rejected because of culling (n=5); use of antimicrobials (n=4); metabolic disease (n=5); missing data (n=10) and exclusion criterion (n=35). Finally, 26 cows were enrolled in the study: 19 met the case definition criteria for cervicitis and 7 met the criterion defined for healthy cows (Figure 1).

2.4 Measurement of cytokines concentration in vaginal and uterine samples.

The concentrations of interleukin 1α (IL1), interleukin 8 (IL8) and α1-acid glycoprotein (AGP) were determined using commercial kits [Bovine IL-1α ELISA Kit Nori®; Bovine IL-8 ELISA Kit Nori® and; bovine AGP ELISA Kit Nori® (GENORISE SCIENTIFIC, INC. Philadelphia, USA)]. All procedures were performed according to the guidelines provided by the manufacturers. Briefly, for IL1α; IL-8 and AGP, 100 µL of uterine mucus, vaginal fornix mucus, or standards (stored in PBS) were added to 96-well microplates and incubated for 1 h at room temperature. After aspiration and washing (with 300 µL of Assay Buffer, three times), 100 µL of the working dilution of Detection Antibody (diluted in Regent Diluent) was added to each well and incubated for 1 h. The plates were washed and then incubated with 100 µL of the working dilution of HRP conjugate solutions for 20 min at room temperature, washed again, and then incubated with 100 µL of Substrate Solution for 5 min. Finally, 50 µL of Stop Solution was added. The optical density of each well was then measured at 450 nm in Microplate Spectrophotometer (SpectraMax® Plus 384 Absorbance Plate Reader, USA).

2.5 Data analysis

Variables included in the data analysis were time of examination (-1w, +1w, +3w, +5w weeks postpartum); sample origin (Vaginal Fornix – Uterus); concentrations of IL1, IL8 and AGP; proportion of polymorphonuclear neutrophils in the vaginal fornix, uterus (percentage of PMN in a 300-cell count), white blood cell count (WBC) and, clinical profile: healthy cow (CH), clinical cervicitis cow (CC), clinical cervicitis + purulent vaginal discharge (CC+CE), clinical cervicitis + subclinical endometritis (CC+SE).

Variables were assessed for normal distribution using histogram with Gaussian distribution graph, Shapiro-Wilk test and coefficient of variation. Based on the data distribution, parametric and nonparametric tests (One-way ANOVA, Mann-Whitney Test) were used for the comparison for each one of the variables. When a significant difference was found, the power to detect a difference (with a 95% confidence interval) was calculated considering the number of cows per group (cervicitis – Healthy) and the mean and standard deviation of cytokines (for IL1, IL8, AGP) found in each comparisons group. The statistical differences found, were reported only when the calculated power was ≥70%.

3. Results

3.1 Clinical characteristics of cervicitis.

Clinical cervicitis (CC) criteria in this research was considered when the cervix had reddening of the supra-vaginal portion of the cervix with edema and prolapse of the second cervical fold. This research showed that n=19 (23% of then cows) at five weeks postpartum (+5w) were categorized as cervicitis. In addition, it was found some cases of CC was concomitant with purulent vaginal discharge (CC+PVD) or subclinical endometritis (CC+SE).

3.2 Percentage of polymorphonuclear neutrophils in vaginal fornix and uterine mucus.

The percentage of PMN in vaginal fornix in CC cows was diverse during the sampling time pre- and post-partum respectively. At -1w, >15.89% (P=0.001), +1w >13.84% (*P*=0.005), +3w 15.8 and +5w >14.05% (*P*=0.004). There were not significant differences between healthy and CC animals at -1w; however, there were significant differences ($p<0.05$) at +1, +3w and 5w respectively. It was noticed that at 3+w was the highest percentage of PMN, however the significant difference at +1 and +5w were the lowest when compare with the healthy animals.

In uterine mucus, cows with CC showed a significant difference ($p<0.05$) in the PMN ($p<0.05$) in all the sampling times except at -1w; the highest percentage was found at +1w. However, the were not differences between CC/CH (Figure 2).

3.3 Cytokine variations by examination time, case/control, and sample origin.

No significant differences were found between sampling time periods. Comparison between CC and CH cows (Figure 3) found statistical differences only for time +3w where the concentrations of IL1α in Fx (1.05±0.11 pg/dl) and Ut (0.65±0.11) of CC cows were significantly higher (P=0.005) than the Fx (0.38±0.19 pg/dl) and Ut (0.35±0.16 pg/dl) of CH cows. In addition, the levels of IL8 in CC cows were more elevated than CH cows in both the Fx (7.39±1.46 pg/dl vs 2.72±1.49 pg/dl, P= 0.043) and uterine (6.07±1.13 vs 3.26±1.63 pg/dl, P= 0.040) respectively. The concentration of AGP in Fx (Figure 4) for CC was 2.76 pg/dl higher than CH (P=0.0018). Comparison between Fx and Ut for CC cows at +3w found that IL1α and AGP concentrations in Fx were 0.39 pg/dl (P= 0.018) and 1,93 pg/dl (P=0.002) higher respectively than in the Ut samples. No significant difference was found between Fx and Ut concentrations of IL1, IL8 and AGP in CH cows at time +3w ($p>0.05$).

3.4 Cytokine variations by clinical characteristics of cervicitis.

Cases of cervicitis (CC) occurred concurrently with other clinical conditions diagnosed only at +5w (Figure 5). At the time -1w, Fx IL1 was 1.03 pg/dl higher in cows with (CC+SE) compared with CH cows (P=0.016). At the time +3w: a) Fx IL1 levels was 0.98 pg/dl higher for (CC+SE) cows compared with CH cows (P=0.022); b) Fx IL8 levels was 11.04 pg/dl higher for (CC+SE) cows compared with CH cows (P=0.028). At the time +5w: a) Fx IL8 levels was 11.54 pg/dl higher for (CC+SE) cows compared with CH cows (P=0.028); b) Fx AGP levels was 5.56 pg/dl higher for (CC+SE) cows compared with CH cows (P=0.025). In uterine samples, at time +3w: a) Ut IL1 levels was 0.76 pg/dl higher for (CC+SE) cows compared with CC+PVD cows (*P*=0.08); b) Ut AGP levels was 2.75 pg/dl) higher for (CC+SE) cows compared with CC+PVD cows (*P*=0.028).

3.5 White blood cell count (WBC). Variations by examination time and case/control.

For white blood cell count ($x10^3$ mm^3) for monocytes (Mon), neutrophils (Neu) eosinophils (Eos), and basophils (Bas), there were no significant differences ($p<0.05$) between clinical cervicitis cows (CC) and healthy cows (CH). At time +1w, WBC was higher compared to -1w; +3w and +5w (Figure 6).

4. Discussion

Innate immunity plays an important role in keeping postpartum reproductive tract microbiota balance where the cervix acts as an anatomical barrier protecting the uterus from external pathogens producing cervical mucus (Deguillaume et al., 2012; Eurell and Frapier, 2006; Dhaliwal et al., 2001). During postpartum, due to puerperal physiological modifications occurring in the vagina or uteri, the cervix is exposed to microorganisms that belong to the local microbiota or pathogen infections,as well as physical damage or obstetrical manipulations

facilitating the presence and growth of pathogens triggering cervical inflammation. In the present study, clinical cervicitis was diagnosed in 23% of the cows examined at five weeks postpartum when the cervix had reddening of the supra-vaginal portion of the cervix with edema and prolapse of the second cervical fold. Moreover, some cases were accompanied by purulent vaginal discharge or subclinical endometritis. We found that the average values of all cytokines tended to be higher in cows with clinical cervicitis. It means that cervicitis is a process initially controlled by innate immunity, characterized for increased amount of uterine tract neutrophils, a major production of pro-inflammatory cytokines (IL-1) and chemokines (IL-8), and recruitment of neutrophils and monocytes.

The aim of the current research is to describe the innate immune response in the uterus and vaginal fornix of cows diagnosed with clinical cervicitis and the variations during the pre- and post-partum periods (-1w, +1w, +3w, +5). The concentrations of IL-1, IL8 and AGP in cervical and uterine mucus, for both CE and CH, shown an increasing pattern from 1 to 3 weeks postpartum. These innate immunity proteins reached the highest levels at +3 weeks followed for a decrease at 5 weeks postpartum. Our results are in agreement with those supporting that IL-1, IL8 and AGP proteins keeps a pattern with bacterial presence of the uterus from vaginal, fecal and environmental microbiota which occurs for two to three weeks postpartum (Jeon et al., 2015; Sheldon et al., 2008). The post-partum period is characterized by calving-associated physical barriers to relaxation including an open cervix and negative pressure events created by repeated uterine contraction and relaxation. As a result there is an enhances bacterial contamination in all bovine uteri after calving, initially by Gram-negative bacteria followed by Gram-positive bacteria decreasing the levels of neutrophils and cytokines (Dadarwal et al., 2017). Therefore, inflammatory cytokines are expressed in the bovine endometrium in a time-related manner

during the postpartum period, with a significant expression peak on a time around the 3 weeks postpartum (Gabler et al., 2010).

The parturition period requires extensive remodeling of the cervix before parturition (cervical softening and cervical ripening) for the transformation of the cervix from a closed rigid structure, to one that opens for birth (Mahendroo, 2012; Timmons et al., 2010). In this study before calving (-1w), we found that a high blood neutrophils and IL-8 concentration, and detectable IL1 - AGP levels. Our results agree with the increase in expression of IL-8 enabling the influx of neutrophils in the cervical tissue that excretes matrix metalloproteinase, which contributes to the softening of the cervix (van Engelen et al., 2007; Van Engelen et al., 2009). Moreover, parturition is associated with the local accumulation of IL8, which acts synergistically with PGE2 to attract polymorphonuclear neutrophils (REF). Hence, activation of placental leukocyte population releases proteolytic enzymes that degrade the caruncle, thus facilitating fetal membrane separation and advancing the progression of labor (Attupuram et al., 2016; van Engelen et al., 2007).

Therefore, fetal membrane separation is directly linked to inflammatory changes at the uteroplacental level. The current study results at 1-week prepartum to 1 week postpartum, might explain the direct connection between increased blood IL-8, and monocytes levels with high uterine concentrations of neutrophils. Increased apoptosis, caruncular degradation, and IL-8 production around parturition trigger a massive influx of neutrophil and mononuclear cells into the uterus and the cervix of cows that expel their placenta normally (Attupuram et al., 2016). As such, the prepartum increased IL8 concentration found in this research could be related to cervical ripening before parturition which is a physiological pro-inflammatory process influenced by regulatory cytokines.

In the first week postpartum, the current research found an increase in both uterine and blood neutrophils percentage, and blood monocytes levels (Figure 2 – 6). This is an expected finding considering that tissue macrophages release IL1 and IL8 that act as a pro-inflammatory and chemo-attracting coordinates the recruitment of neutrophils to the site of infection enhancing an inflammatory repairing response, also blood monocytes (an increase of blood monocytes at 1-week postpartum can be observed in figure 6) are recruiting during inflammation to differentiated in tissue macrophages for an effective control and clearance of pathogens (Sheldon et al., 2018; Singh et al., 2008). Our results agree with the important role of the expression of pro-inflammatory cytokines for calving and placenta expulsion where IL1 and IL8 concentrations are increased in the cervix (at least eightfold higher) at parturition compared to bovine gestation (Van Engelen et al., 2009). And also, the abundance of monocyte increases in the placentome around parturition, that as a macrophages participates in the recognition and phagocytosis of cells undergoing apoptosis and capture of bacterial lipopolysaccharides (Streyl et al., 2012).

In the current research, during all the time-course analysis, the results shown an increased levels for AGP in relation to IL1 and IL8. The α1-acid alpha glycoprotein AGP is an acute phase protein normally expressed by the liver and usually found in blood; however extrahepatic expression of bovine AGP has been reported in tissues like uteri and ovaries (Fournier et al., 2000; Lecchi et al., 2009). AGP possesses an immunomodulatory activity and it is believed to play an important role in the regulation of local inflammatory reaction by reducing the tissue damages caused by an excessive activation of complement, modulate apoptosis in bovine monocytes and modulate the degranulation of neutrophils involved in the fine tuning of neutrophil activity in the inflammatory environment (Ceciliani et al., 2007; Miranda-Ribera et

al., 2010; Rahman et al., 2015, 2008). In the study, AGP concentrations increased during cervicitis, mainly at 3 week postpartum and higher levels were found when cervicitis was concomitant with subclinical endometritis (Figure 4). Our results agree with previous work that demonstrated a higher level of plasma AGP in cows developing uterine infection in comparison with cows without endometritis (Cairoli et al., 2006). AGP is mainly synthesized by the liver, but it can be localized in several other bovine tissues.

AGP exerts a sort of protective activity by reducing the apoptosis rate in some inflamed tissues and by increasing the lifespan of monocytes, at least in the bovine species (Miranda-Ribera et al., 2010; Rahman et al., 2008; Theilgaard-Mönch et al., 2005).

Acute phase proteins can reach the inflammatory site through the inflammatory exudate, but it has also been demonstrated an extrahepatic production, specifically AGP, can be produced in organs such subcutaneous adipose tissue, lymph nodes, uterus and ovary of dairy cattle (Lecchi et al., 2009; Rahman et al., 2015, 2008). In general, AGP can modulate locally the uterine innate immune response in different cases such as normal uterine involution, cervicitis or endometritis without the presence of a systemic inflammatory process. Therefore, our results allow us to infer that endometrial production of AGP has an important role in neutrophil function in both in healthy and infected cows. However, there is limited information related with the role of AGP in postpartum uterine disease in dairy cows, but we know that the APPs work as a predictor of postpartum uterine infections during the transition period (Manimaran et al., 2016), therefore more research is needed.

In conclusion, the present study describes a cytokines and APP characterization profile of local innate immune response in the cervix during the postpartum period. The clinical findings of the present study allow diagnosing the presence of a regulated proinflammatory process in the

external section of the cervix with certain reliability, particularly, the prolapse of the second cervical fold could be related with an inflammatory process in the inner part of the cervix also (clinical cervicitis). The 3-week postpartum was found a critical point showing a significant increase of the concentration for all molecules studied. Variations of IL1 and IL8 during the time-course keeps a relation with the neutrophils function as a normal homeostatic process. During all the time-course analysis, thesethese cytokines had major concentration in cows with clinical cervicitis than healthy cows. With a proinflammatory protective effect, IL1, IL8 and AGP can modulate locally the uterine innate immune response involving neutrophils in normal uterine involution, cervicitis or endometritis, without the presence of a systemic inflammatory process. Cervicitis can occur in concomitance with other recognized postpartum uterine clinical conditions (clinical - subclinical endometritis) changing the number of open days. Nevertheless, considering that the studies on this topic are scarce, and there are significant differences in disease definitions and research methods, further information is needed to understand better the impact of cervicitis on postpartum uterine disease and fertility.

5.Conflict of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality in conducting the experiment and publishing the manuscript.

6. Acknowledgments

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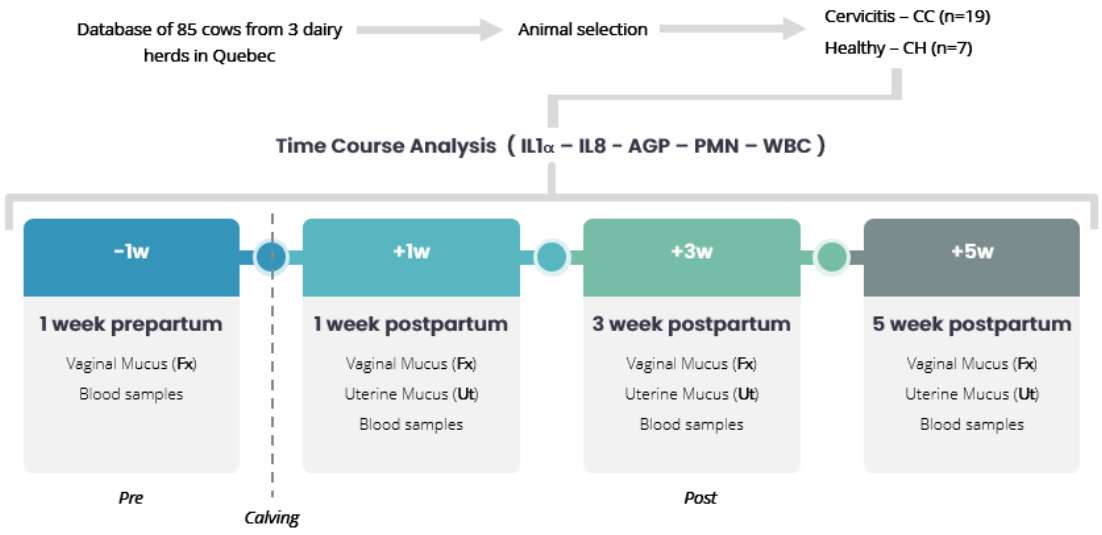
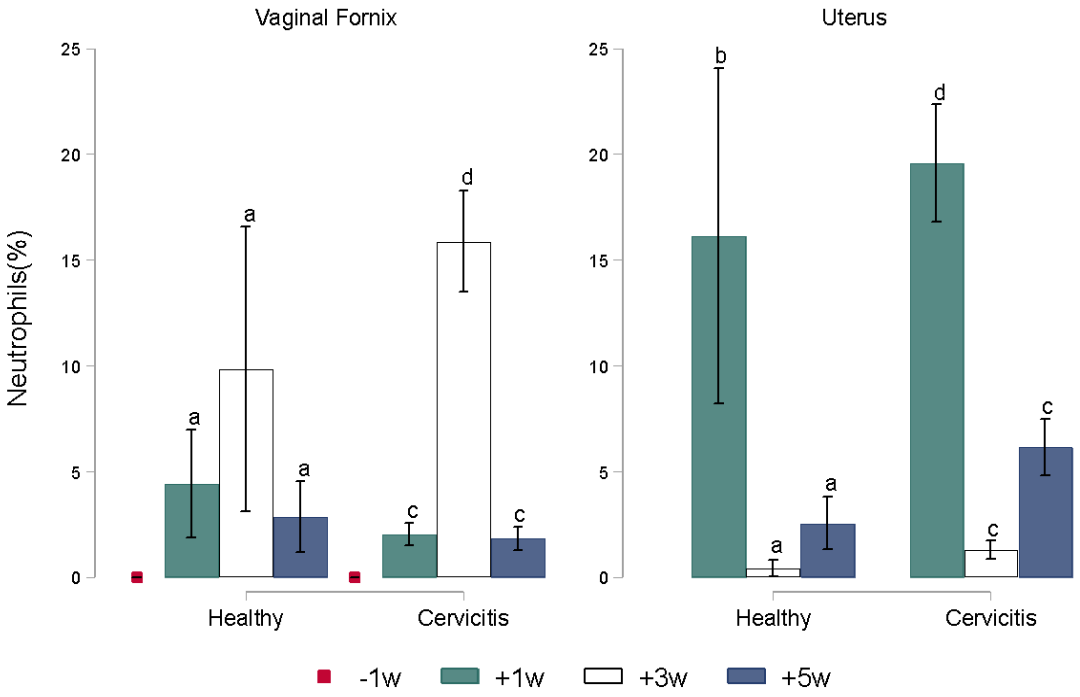
Figure 1. Study designe. Time-course analisys of innate immune responses of clinical cervicitis in pre- and post-partum dairy cows (n=26) from 3 dairy herds in Quebec in 2016.



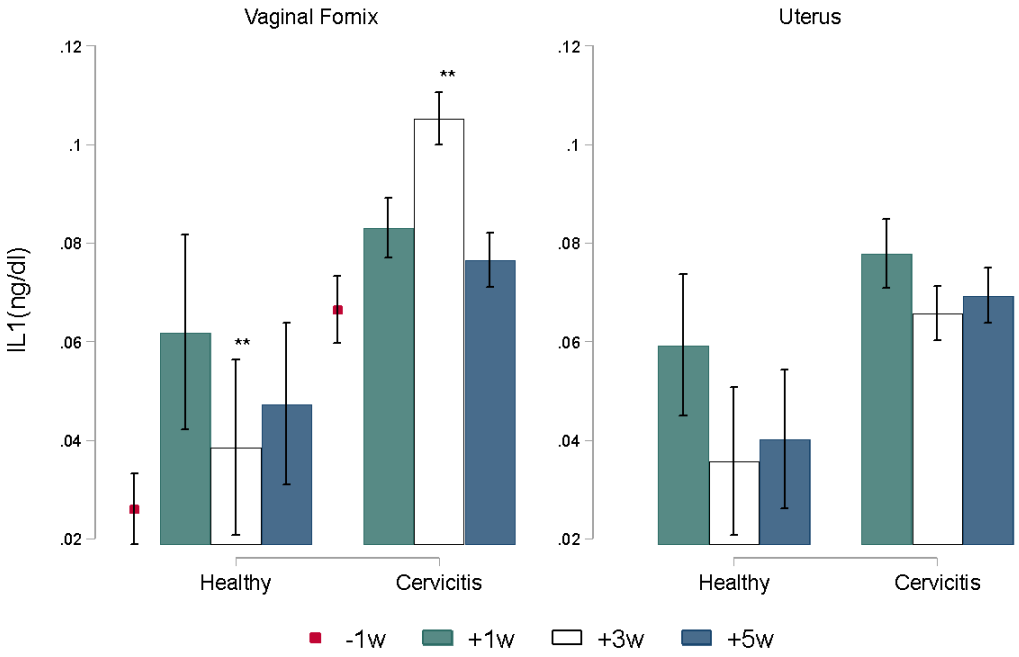
A retrospective study involving database and stored blood and mucus (vaginal fornix, Fx – uterine, Ut) sampling of 26 dairy cows from three commercial dairy herds in Quebec. Cows diagnosed with clinical cervicitis (n=19) and healthy (n=7) were involved in a time-course analysis from 1 week before calving to 5 weeks after calving to establish variations for withe blood cell count (WBC), uterine PMN percentage, and vaginal fornix and uterine mucus cytokines (IL1 – IL8 – AGP).

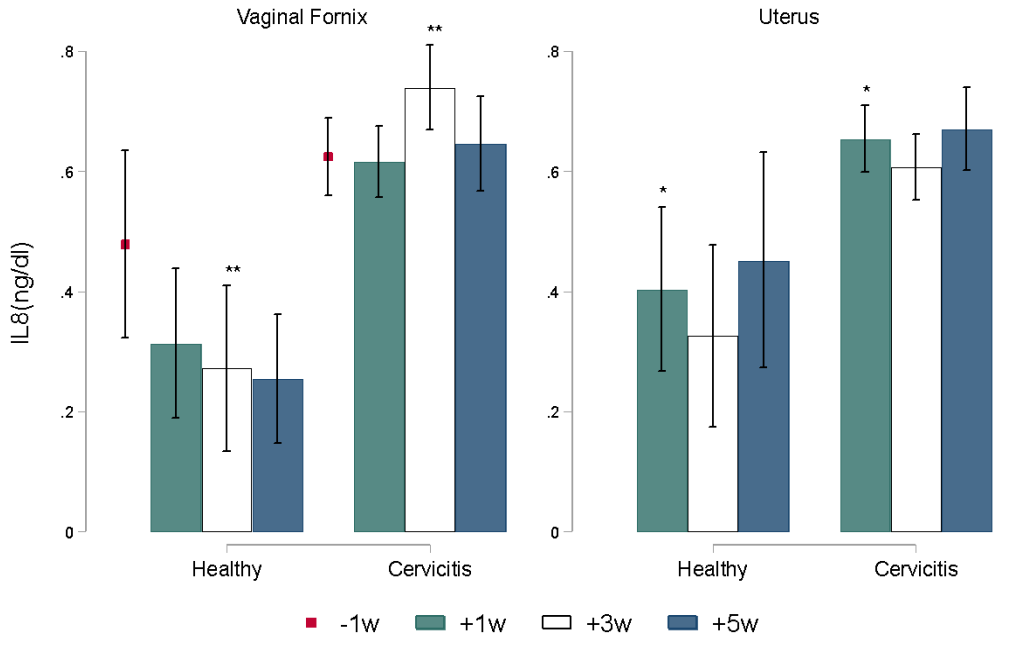
Figure 2. Time-course variations in pre- and post-partum percentage of neutrophils in the vaginal fornix and uterine mucus of cows (n=26) defined as clinical cervicitis cows (n=19) and healthy cows (n=7) from 3 dairy herds in Quebec in 2016.



Time-course variations (weeks -1w +1w +3w +5w) of neutrophils percentage, time-course differences are indicated with superscript a-b (for healthy cows), and c-d (for clinical cervicitis cows). Means without a common superscript differed ($P < 0.05$).

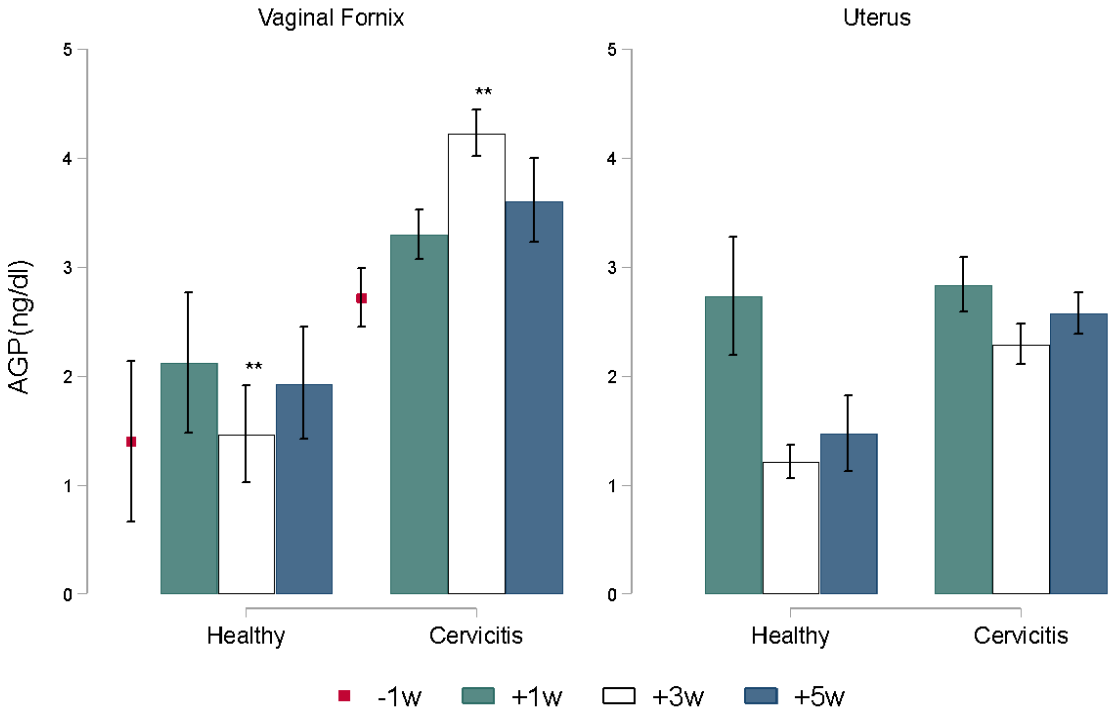
Figure 3. Time-course variations (mean ± SEM) in pre- and post-partum concentrations of IL1 and IL8 in the vaginal fornix and uterine mucus of cows (n=26) defined as clinical cervicitis cows (n=19) and healthy cows (n=7) from 3 dairy herds in Quebec in 2016.





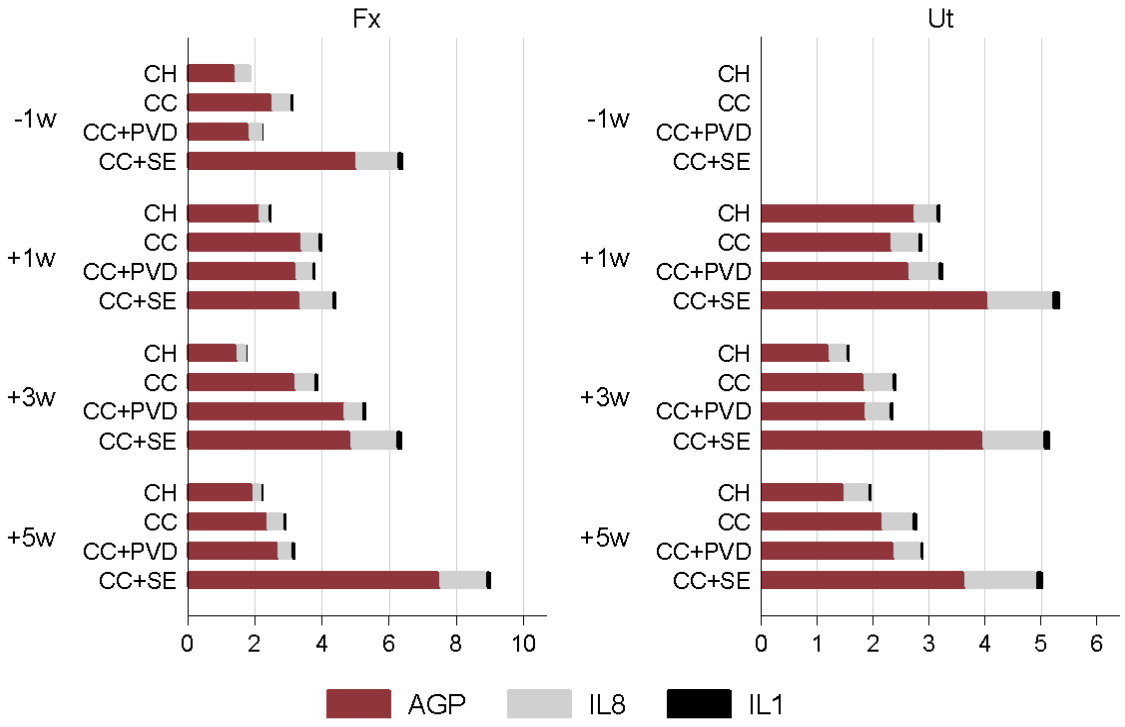
Time-course variations (weeks -1w +1w +3w +5w) of Interleukin 1α (pg/ml) and Interleukin 8 (pg/ml) concentrations (mean ± SEM). Mann-Whitney Test (clinical cervicitis cows ≠ healthy cows at $P<0.05$) indicate a significant difference at $p\leq0.05$ (*) and $p\leq0.01$ (**).

Figure 4. Time-course variations (mean ± SEM) in pre and post-partum concentrations of AGP in the vaginal fornix and uterine mucus of cows (n=26) defined as clinical cervicitis cows (n=19) and healthy cows (n=7) from 3 dairy herds in Quebec in 2016.



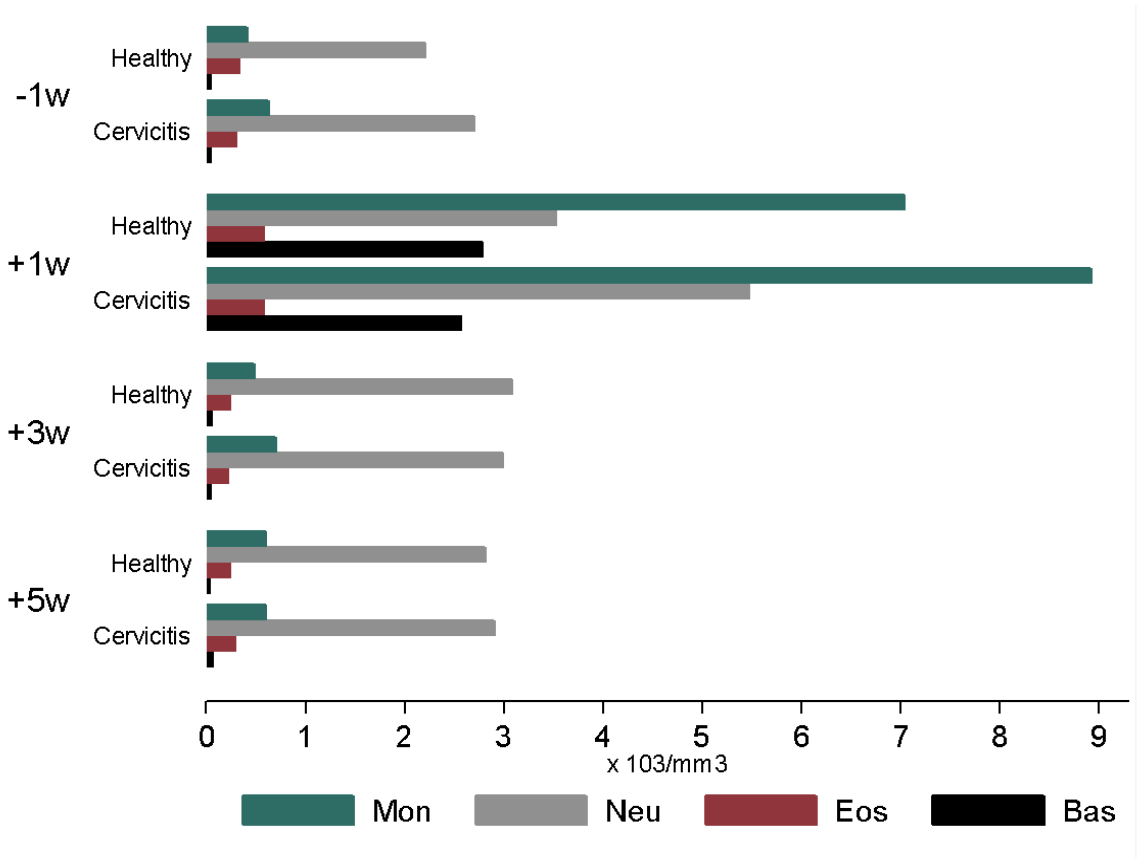
Time-course variations (weeks -1w +1w +3w +5w) of α1-acid glycoprotein (pg/ml) concentrations (mean ± SEM). Mann-Whitney Test (clinical cervicitis cows ≠ healthy cows at $P<0.05$) indicate a significant difference at $p \leq 0.05$ (*) and $p \leq 0.01$ (**).

Figure 5. Time-course variations (mean ± SEM) in pre and post-partum concentrations of AGP, IL1 and IL8 in the vaginal fornix and uterine mucus of cows (n=26) defined as healthy cows, clinical cervicitis cows and clinical cervicitis cows plus additional uterine disease conditions.



Time-course variations (weeks -1w +1w +3w +5w) of concentrations (mean ± SEM) of IL1 (ng/ml), IL8 (ng/ml), and AGP (pg/ml) in vaginal fornix (Fx) and uterine mucus (Ut) of healthy cows (CH), clinical cervicitis cows (CC), cervicitis + purulent vaginal discharge (CC+PVD) and, cervicitis + subclinical endometritis (CC+SE).

Figure 6. Time-course variations in pre and post-partum white blood cell count of cows (n=26) defined as clinical cervicitis cows (n=19) and healthy cows (n=7) from 3 dairy herds in Quebec in 2016.



Time-course variations (weeks -1w +1w +3w +5w) of blood cell count (x10^3 mm^3) for monocytes (Mo), Neutrophils (Neu), Eosinophils (Eos), Basophiles (Bas).

CHAPTER 3: ARTICLE 2

Diagnosis of clinical cervicitis and vaginitis in dairy cows in relation to different postpartum uterine disorders

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1. Abstract

Postpartum uterine diseases have a high prevalence in dairy cows and their impact on subsequent reproductive performance is significant. The resulting infertility is directly linked to changes in the uterine environment and ovarian function. However, there is poor agreement between clinical findings and the results of diagnostic tests, which may be explained at least in part by the presence of inflammation and infection at sites other than the endometrium, such as the cervix uteri and the vagina. We hypothesize that clinical cervicitis and vaginitis are components of reproductive tract inflammatory disorders in postpartum dairy cows. The goal of this study was to diagnose clinical cervicitis and vaginitis in dairy cows and evaluate their association with other postpartum uterine diseases. A total of 61 dairy cows in the postpartum period were enrolled in this nested case-control design study. Calving history, periparturient disease occurrence at one week (1w), three weeks (3w) and five weeks (5w) postpartum were recorded. The co-occurrence of the different disease conditions was assessed using Kappa coefficients, and associations between clinical cervicitis and the other postpartum diseases were assessed using contingency tables and chi-square tests. Clinical cervicitis was diagnosed in 36.7% (22/61), 40.1% (25/61) and 31.1% (19/61) of the cows at 1w, 3w and 5w postpartum, respectively. About 64% (20/32) of cows with clinical endometritis at 5w postpartum also had clinical cervicitis ($P \leq 0.05$), and 30% (3/10) of cows with cytological vaginitis at 3w postpartum had clinical cervicitis ($P > 0.05$). The prevalence of clinical and cytological vaginitis was 0% (0/61) at 5w postpartum. In terms of reproductive efficiency, average open days was 110 for healthy cows, 117 for cows with clinical cervicitis ($P < 0.001$), 145 for cows with clinical cervicitis and clinical endometritis ($P < 0.005$), and 199 for cows with clinical cervicitis and cytological endometritis ($P < 0.001$).The high prevalence of clinical cervicitis with its association with clinical endometritis and longer average open days suggests that it would be advantageous to conduct a visual vaginal examination at 5 weeks as part of a complete postpartum genital examination. Larger observational studies need to be done to confirm this finding. Given its low prevalence, it is less likely that vaginitis plays an important role in postpartum uterine diseases.

Key words: Uterine diseases, neutrophils, uterine inflammation and puerperal period

2. Introduction

Postparturient cows sustain significant damage to the endometrium along with bacterial contamination of the uterus. This triggers an active inflammatory process that clears cellular debris and bacteria, as well as repairs the endometrium, as part of normal uterine involution. In healthy cows, uterine inflammation eventually subsides and bacteria are eliminated by 4 to 6 weeks postpartum [1]. In the scenario where pathogenic bacteria persist in the uterus, the genital tract inflammation is prolonged and plays a significant role in the pathogenesis of postpartum uterine diseases [2,3]. In dairy cows, the occurrence of postpartum uterine diseases (PUDs) is high: up to 40% [4]. PUDs include clinical conditions like retained placenta, metritis, clinical endometritis and pyometra, as well as non-clinically evident conditions like subclinical endometritis. All of these conditions have a negative effect on fertility, causing a decrease in pregnancy rates and an increase in the number of services per pregnancy and days open [5]. The infertility is directly linked to changes in the uterine environment and ovarian function. The uterine inflammatory response and the presence of bacterial toxins like lipopolysaccharides (*Escherichia coli*) and pyolysin (*Trueperella pyogenes*) have a negative effect on ovarian function, resulting in the establishment of anovulatory conditions and postpartum anestrus [2,6]. Of course, the distinction between physiological and pathological uterine involution depends on the severity of the disorder, postpartum stage, the duration of the inflammatory process and, most importantly, whether it impairs cow fertility at the end of the voluntary waiting period.

Diagnosing PUDs is complicated by a lack of clarity in case definition, the use of different gold standard tests, the reduced sensitivity and specificity of diagnostic tests, and simply the difficulty inherent in exploring the bovine reproductive tract [7,8]. Practically speaking, several of the techniques used for diagnostic purposes are not suitable for widespread use in clinical practice because they are not rapid, easy to use or cost effective. Sometimes there is poor agreement between clinical findings and the results of the diagnostic test because of the presence of an inflammatory process at a site other than the endometrium, such as the cervix uteri or vagina.

The cervix is a self-contained organ of the reproductive tract that serves as an anatomical and functional barrier between the vagina and uterus. However, our understanding of cervical inflammation and bacterial contamination, and its subsequent influence on reproductive efficiency in dairy cows, is incomplete. Research has suggested that cervicitis is a distinct condition that has both a separate and additive effect on the reproductive performance of dairy

cows [9]. Cervicitis has a prevalence of 11 to 30% in dairy cows, and about 75% of cows with cervicitis also exhibit clinical or subclinical endometritis [10]. In women of reproductive age, diagnosing cervicitis is difficult, partly because it is frequently asymptomatic and so remains undiagnosed [11,12]. The literature reports a prevalence of about 20 to 25% in women and that cervicitis is associated with an increased risk of pelvic inflammatory disease and adverse pregnancy outcomes [11].

The vagina is the female copulatory organ and extends from the caudal extent of the cervix to the border of the vestibule at the external urethral orifice. One important function of the vagina (like the cervix) is to serve as a line of defence against bacterial invasion, which it does by secreting fluids that inhibit the growth of undesirable bacteria. Very little information is available on vaginitis in postpartum dairy cows. The objective of the present study was to investigate whether cervicitis and vaginitis are potentially a significant problem in high-producing dairy cows postparturient. We examined the occurrence of these two disorders during the postpartum period and their associations with other PUDs.

3.Materials and methods

3.1. Study population and experimental design. This prospective observational longitudinal cohort study was carried out in postpartum dairy cows (n=61) from three convenient commercial dairy herds in Saint-Hyacinthe, Quebec (Canada). We used a nested case-control design and cows were enrolled systematically based on calving date. Cows were housed in a tie-stall barn and milked twice daily. The rolling herd average for milk production was 9000 kg. Cows were fed a total mixed ration of corn and hay silage to meet the nutrient requirements of dairy cows as recommended by the National Research Council (NRC, 2011). Farms were visited weekly by the same veterinarian, and all cows were vaccinated twice against *E. coli* before calving (2 ml IM, on D-40 and D-26 before parturition; J-VAC®, Boehringer Ingelheim, Burlington, Ontario, Canada), and once against IBR, BVD type 1 and 2, PI3 and BRSV after calving (2 ml IM, between D-10 and D-30 postpartum; Bovi-shield GOLD5® FPTM 5 L5, Zoetis, Kirkland, Quebec, Canada). In addition, all pregnant cows were injected with selenium 5.0 ml on D-60 before calving (MU-SE, Intervet, Merk, Kirkland, Quebec, Canada).

3.2. Blood and cytological sampling, and follow-up period. The follow-up took place after calving, from the first to the fifth week postpartum. Cows were examined three times: at one week postpartum (1w), 3 weeks postpartum (3w) and 5 weeks postpartum (5w) postpartum. At each examination, a complete clinical and reproductive examination was performed. A blood sample was also taken via coccygeal venipuncture for determination of complete blood cell counts, and of beta-hydroxybutyrate (BHB) concentration using a portable device (Freestyle Precision™ Blood Glucose and Ketone Monitoring System). The reproductive assessment comprised: 1) a vaginoscopy to assess cervical and vaginal appearance as well as the presence of purulent vaginal discharge; 2) a transrectal examination to determine ovarian status; and 3) an ultrasonographic examination to assess uterine horn symmetry and fluid.

To diagnose subclinical conditions, the cytobrush technique [8] was used to collect samples of mucosa from the vagina (in the fornix against the cervix; 1w, 3w and 5w postpartum), the uterus (1w, 3w, and 5w) and the cervix (5w). Briefly, cytobrush samples were collected using a modified Casou cannula. The double-protected Casou containing the cytobrush was inserted into the fornix of the vagina and the uterus. At each location, the cytobrush was exposed and rolled over the mucosa before being recovered in the double-protected Casou. A new brush and protective sheet were used for each sampling location. Once withdrawn from the reproductive tract, the cytobrush was rolled over a sterile slide, fixed and stained. Smears were examined under the microscope at 400x magnification. A total of 200 cells were counted and characterized [13]. The percentage of polymorphonuclear neutrophils (PMNs) was determined for each of the three sampling sites (uterus, cervix, vagina).

3.3. Uterine bacterial culture and identification. Uterine cytobrush samples for bacteriological examination, which were only collected at 3w after calving, were subject to aerobic and anaerobic culturing using standard bacteriology testing methods (API system, Biomérieux, Marcy Étoile, France). The bacteriological samples were stored in a culture tube on ice (Starplex Scientific Inc., Etobicoke, Ontario, Canada) and transported at room temperature to the Faculty of Veterinary Medicine's diagnostic laboratory within 3 hours. For microbiological analysis, the brushes were plated onto sheep blood agar (soy agar with 5% sheep blood; Becton, Dickinson and Co., Sparks, Maryland, USA) using a sterile disposable innoculating loop within six hours of arrival. Plates were incubated for 48 h at 35°C under aerobic conditions and then examined.

When growth was observed, colony type was identified based on morphology, pigmentation and hemolytic pattern. Very small beta-hemolytic, catalase-negative colonies consisting of Gram-positive coryneform rods were identified as *T. pyogenes*. Briefly, swabs were plated on Brucella agar containing neomycin (100 g/ml) and incubated anaerobically at 35°C for 5 days. When Gram-negative rods were observed, colonies were examined using the API 20 A gallery for identification of *F. necrophorum* and *P. melaninogenica*. For isolation of *Escherichia coli*, swabs were plated on blood agar and MacConkey agar (OXOID, Ottawa, Ontario, Canada) at 37°C at the OIE Reference Laboratory for *Escherichia coli* (EcL – Faculty of Veterinary Medicine, University of Montreal). For confirmation of *E. coli*, isolates were submitted to three biochemical tests: indole spot, Simon citrate, and motility.

3.4. Disease definitions, and selection of cases and controls. In total, the dairy cows were examined for seven PUDs, which were defined as follows. 1) Clinical metritis (MET): an abnormally enlarged uterus and purulent uterine discharge at 1 weeks postpartum (1w) [14]. 2) Clinical endometritis (CE): purulent vaginal discharge (PVD) of uterine origin at 3 weeks postpartum (3w) with normal-sized uterus and no fluid [15]. 3) Cytological endometritis (CYTOe): absence of PVD (3w and 5w) plus the presence of PMNs exceeding 5% on cytobrush examination, a normal-sized uterus and no fluid in the uterus [16]. 4) Clinical cervicitis (CC): Grade 2 cervical fold on vaginoscopy at 5w, where Grade 0 = normal cervical fold, Grade 1 = second cervical fold swollen without redness and protruding through the first cervical fold, and Grade 2 = second fold swollen with redness and protruding through the first cervical fold [10](Hartmann et al., 2016). 5) Cytological cervicitis (CYTOc): PMNs exceeding 5% on cytobrush sampling at 5w. 6) Cytological vaginitis (CYTOv): PMNs exceeding 5% on cytobrush sampling of the vaginal fornix at 5w without purulent discharge. 7) Clinical vaginitis: red and edematous mucosa on vaginoscopy without purulent discharge. Note that all of these postpartum reproductive tract diseases were not associated with systemic clinical signs.

3.5. Variable definitions The following variables were included in the analysis: a) herd variables: average parity and herd effect; b) disease variables: metritis, clinical endometritis, cytological endometritis, clinical and cytological cervicitis (5w postpartum), and clinical and cytological vaginitis (at 1w, 3w , and 5w); and c) clinical variables: body condition score; subclinical

ketosis; cervical, vaginal fornix and uterine PMN counts; grade of vaginal discharge; and grade of uterine asymmetry.

3.6. Statistical analysis. A descriptive analysis was performed using frequency tables to determine the percentage of each variable category in the study population. The percentage of cows affected by the different postpartum conditions (MET, CE, CYTOe, CC, CYTOc and CYTOv) were calculated by dividing the number of cows with the condition by the number of cows in the study. The co-occurrence of CYTOv and CYTOc, CYTOv and CYTOe, and CYTOc and CYTOe was assessed using Kappa coefficients. Associations between clinical cervicitis and the other postpartum diseases were assessed using contingency tables and chi-square tests. We used a logistic regression model to explore the association between risk factors and the occurrence of CC. We first conducted a univariate analysis for each independent variable (herd, clinical and disease variables), with clinical cervicitis or cytological vaginitis as the outcome variable (separate models for each outcome variable). Variables with a P-value <0.15 were considered for the multivariate analysis, which was conducted using backward selection with a P-value of 0.05. We examined confounding and interaction effects by looking at non-causal exposure-outcome associations [17]. The variables parity and body condition score were considered potential confounding variables. We explored the random herd effect in a mixed-effects logistic regression model. The Hosmer–Lemeshow test was used to determine the goodness of fit of the logistic regression models. All analyses were conducted using Stata® Statistical Software (Release 15, StataCorp LLC, College Station, Texas, USA).

3.7. Ethical Statement. This research was performed in compliance with the experimental practices and standards approved by the animal care committee of the University of Montreal (211-03), and all efforts were made to minimize animal suffering in compliance with Canadian Council of Animal Care Guidelines.

4. Results

The study population consisted of 85 cows, but an initial exclusion resulted in the removal of some cows due to culling (n = 5), the use of antimicrobials (n = 4), metabolic disease (n = 5) and missing data (n = 10). A total of 61 cows ended the follow-up period without symptoms related to systemic illness. Clinical cervicitis were diagnosed in the 36.7%, 40.1% and 31.1% of the cows at 1w, 3w and 5w postpartum, respectively. Many cases of cervicitis occurred concurrently with another postpartum condition. In total, 47% of cases of clinical cervicitis (CC) were associated with purulent vaginal discharge (PVD), and 21% with cytological endometritis (CYTOe). Only 32% of cases of CC did not co-occur with another clinical condition. Table 1 shows the association between cervicitis at 5w and other postpartum diseases at 1w, 3w and 5w. It shows that 60% (n=15) of cows with CC and 37.5% (n=12) of cows with clinical endometritis at 3w postpartum had clinical cervicitis at 5w postpartum. However, the association was not significant. Cows with clinical endometritis at 5w postpartum (64.2%, n=9) had clinical cervicitis (P = 0.002). CYTOv, CYTOc and CYTOe (3w and 5w) were not associated with CC at 5w (P > 0.05). Average open days were: 110 for healthy cows, 117 for cows with CC, 145 for cows with cervicitis plus purulent vaginal discharge, and 199 for cows with clinical cervicitis plus CYTOe. The data show low to very low levels of co-occurrence between clinical cervicitis and CYTOv (Kappa = 0.024), CYTOc (Kappa = 0.077) and CYTOe (Kappa = 0.132). The results were similar for the co-occurrence of CYTOe and CYTOv (Kappa = 0.041) and CYTOc (Kappa = 0.253). There was slight agreement between vaginal and cervical findings and the results of the cytological evaluation (Kappa = 0.211).

We found no significant herd effect when herd was used as a random effect in a mixed effects logistic regression model. The univariate logistic regression analysis showed that the variables parity, body condition score at 1w postpartum, diagnosis of subclinical ketosis (>1.2 mmol/l) at 1w postpartum, high uterine PMN count at 1w postpartum (> 5%), and positive culture for *T. pyogenes* (15/61) at 3 weeks postpartum were statistically associated with clinical cervicitis at 5w postpartum (P<0.15). No other bacteria were found to be present in a significant amount. The univariate analysis did not find any associations between the independent variables and vaginitis (P<0.15) and so vaginitis was not included as an outcome variable in the multivariate analysis.

Table 2 shows the results of the multivariate logistic regression model for CC. Using a backward elimination process in the final model, we found that body condition score at 1w postpartum was

a confounder and was forced in the model. In the model, subclinical ketosis at 1w postpartum increased the odds of CC compared with cervicitis-free cows (OR: 5.2, 95% IC: 1.3 – 19.8), and a positive culture for *T. pyogenes* at 3w postpartum increased the odds of cervicitis compared with cows with a negative culture (OR: 4.2, 95% IC: 1.0 – 16.7).

5.Discussion

The bovine cervix is a thick-walled cylindrical structure composed of 3 or 4 annular folds that separate the uterus from the vagina. It forms a recess in the vagina known as the fornix [18]. Because of its strategic position, it is exposed to changes that occur in the vagina or uterus, such as infection/inflammation [19]. For that reason, fornix and cervical status can be a good indicator of PUDs. However, in this study we found low levels of co-occurrence between CC and CYTOv and CYTOe, and between CYTOe and CYTOv, which indicates a significant compartmentalization of the reproductive genital tract in cows. Based on the assumption that cervical involution is slower in the cervix than it is in the rest of the genital tract, cervical status at 5w may be an important element in attempts to develop more sensitive, specific and easier-to-use diagnostic tools for postpartum uterine diseases like CC and CYTOe. The weak associations between purulent vaginal discharge (resulting in colonization by putative bacteria), the presence of endometrial inflammation (as measured by PMNs in the uterus), and the absence of visible inflammation of the vaginal mucosa (clinical vaginitis) in postpartum cows (Tyson and Lefebvre, unpublished data), coupled with the strong compartmentalization of the genital tract, all point to the need for a better understanding of genital tract involution.

This study examines cervicitis and vaginitis in postpartum dairy cows as diagnosed by visual and cytological examination, respectively, in established cases of CE and CYTOe in the absence of exclusion criteria. The occurrence of CC in high-producing dairy cows at 5w was 31.2% (35-39 DIM), which is similar to that found in a previous study (26.1% 42-50 DIM; [10]). Similar to our findings for CE and CYTOe in the present study, as well as the results of other studies [20,13], we found a decrease of about 22% in the percentage of cows with CC between 3w and 5w (Table 1). This drop is less than that observed for CE (56%).

Our study is the first to report changes in the percentage of cows diagnosed with CC over the postpartum period: 36.7% at 1w, 40.1% at 3w and 31.1% at 5w. Due to the phenomenon of spontaneous clinical cure during physiological uterine involution, the prevalence of postpartum

reproductive tract diseases is expected to decrease with number of days in milk. As cervical involution is slower than uterine involution [21], one would also expect the rate of spontaneous clinical recovery of the cervix to be lower than that of the uterine horn in postpartum cows. The prevalence of CYTOc at 5w in the present study was 6.6%, which is much lower than the 42% found in a previous study for a similar postpartum period (less than 35 DIM; [9]). In the latter study, the high prevalence of CYTOc may have been associated with undiagnosed CE given that no assessment of vaginal purulent discharge was performed. This could also explain the presence of bacteria on their endometrial cytology samples. Like that study, we used a threshold of 5% PMNs to diagnose CYTOc. Note that we performed the cervical cytology only at 5w.

Clinical cervicitis occurs in conjunction with various PUDs (Figure 1), and our final model shows that cases of CC increased when the cow had a positive culture for *T. pyogenes* (OR=3.8 $P = 0.05$). There was concomitant CE and CYTOe in 47% and 21% of CC cases, respectively. In a previous study, approximately 12% of cows with CC between 42 and 50 days postpartum also had cytological endometritis [10]. In that study, cows with CE and abnormal uterine content were excluded. In another study where no exclusion criteria were applied, 75% of cows with CYTOe also had CYTOc, and approximately half of all cows with CE had CYTOc [9].

Numerous risk factors have been documented for postpartum uterine diseases like MET, CE and CYTOe. However, because studies often have limited statistical power, as is true of this study, it is difficult to rank all possible risk factors. In the case of CE, dystocia, twin calves, stillbirth, male calves, retained placenta and puerperal metritis have been reported as important risk factors [22]. Taking into account independent variables like herd, clinical variables and disease variables, CC at 5w was significantly associated with CE 5w, which implicates the involution process. If CC is associated with trauma during calving, the condition should be visible earlier in the postpartum period (e.g., at 3w). In addition, we found that PMNs of more than 5% in the uterus at 1w was associated with CC at 5w ($P < 0.001$). A threshold of 18% may have been more appropriate at 1w and could have changed the results. In the same way, the presence of *T. pyogenes* at 3w was associated with CC ($P < 0.001$). The presence of PMNs of more than 5% in the vagina at 3w was not associated with CC at 5w ($P > 0.05$).

In the present study, cows with CC and CYTOe accumulated 89 additional days open compared to cows with only CC. The effect of CC on reproductive efficiency is amplified when associated with other postpartum uterine diseases ($P < 0.001$), thus supporting previous reports for different

PUDs [23]. This translates into a significant economic loss for individual dairy farms and for the industry as a whole. Cows affected by CE were 1.7 times more likely to be culled compared to cows without endometritis [24]. Cows with postpartum clinical metritis exhibited reduced conception rates, and time to first insemination was extended by 7.2 days, ultimately leading to subfertility [25](LeBlanc, 2008). In addition, postpartum dairy cows with more than one disease have poorer reproductive performance. Dubuc et al. (2011) showed that cows with CE and CYTOe have worst reproductive performance than those with only one pathological condition. In the same way, CC reduces reproductive performance [10], and its negative effect is amplified when the disease is combined with other uterine diseases.

It is believed that postpartum uterine infection/inflammation is caused by bacteria ascending from the vagina or travelling through the vagina from the outside environment when the cervix opens during parturition [26], and that this state continues until the uterus returns to normal functioning. However, this concept is not necessarily associated with infection of the vagina, as shown by the very low prevalence of CYTOv (3%) and the absence of clinical vaginitis in the cows we studied. The vaginal microbiota in dairy cows have been shown to harbour the main uterine pathogens [27]. In addition, it has been hypothesized that the vagina is responsible for a portion of the purulent vaginal discharge observed in postpartum endometritis in dairy cows. We found no clear visual evidence of this in the present study.

6. Conclusion

The prevalence of CC and CYTOc at 5 weeks postpartum in high-producing dairy cows was 31.1% and 6.6%, respectively. CC occurred concomitantly with other common uterine conditions like endometritis and metritis, with only 32% of cows exhibiting CC alone. The high prevalence of clinical cervicitis with its association with clinical endometritis and longer average open days suggests that it would be advantageous to conduct a visual vaginal examination at 5 weeks as part of a complete postpartum genital examination. The absence of clinical vaginitis and the low prevalence of CYTOv in the cows we studied suggests that these conditions do not play an important direct role in PUDs in high-producing dairy cows. In the future, a better understanding of the interaction between CC and the other PUDs will be possible with larger studies.

7. Conflicts of interest

The authors declare that they have no conflicts of interest that would prejudice their impartiality in conducting this research or in publishing the results.

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Table 1. Contingency table and chi-square test results: clinical cervicitis diagnosed at 5 weeks postpartum in relation to postpartum uterine disorders diagnosed at 1, 3 and 5 weeks postpartum in cows (n = 61) from three commercial dairy herds in Quebec (Canada).

Weeks postpartum	Disease		Clinical cervicitis (5w PP)	
			No	Yes
1w PP	Metritis	No	26 (72.2%)	10 (27.8%)
		Yes	15 (62.5%)	9 (37.5%)
	Grade 2 cervix	No	25 (65.8%)	13 (34.2%)
		Yes	16 (72.7%)	6 (27.3%)
3w PP	Clinical endometritis	No	22 (75.9%)	7 (24.1%)
		Yes	20 (62.5%)	12 (37.5%)
	Grade 2 cervix	No	32 (88.9%)	4 (11.1%)
		Yes	10 (40%)	15 (60%)
	CYTOv	No	35 (68.6%)	16 (31.4%)
		Yes	7 (70%)	3 (30%)
	CYTOe	No	21 (72.4%)	8 (27.6%)
		Yes	21 (65.6%)	11 (31.4%)
5w PP	Clinical endometritis*	No	37 (78.7%)	10 (21.3%)
		Yes	5 (68.8%)	9 (64.2%)
	CYTOc	No	28 (73.7%)	10 (26.3%)
		Yes	14 (60.9%)	9 (35.1%)
	CYTOe	No	39 (68.4%)	18 (31.6%)
		Yes	3 (75%)	1 (25%)

PP = Postpartum CYTOc = Cytological cervicitis.

CYTOv = Cytological vaginitis CYTOe = Cytological endometritis

Table 2. Results for multivariate logistic regression model of clinical findings associated with clinical cervicitis to 35 days in milk in cows (n = 85) from three commercial dairy herds in Quebec (Canada).

Disease	Variable	OR	Std. Err.	P	95% CI
Clinical cervicitis	Intercept	0.18	0.03	<0.001	(0.12 – 0.28)
	Body Condition Score 1w				
	< 3.0 / 5.0	Ref.	-	-	-
	3.0 – 3.25 / 5.0	0.51	0.46	0.468	(0.08 – 3.04)
	3.5 – 3.75 / 5.0	0.19	0.16	0.062	(0.03 – 1.08)
	≥ 4.0 / 5.0	0.08	0.12	0.079	(0.005 – 1.33)
	Subclinical ketosis 1w				
	No	Ref	-	-	-
	Yes	5.22	3.55	0.015	(1.37 – 19.85)
	Trueperella pyogenes 3w				
	Negative culture	Ref	-	-	-
	Positive culture	4.25	2.96	0.038	(1.08 – 16.70)

DIM = Days in Milk

CHAPTER 4: ARTICLE 3

VAGINAL MICROBIOTA BEFORE CALVING AND THE UTERUS IN POSTPARTUM IN DAIRY COWS WITH ENDOMETRITIS

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1. Abstract

The loss of the cervical barriers after calving allows the ascent of bacteria into the uterus and rapid colonization resulting in a unique endometrial microbiota within 20 minutes after calving. During the normal uterine involution, the uterus clears bacteria in 6 weeks after calving but 40% of dairy cows are not able to eliminate the infection, which delays the uterine involution and increases the risk of postpartum endometritis. The culture-independent approaches based on the analysis of the 16S rRNA gene have shown that cows with endometritis have reduced richness and diversity in the reproductive tract compared to healthy animals. The goal of the present study was to characterize the vaginal bacterial microbiota before calving and the uterine microbiota after calving in cows that had or had not developed endometritis. We also aimed to compare the results of DNA sequencing with conventional culture methods in uterine samples collected at 21 days after calving. For this prospective study, a total of 61 multiparous cows were sampled with a cytobrush in the vagina 7 days before calving (D-7) and in the uterus 7 days (D+7), 21 days (D+21), and 35 days (D+35) after calving. Samples of healthy cows (n=11) and cows with endometritis (n=11) were selected for DNA extraction and 16S rRNA sequencing. Richness and diversity measures in the uterine microbiota were not significantly different between healthy and disease groups (Chao's $p = 0.17$, Simpson $p = 0.29$). The uterine microbiota composition was different between groups three weeks after calving but not at the other sampling time. Disease cows had a lesser relative abundance of *Firmicutes* and *Bacteroidetes* than healthy cows and disease cows had a greater relative abundance of *Actinobacteria* than healthy cows. *Trueperella spp*, *Peptoniphilus spp*., and *Helcococcus spp*. were numerically more abundant in cows with clinical endometritis.

2. Introduction

Approximately 95% of cows present some degree of uterine contamination with bacteria after calving, regardless of the presence of signs of disease (Foldi et al., 2006; Paisley et al., 1986; Sheldon and Dobson, 2004). The loss of anatomical barriers and the negative pressure created by the phenomenon of the uterine contraction and relaxation enhances the vacuum effect and the ascent of bacteria into the uterus, allowing for rapid colonization within minutes (Sheldon IM et al., 2020, Jeon SJ et al., 2015). There is also evidence that bacteria could reach the uterus through blood circulation (Jeon, S.J. et al.,2017). Gram-negative bacteria are dominant

during the first week after calving, being gradually replaced by Gram-positive organisms by day 15 postpartum when 78% of cows still have bacteria in the uterus (Wira et al., 2005). During normal uterine involution, the uterus clears bacteria in week 6 after calving (Sheldon et al., 2002), but 40% of dairy cows are not able to eliminate the infection (Sheldon et al., 2008). The persistence of pathogenic bacteria in the uterus is associated with delayed uterine involution, postpartum uterine diseases (PUD), and reduced reproductive efficiency (Bonnett et al., 1993; LeBlanc et al., 2002; Ribeiro et al., 2013).

Based on culture-dependent studies, the most common bacteria involved in PUD are *Trueperella pyogenes, Escherichia coli, Fusobacterium necrophorum, Prevotella melaninogenica* and *Bacteroides spp*. (Griffin et al., 1974; Studer and Morrow, 1978; Ruder et al., 1981; Olson et al., 1984; Bonnett et al., 1993; Dohmen et al., 1995; Huszenicza et al., 1999; Dohmen et al., 2000; Williams et al., 2005; Sheldon et al., 2009b).

Culture-independent approaches based on the analysis of the 16S rRNA gene (PCR, metagenomic DNA sequencing techniques) have shown that dairy cows with PUD present different microbial profiles, and reduced richness and diversity compared to healthy cows (Bicalho et al., 2017; Machado et al., 2012; Peng et al., 2013; Knudsen et al., 2015; Wagener et al., 2015). New pathogens have been identified by next-generation sequencing (NGS), such as species of *Bacteroides* and *Helcococcus*, while other bacteria normally associated with diseases have been found abundant in healthy postpartum cows, such as *E. coli*, *Mycoplasma* and *Ureaplasma* (Galväo et al., 2019). Currently, it is unclear whether changes in the normal composition of the uterine microbiota (dysbiosis) are associated with the occurrence of PUD (Sheldon et al., 2019), and what is the real etiological importance of the unknown bacteria.

In the face of the high prevalence of PUD in dairy cows, most studies have focused on the early postpartum period before the completion of the uterine involution and the reestablishment of a complete partitioning of the reproductive tract. Since the major source of uterine bacterial contamination after calving is most likely the vagina, joint studies of the vagina (prepartum period) and uterus (postpartum) microbiota can increase our understanding of this important interaction.

In this present study, the authors hypothesized that the vaginal microbiota in the prepartum and the uterine microbiota in postpartum would differ between healthy and diseased dairy cows. The objective was to use 16s rRNA to characterize bacterial

communities in the genital tract of healthy dairy cows during the peripartum period and make associations with the occurrence of clinical endometritis. We also aimed to compare the uterine bacteria recovered with conventional culture methods with the metagenomic profile of samples collected 21 days after calving.

3. Material and methods

3.1. Animals and Management

This research was performed in compliance with the experimental practices and standards approved by the animal care committee of the University of Montreal (211-03), and all efforts were made to minimize animal suffering in compliance with the Canadian Council of Animal Care Guidelines. A total of three different commercial dairy herds located in the same region of Quebec (Canada) were recruited and 61 multiparous cows were included in the study between June 2016 to February 2017. Reproductive and health data were compiled in a databank using health record management software (DSAHR, Saint-Hyacinthe, Québec, Canada J2S 3A5). The rolling herd average milk production was about 9000 kg. Cows from tied stall barns were milked twice daily and similarly fed a total mixed ration formulated mainly with corn and hay silage to meet the dietary requirements for a lactating dairy cow (NRC, 2011).

Farms were visited weekly by the same veterinarians and all cows were vaccinated twice against *E. coli* (J-VAC, Merial Inc., Athens, GA, USA) before calving (2 ml intramuscularly, D–40 and D–26 before parturition) and once against BVD types 1 and 2, IBR, PI-3, and BRSV (Bovi-Shield GOLD FPTM 5 L5, Zoetis, Parsippany, New Jersey 07054, USA) (2 ml intramuscularly, D15-40 after calving), and injected with Se (5.0 ml, D–60, MU-SE, Intervet Canada Corp., a subsidiary of Merck and Co. Inc, Kirkland, QC, Canada H9H 4M7).

3.2. Sampling and experimental design

This was an observational prospective cohort in which all cows (n=88) from three convenient commercial dairy herds in Saint-Hyacinthe, Quebec (Canada) were systematically and consecutively enrolled during the dry-off period and then examined four times between one week before to five weeks after calving.

Exams were carried out on cows one week before calving (-1w) and one week after (+1w), three weeks after (+3w), and five weeks after (+5w) calving (Figure 1). All four exams included an assessment of lameness, cyclicity, body condition, the collection of milk (somatic cell count) and blood samples (hematology profile), a transrectal (TE) and vaginal exam (VE: vaginal discharge (Tison et al., 2017) and visual cervical assessment (Hartmann et al., 2016)). Before calving, a TE was performed in order to confirm the pregnancy; it involved the assessment of placentomes, fetus viability and the uterine artery (fremitus). After calving, a TE was performed in conjunction with ultrasonography to determine the diameter of the cervix and uterine horns, to assess the presence of fluid in the uterus, and the ovarian structures (corpus luteum, dominant follicle, and follicular cyst). In addition, vaginal cytobrush was collected one week before calving (fornix) and endometrial cytobrush samples were collected at one, three and five weeks after calving. After cleansing the perineal area of the cow with water and soap, 70% isopropyl alcohol was sprayed and dried using paper towels. The visual cervical assessment was done before a sterile cytobrush rod (covered with a sterile sanitary sheath) was introduced into the vagina and guided through the cervix per rectum (Dario et al., 2022). Once the tip of the rod reached the uterine body, the sanitary sheath was pulled back, and the cytobrush was exposed from the rod and rotated against the dorsal wall of the uterine body with gentle pressure of the index finger through the rectum. The cytobrush was retracted into the rod and sanitary sheath before being removed from the vagina. Once outside the genital tract, the cytobrush was gently rolled onto a sterilized microscope slide. The cytobrush was then cut with sterile scissors, placed in a sterile 2 mL cryovial, and stored at − 80 °C within 5 min. For only the sampling at 21 days after calving that an additional cytobrush sample was collected and transferred in a tube containing anaerobic transport medium (BBL Culture Swab, BD, Mississauga, ON, Canada), placed on ice, and processed in the bacteriology laboratory within 2 h of collection. Cytology slides were stained using May-Grunwald-Giemsa stain, 300 cells (neutrophils and endometrial cells) were counted per slide in multiple fields, and the number of polymorphonuclear neutrophils (PMNs) was assessed. Cows were not enrolled in a systematic synchronization protocol, and reproductive data from the cows were collected for at least 300 days after calving.

3.3. Case definition

No antibiotic was used before and during the sampling period. Disease cows (n = 11) were identified based on two criteria at five weeks after calving: 1) cows with purulent discharge (PVD) of grade ≥2 (Tison et al., 2017), 2) and with > 5% PMNs on endometrial cytology (number of PMNs/number of total cells (Gilbert et al., 2016)). The control animals (n = 11) were characterized by the absence of all criteria (PVD < 2 and PMNs < 5%).

3.4. DNA extraction, 16S rRNA sequencing:

Cytobrushes were stored at −80°C after collection until DNA extraction. Frozen samples were dipped in 1 ml of phosphate-buffered saline (PBS) and DNA was extracted from the PBS suspension samples using the PowerSoil DNA isolation kit (MoBio, Carslbad, CA) according to the manufacturer's protocol. The V4 region of the 16S rRNA gene was amplified by PCR using the forward 515F (GTGCCAGCMGCCGCGGTAA) and reverse 806R (GGACTACHVGGGTWTCTAAT) (Walter, William et al., 2015) primers and the following conditions: denaturing for 3 min at 94°C, followed by 35 cycles of 45 seconds at 94°C, 60 seconds at 50°C, and 90 seconds in 72°C, with a final elongation step of 72°C for 10 minutes. The PCR products were sequenced in an Illumina MiSeq platform using reagents for 2x250 cycles at the Genome Quebec McGill Innovation Centre.

The Contig assembly was made from the original fastq files, excluding sequences longer than 300 bp, containing base pair ambiguities, and having polymers longer than 8 bp. Sequences were aligned using the SILVA 16S rRNA reference database and clustered at 97% similarity before outliers were removed (Quast, C., et al., 2013).

The software mothur was used to perform the bioinformatic analysis (Kozich JJ. Et al., 2013). The total number of OTUs per sample and the Simpson's index were used respectively as measures of richness (number of species present in a community) and diversity (Costa, Marcio et al., 2021) were clustered.

3.5. Uterine Bacterial Culture and Identification

Uterine cytobrush samples were collected 21 days after calving for routine bacterial culture (aerobic and anaerobic) using standard methods for bacteriological testing (API system,

bioMrieux, Marcy l'Etoile, France). Cytobrush samples were stored in a culture tube (BBL Culture Swab, BD, Mississauga, ON, Canada), placed on ice, and transported to the diagnostic laboratory of the Faculty of Veterinary Medicine within 2 hours. For microbiological analysis, the brushes were plated onto sheep blood agar with a sterile disposable plastic eye (soy agar with 5% sheep blood, Becton, Dickinson and Co., Sparks, MD, USA). Plates were incubated for 48 h at 35 °C under aerobic conditions and then examined. When growth was observed, the number of colonies was graded as rare (1 colony), few, +1 (between 2 to 5 colonies), +2 (> 5 colonies after dilution), and +3 (> 5 colonies after a second dilution), while colony types were identified based on morphology, pigmentation and hemolytic patterns.

Beta hemolytic, catalase-negative minuscule colonies demonstrating coliform Gram-positive rods were identified as *T. pyogenes* and were isolated by the standard procedure used at the diagnostic laboratory of the Faculty of Veterinary Medicine (PON-BAC-019). For other bacterial species, cytobrush samples were plated on Brucella agar containing neomycin (100 g/ml) and incubated anaerobically at 35 °C for 5 days.

When Gram-negative rods were observed, colonies were examined using the API 20 A gallery system for the identification of *F. necrophorum* and *P. melaninogenicus*. For isolation of *E. coli*, cytobrush samples were plated on blood agar and MacConkey agar (Oxoid Inc., Ottawa, ON, Canada) at 37 C. At the OIE Reference Laboratory for *Escherichia coli* (EcL; Faculty of Veterinary Medicine, University of Montreal), 5 typical lactose-positive *E. coli* colonies from the

MacConkey agar plates were streaked with blood agar for isolation and further identification Isolates were submitted to three biochemical tests (indole spot, Simon's citrate and motility) for confirmation of *E. coli*. Isolates of *E. coli* were stored in tryptic soy broth containing 30% glycerol at –80 °C (Becton, Dickinson and Co., Sparks, Maryland, USA).

3.6. Collection and Analysis of Blood

Blood samples were collected aseptically by coccygeal venipuncture from individual cows into collection tubes containing EDTA for one week before to five weeks after every two weeks. The collected blood was used for hematological studies. The hematological parameters including RBC count, hemoglobin concentration (Hb), packed cell volume (PCV), erythrocyte

sedimentation rate (ESR), corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), total leukocytic count (TLC) and differential leukocytic count (DLC) including, neutrophils, lymphocytes, monocytes, eosinophils and basophils were studied by standard methods of the veterinary hospital.

3.7. Statistical analysis

Downstream analyses were carried out in RStudio (version 3.6.3; R Core Team, Vienna, Austria) using the packages ggplot2, vegan, FactoMineR, dplyr, tidyvers, and phyloseq unless otherwise stated. Uterine and vaginal alpha diversity metrics were checked for normal distribution. Repeated measures analysis was performed on all alpha diversity indices, with fixed effects of the day, status, and their interaction with cows. Richness (total number of species) was estimated by the Chao1 index, and diversity was measured by the Simpson index (number of species present accounting for their evenness of distribution). For beta diversity (phylum and genera levels), principal coordinate analysis (Bray–Curtis) was used to assess differences in uterine bacterial composition by reproductive tract inflammatory disease condition and days after calving at sampling, and their outcomes were evaluated using non-parametric multivariate analysis of variance (PERMANOVA) accounting for repeated measures. Linear discriminant analysis effect size (LEfSe) was used to describe the statistical significance and biological relevance among healthy and diseased cows at the phylum and genera levels. The LEfSe was performed using the online Galaxy interface https://huttenhower.sph.harvard.edu/galaxy/)27with uterine health status as the main class, DIM at sampling as the subclass, and the cow as the subject, using an alpha of 0.05 and an effect size threshold of 3.5. For all statistical tests, the significance level was set as p-value < 0.05.

4. Results

4.1. Descriptive Statistics

The study population consisted of 88 cows, but an initial exclusion resulted in the removal of cows due to culling (n = 5), the use of antimicrobials (n = 4), metabolic diseases (n = 5) and missing data (n = 10). A total of 61 cows ended the follow-up period without symptoms related to systemic illness. A total of 88 samples were processed for DNA sequencing, including vaginal (prepartum) and uterine (postpartum) samples from 22 cows classified as healthy (n = 11; parity 1.7 ± 0.5 (mean ± SD) and BCS 3.8 ± 0.2) and diseased cows (n = 11; parity 1.9 ± 0.5

(mean ± SD) and BCS 3.7 ± 0.3). One healthy (9%) and five diseased (45%) cows had cervicitis grade #2 at 5 weeks after calving, no significant hematological changes were observed during the sampling period.

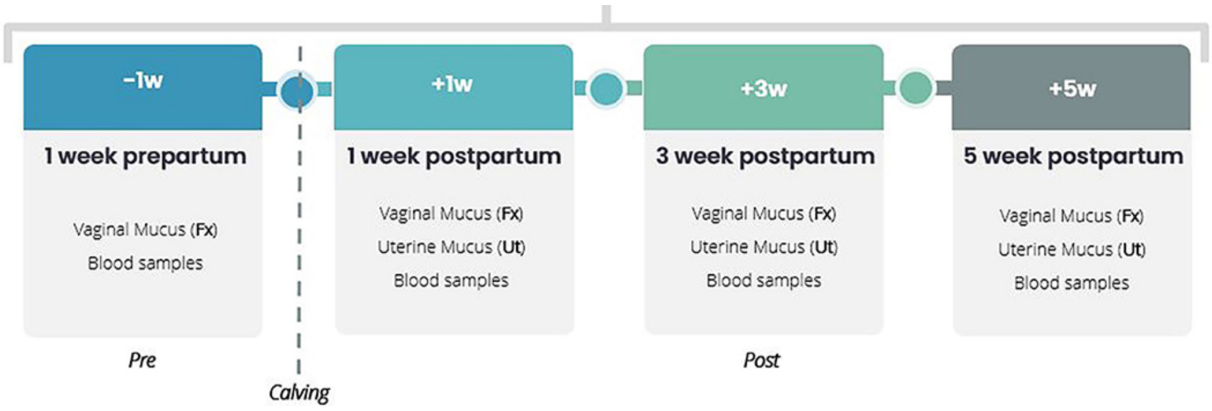

Figure 1: Timeline of sampling and genital examination of pre-and post-partum dairy cows (n = 22) from three dairy herds in Quebec

4.2. Microbial Community Analyses: α and β Diversity

A total of 9,480,588 paired-end reads were obtained of which, 7,820,890 passed all quality control after bioinformatic analysis. Figure 2 represents results from the alpha diversity of microbiota of the healthy and diseased cows between one week before calving to five weeks after calving. Alpha diversity of the uterine microbiota was not significantly different between healthy and disease groups. (Chao's p-value = 0.17, Invsimpson's p-value = 0.29). The principal coordinate analysis addressing the microbial composition revealed distinct microbiota profiles between healthy and disease groups at three weeks after calving (Figure 3). No significant difference (p = 0.349) was observed for the other sampling times. Effects of reproductive status and time of sampling were assessed by nonparametric multivariate analysis of variance (PERMANOVA) accounting for repeated measures. β diversity was not different at the phylotypes during the same period between healthy and diseased cows, but the phylum and

genera level of uterine microbiota in the healthy and diseased groups were clustered and separated at three weeks after calving (figure 3A and 3B respectively).

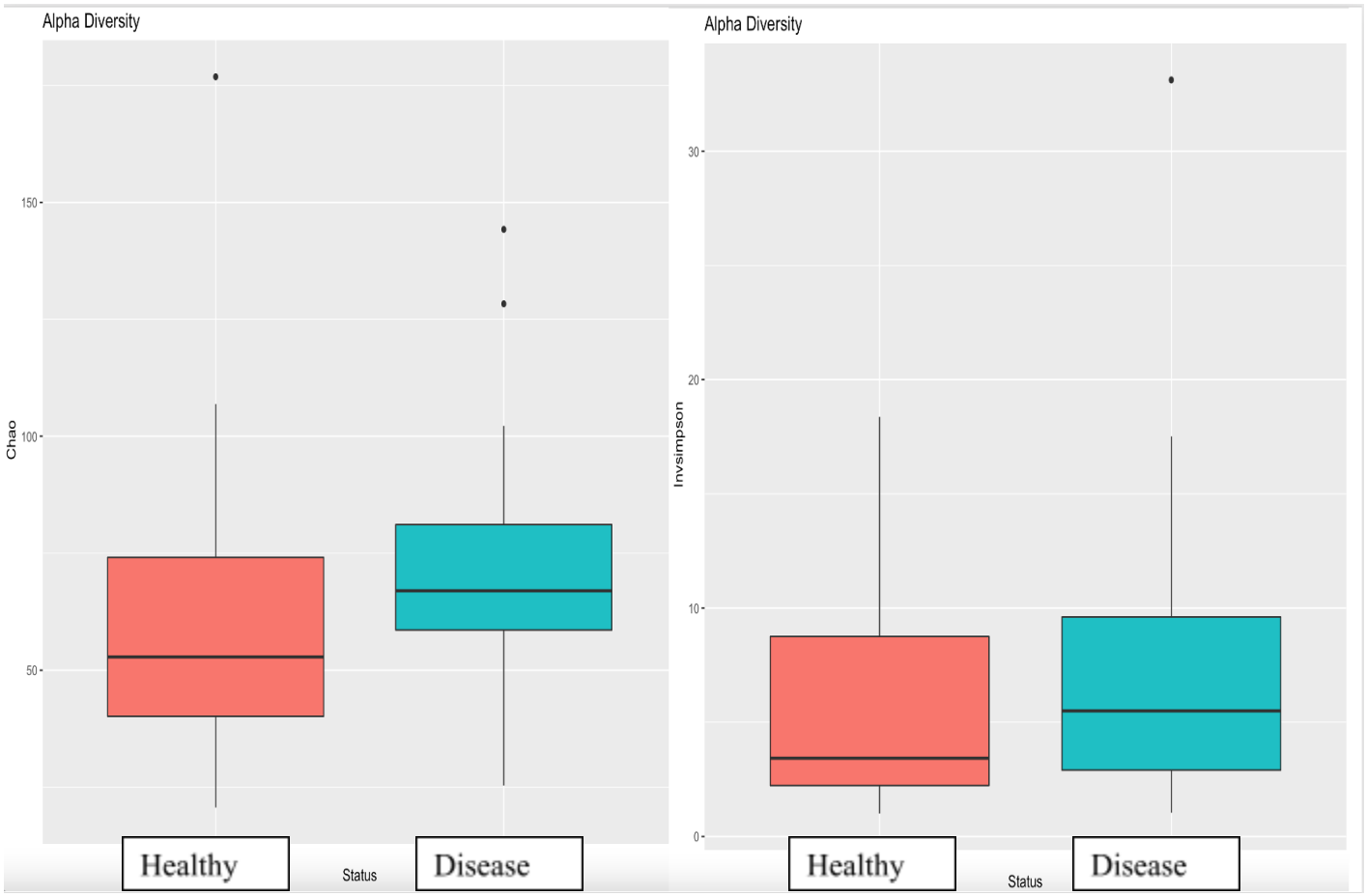

Figure 2. Box plot of the distribution of Alpha diversity indices. Chao richness estimator (A) and the Simpson diversity index (B) of the uterine microbiota postpartum (n-22). Healthy (red, n=11) and diseased (blue, n=11) cows were identified retrospectively on the vaginal discharge and the number of neutrophils on endometrial cytobrush. The bar inside the box marks the median. Alpha diversity for bacterial genera and phyla were similar between healthy and diseased cows. Repeated measures analysis was performed on all alpha diversity, with fixed effects of the day, status, and their interaction with cows. Bars represent SD ($p \leq 0.05$).

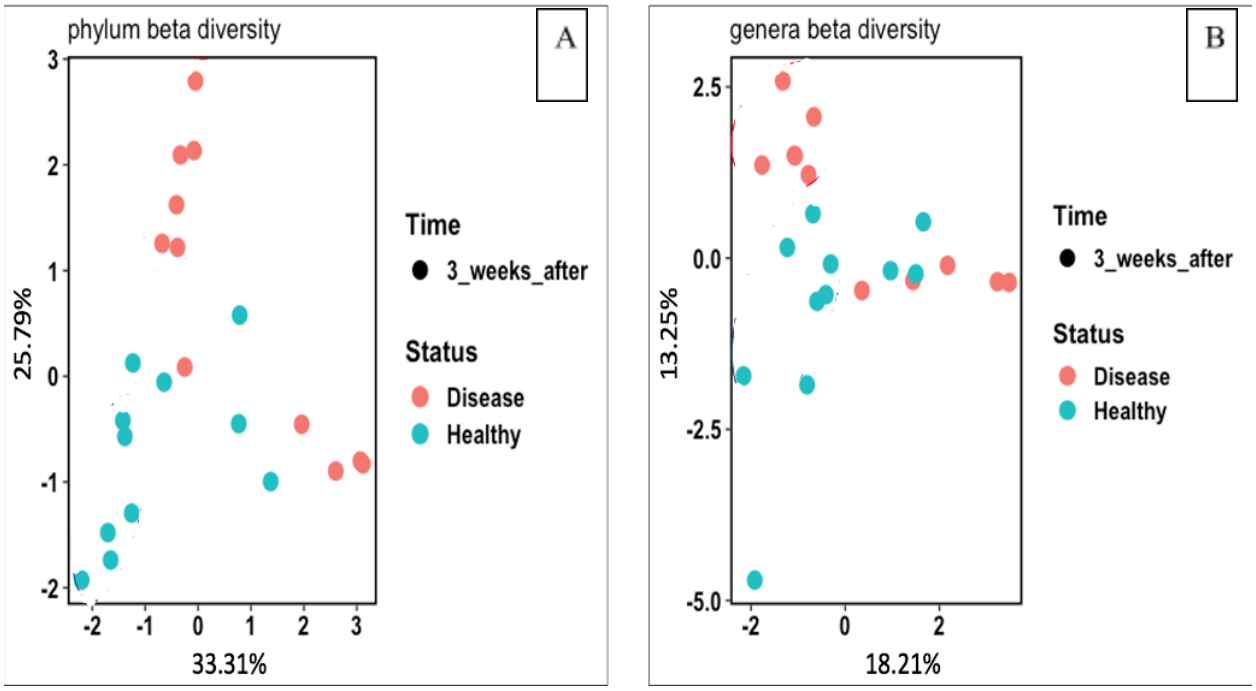

Figure 3. Beta diversity or bacteria phyla (A) and genera (B) level at three weeks after calving

4.3. Taxonomic Composition

The relative abundance of the most abundant genera is shown in Figure 4 and Figure 5 (phylum and genera levels, respectively). According to taxonomical annotation, the most common bacterial phyla in the uterine microbiota in both groups of cows were *Bacteroidetes, Firmicutes, Actinobacteria, Fusobacteria, Proteobacteria and Tenericutes* (Fig. 4). Endometritis cows had lesser ($P < 0.05$) relative abundance of *Firmicutes* and *Bacteroidetes* and greater abundances of *Actinobacteria* than healthy cows (Fig. 4). The number of unclassified bacteria was less than 2 percent in the whole time of sampling.

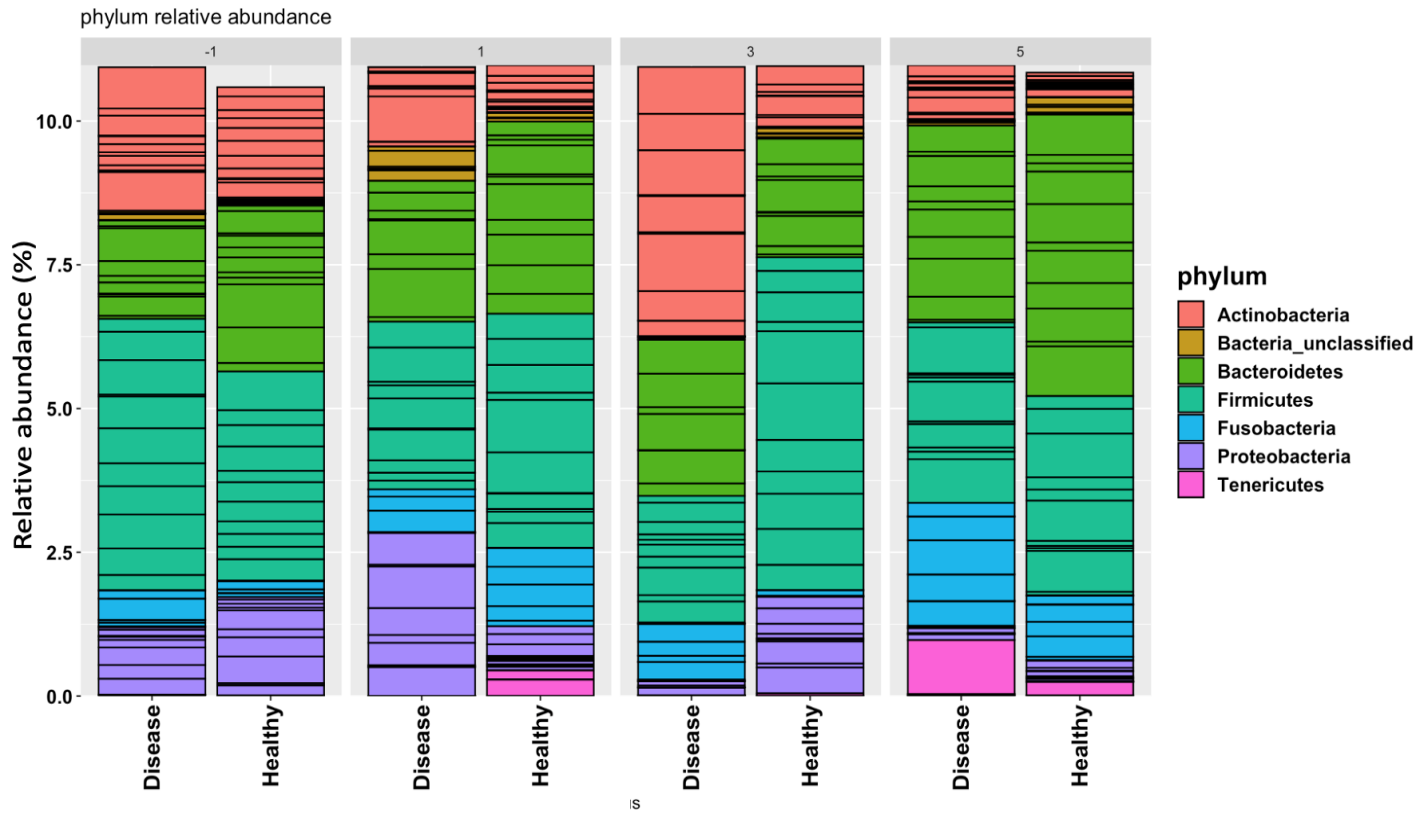

Figure 4. Colour-coded bar plot showing the relative abundance of predominant bacteria at the phylum levels. Relative abundance of the most dominant bacterial phyla in dairy cows (n = 22) in samples collected 1 week before calving, one week, three weeks and five weeks after calving. Only the 7 most common phyla are represented (98% of the reads). Based on their uterine health status in the fifth week postpartum, cows were retrospectively selected and classified as healthy and diseased groups.

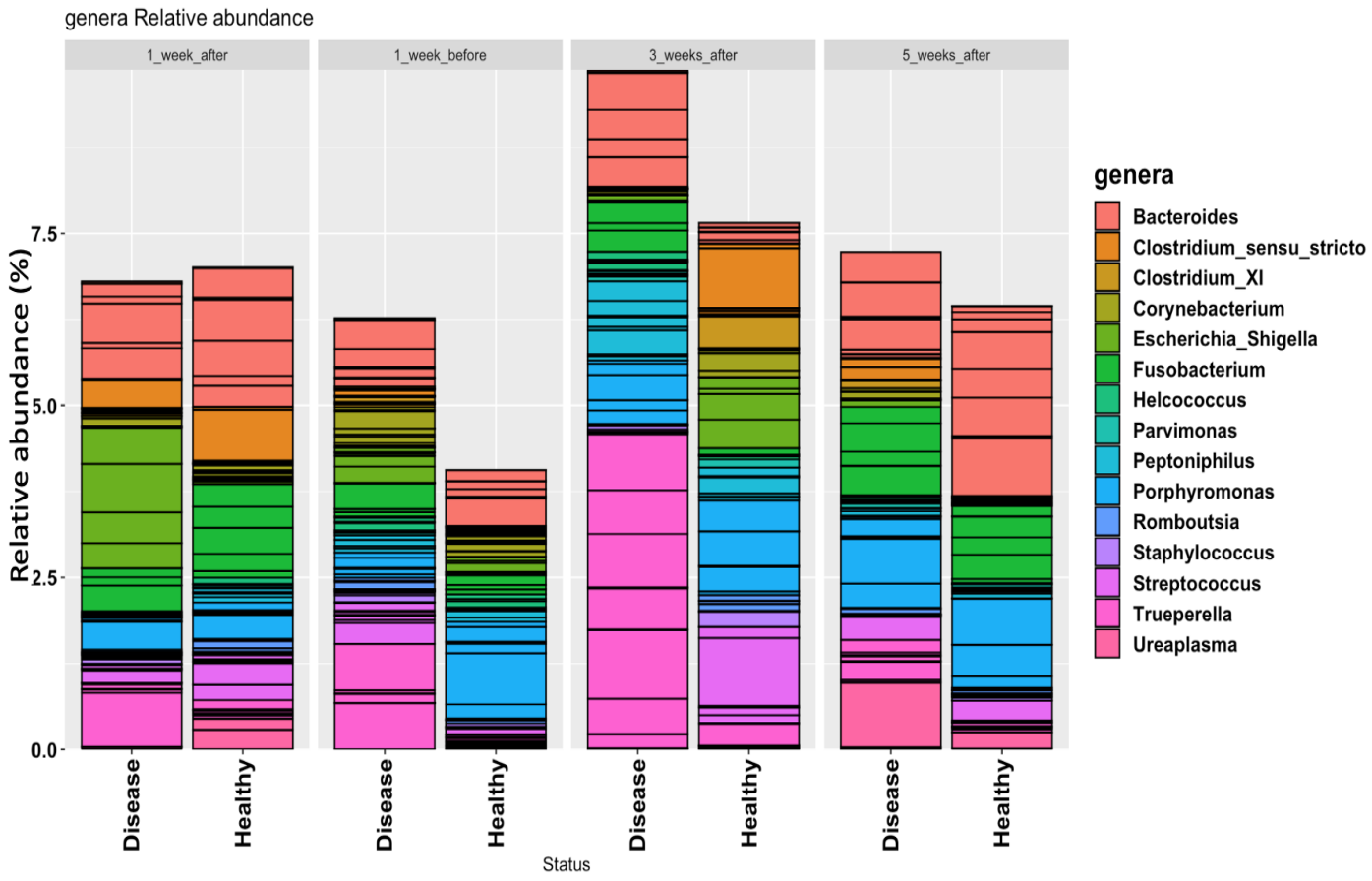

Figure 5. Relative abundance of predominant bacteria in the genus level. Samples were collected one week before and one, three and five weeks after calving. Only the 14 most common genera are represented. Based on their uterine health status in the fifth week postpartum, cows were retrospectively selected and classified as healthy and diseased groups.

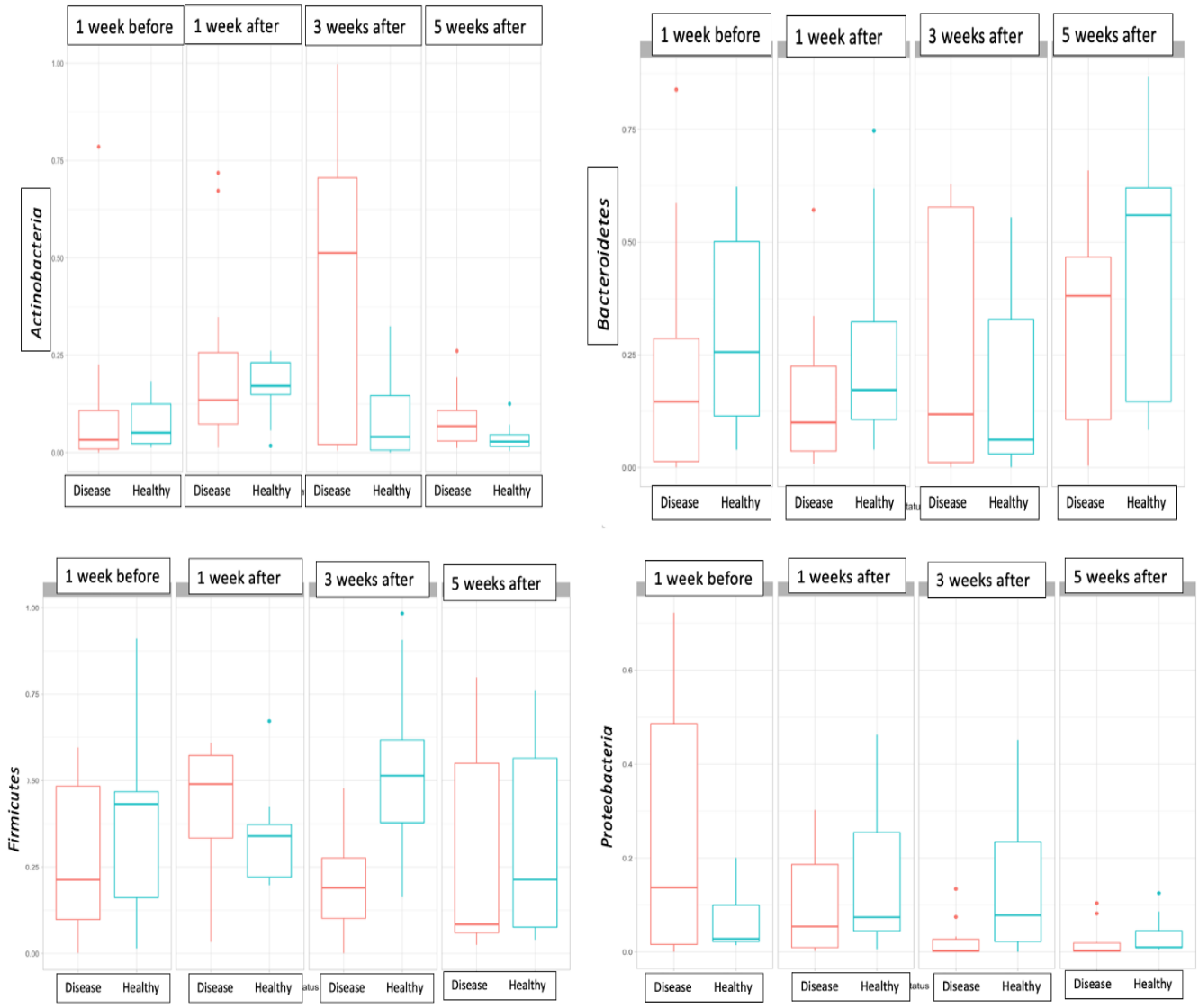

Figure 6: Relative abundance of the most dominant bacterial phyla in healthy and disease postpartum dairy cows (n = 22) in samples collected 1 week before, 1 week after, three weeks after and five weeks after calving. Cows were retrospectively selected based on their uterine health status in the fifth week after calving and classified as healthy (n = 11), and disease cows with clinical and subclinical endometritis (n = 11; > 50% purulent vaginal discharge and > 5% endometrial PMN). No differences in relative abundance in bacteria phyla were found between healthy and diseased cows (*P*= 0.58).

Taxonomic assignment showed that the dominant uterine bacterial genera were *Bacteroides, Trueperella, Porphyromonas, Fusobacterium, Escherichia, Streptococcus* and *Clostridium_sensu_stricto*. At the genus level, disease cows had a greater relative abundance of *Bacteroides, Trueperella, Fusobacterium* and *Peptoniphilus* than healthy cows at 3 weeks after calving ($P = 0.03$). Also, diseased cows had a greater relative abundance of *Trueperella* from one week before to 5 weeks after calving than healthy cows (Figs. 6 and 7).

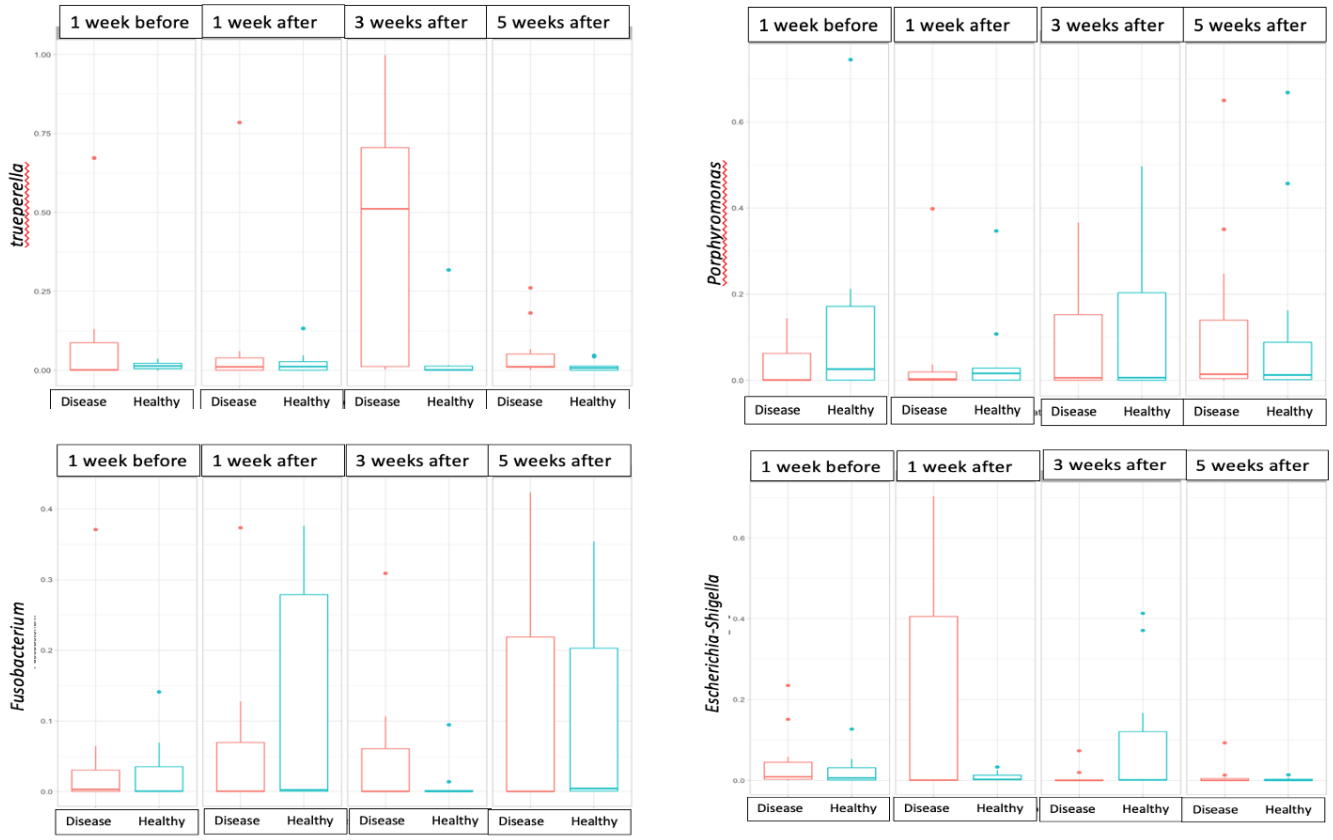

Figure 7: Relative abundance of the most dominant bacterial genera in healthy and disease postpartum dairy cows (n = 22) in samples collected 1 week before calving, 1 week after, three weeks and five weeks after calving. Cows were retrospectively selected based on their uterine health status in the fifth week postpartum and classified as healthy (n = 11), and disease cows with clinical and subclinical endometritis (n = 11; > 50% purulent vaginal discharge and > 5% endometrial PMN). No differences in relative abundance in bacteria genera were found between healthy and diseased cows ($P > 0.20$).

4.4. Linear discriminant analysis effect size

Diseases cows had discriminately greater abundance (LDA sores > 3.6) of *Helcococcus*, *Peptoniphilus* and *Trueperella* than healthy cows at three weeks after calving (Figure 8). At the genera level, no significant differences between healthy and diseased cows were found at one week before, one week after and five weeks after calving.

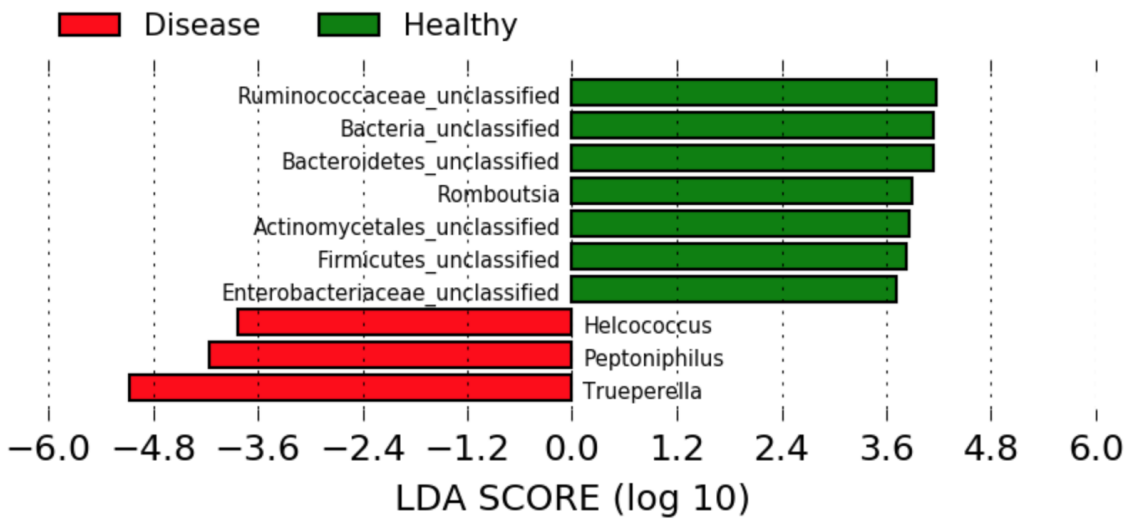

Figure 8:

Linear discriminant analysis (LDA) effect size plots showing the differences in uterine microbiota among cows diagnosed in the 5 weeks after calving as healthy (n = 11), and diseases cows (n = 11; > 50% purulent vaginal discharge and > 5% endometrial PMN). Histograms show the LDA effect size computed for features at the genera levels. Enriched features for healthy cows are indicated with positive LDA scores (green), and enriched features in diseased cows are indicated with negative LDA scores (red). Only features with $P > 0.05$ and an effect size cut-off of 3.5 are plotted.

4.5. Bacterial Culture

Gram-positive and gram-negative bacteria were cultured from the cytobrushes of healthy and diseased cows three weeks after calving. Most of the disease's cows had *T. pyogeneses* in large amounts (9 out of 11, more than 5 colonies) and small amounts of *E. coli* (3 out of 11, one colony), *Enterococcus* (3 out of 11, one colony), and *Streptococcus uberis* (2 out of 11, one colony). Amongst healthy cows, only 4 out of 11 had few colonies of *Staphylococcus spp*. (one colony), *Corynebacterium* (one colony), and *Bacillus spp*. (one colony).

5. DISCUSSION

During the transition period (3 weeks before to 3 weeks after calving), dairy cows experience a stressful period characterized by a negative energy balance, a dysfunctional innate immunity and the arrival of a large number of bacteria from the vagina, driving the proliferation of uterine bacteria and eventually resulting in the uterine dysbiosis and PUDs. However, multiple confounding postpartum conditions come into play and complicate understanding the etiopathology of these uterine diseases. The development of PUDs and the reproductive tract microbiota behaviors are not very well understood but they are most likely dynamically related. The objective of this study was to use DNA sequencing to investigate the bacterial dynamics in the vagina (prepartum) and in the uterus (postpartum) of cows that remained healthy with those that develop endometritis during the transition period.

Our results showed that the uterine microbiota was stable during the transition period in healthy and endometritis cows, as alpha and beta diversities were similar between groups from one week before to five weeks after calving. Our data confirm that known uterine pathogens are associated with clinical endometritis. Pascottini et al. (2020) showed similar results in phylum and genera levels in cows with clinical and subclinical endometritis compared to healthy cows in equivalent times (10, 21, and 35 days postpartum). Our results from next-generation sequencing of the 16S rRNA gene support findings of loss of bacterial diversity in endometritis cows. However, cows with endometritis had an increased relative abundance of *Bacteroidetes* and *Actinobacteria* at the phylum level, and *Bacteroides* and *Trueperella Spp* at the genus level with *Bacteroidetes* and

Fusobacteria representing the most prevalent phyla and *Truprella spp* was the most abundant genera. In the present study, *T. pyogenes* was the most abundant bacteria in nine of the eleven diseased cows in the culture-dependent technique. Other studies showed that *T. pyogenes* was observed in the uterus of the majority of the postpartum cows with endometritis using culture-independent methods (Machado et al., 2012) and culture-dependent methods (Sheldon et al., 2018). Additionally, intrauterine infusion of *T.pyogenes* with scarification of the endometrium in Holstein heifers has been shown to cause clinical endometritis (Piesanti et al., 2019). Many factors influence bacterial pathogenicity, including bacterial load, the presence of virulence factors, and positive interactions between species (Dadarwal et al., 2017). In fact, *T. pyogenes* has a synergic interaction with *F. necrophorum* and *p.melaninogenica* to support their growth and colonization of the uterine cavity around 3 weeks postpartum (Dadarwal et al., 2017).

In addition, *Truperella Spp, Peptoniphilus and Helcococcus* were discriminately greater (LDA sores > 3.5) in the disease group in comparison to the healthy group at three weeks after calving (fig. 8). As *Helcococus* and *Peptoniphilis* belong to the *Fusobacterium* co-occurrence group, they may act synergistically with *T. pyogenes* and facilitate the uterine dysbiosis observed in day 21 post-partum and increase the risk of clinical endometritis. This synergic interaction between *Trueperella* and the Gram-negative anaerobes like *Fusobacterium* has been suggested as a mechanism to overcome uterine defence culminating with endometritis (Bonnett et al., 1991, Prunner et al.,2014, Dadarwal et al., 2017 and Ballas et al., 2023) showed a strong co-occurrence between obligate anaerobes like *Peptoniphilus spp*. in postpartum and more specifically around 35 days after calving (Pascottini et al., 2020). In addition to the bacterial load and the presence of virulence factors, the sequence at which bacteria grow in the uterus may affect the risk of PUDs. However, other authors like Wang et al., (2018) showed no association between *T.pyogenes* and *F.necrophorum* in cows with clinical endometritis at five weeks after calving. Therefore, larger studies are needed.

E. coli emerges in the uterus within a few days after calving before the invasion of other bacteria and is more associated with metritis in early postpartum than endometritis. Using culture-dependent methods and PCR-based methods, *E. coli* were isolated between 1 and 3 days after calving (Sheldon et al., 2002, Bicalho et al., 2012). In the present study, healthy cows had a greater relative abundance of *Escherichia/Shigella* than diseased cows one week after calving.

Likewise, Jeon SJ et al., (2015) found rare *E. coli* in the uterus of dairy cows with metritis by metagenomic sequencing analysis and was more rather associated with uterine health. In other studies, *E. Coli* was found more frequently in animals with metritis cows than in healthy cows (Wang ML et al., 2018). The lack of knowledge on the interaction between *E. coli* and other bacteria like *T. pyogenes* and *F. necrophorum* and the diversity of *E. coli* strains (virulent factors) may explain the conflicting results. In the present study, virulent factors of E. coli were not assessed.

Based on the rationale that the ascending colonization of the uterus from the vagina is the most evident pathway for PUDs, the investigation of the vaginal microbiota before calving may improve our understanding of the sequential events leading to clinical endometritis. *Frimicutes, Bacteroidetes, Proteobacteria* and *Actinobacteria* (Clemens et al., 2017, Nesengani et al., 2017, Laguadia-Nascimento et al., 2015, Giannattasio-Ferraz et al., 2019, Quadros et al., 2020) were found in the vagina of cows during the postpartum period. In the present study, the dominant bacterial genera in the vagina were *Bacteroides, Trueperella, Porphyromonas, Fusobacterium, Escherichia/Shigella, Streptococcus and Clostridium_sensu_stricto.* Rodrigues et al., (2015) showed that the vaginal microbiota in dairy cows was associated with the occurrence of reproductive tract diseases. The vagina microbiota diversity varies between species with cows exhibiting greater diversity compare to the ewe and women (Swartz et al., 2014). Contrary to cows and ewes, *lactobacilli* comprise around 70% of the vaginal microbiota in women (Miller et al., 2016). However, an important point of the present results is that *Truperella Spp* and *Fusobacterium* were present in the vagina one week before calving sustaining the importance of the theses two species in the ascending colonization of the uterus after calving and their role in the etiology of endometritis in dairy cows in the postpartum period. These results are in line with the findings of Bicalho et al. (2017) who found *Fusobacteria* in the vagina of dairy cows 7 days before calving but the total bacterial load was less important than 7 days postpartum and was not associated with PUDs. Even though the present results are not statistically significant, the results of the present study would support the point that bacteria from the vagina would be transferred to the uterus after calving making the cow more susceptible to PUDs. A significant limitation of the present study is the small sample size and the short sampling time performed before calving. Nevertheless, the presence of *Truprella spp* in the vagina of cows before calving with its increased relative abundance in the uterus at 3 weeks after calving suggests that *Truprella spp*

could potentially be used as a predictor of postpartum clinical endometritis in dairy cows and could justify larger prospective studies to test the ability of this marker to predict endometritis in a broader population and eventually be used as an accurate and rapid test for prognosis of PUDs. Even though the genital microbiome may vary between species and even individuals of the same species (Lacroix G et al., 2020, Jeon SJ et al., 2015), with nutrition and farm management (Galvao KN et al., 2019), and estrous status (Tang G et al., 2008), the three farms of the present study were very close to each other and had very similar herd health management and feeding management. All animals were of the same breed (Holstein) and had similar body conditions (2.75-3.75) at the sampling time. In the case of the vagina, different breeds under different management can also have very similar vaginal microbiota (Giannattasio-Ferraz et al. 2019). Interestingly, bacteria that grew in culture were often present within the most abundant bacteria found in the16S rRNA gene sequencing. The culture-dependent results have identified several genera (*E. coli, Enterococcus, Streptococcus uberis, Staphylococcus spp., Corynebacterium and Bacillus spp.)* in a very small number (one colony). These bacteria are among the most common isolated intrauterine bacteria and have been described as potential or opportunistic pathogens (Wagener et al., 2014, 2015; Carneiro et al. 2016 and Ballas et al., 2023). Conversely, Williams et al. (2005) isolated *streptococci* and *staphylococci* in postpartum cows with a reduced risk of endometritis. Postpartum dairy cows with intrauterine *a-hemolytic streptococci* had improved reproductive performance (Gilbert and Santos. 2016). Even though in small amounts, these bacteria may interact with recognized pathogenic members of the uterine microbiota to favour a healthy or a diseased uterine cavity depending on the local environment.

6. Conclusion:

Alpha and β-diversity of the uterine microbiota were not significantly different between healthy and diseased cows. Disease cows had a lesser relative abundance of *Firmicutes* and *Bacteroidetes* than healthy cows and diseased cows had a greater relative abundance of *Actinobacteria* than healthy cows. In cows with clinical endometritis, *T. pyogenes*, *Peptoniphilus,* and *Helcococcus* were discriminately greater than in healthy cows. The presence of *T.pyogenes* and *E.coli* in the vagina of cows before calving with its increased relative abundance in the uterus at 3 weeks after calving suggests that the bacterial diversity before calving could potentially be used as a predictor of postpartum clinical endometritis in dairy cows

and justify larger prospective studies to test the ability of this marker to predict endometritis. Uterine bacterial composition was not different between healthy and diseased cows. Therefore, the coexistence and interactions of a number of uterine pathogens appear to be very important for the development of PUD. However, the interaction between bacteria and bacteria with the host and uterine environment requires further study, indicating the alternative hypothesis that regulation of uterine inflammation is worth pursuing prevention. and treatment of postpartum uterine disease.

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7. Conflicts of interest

The authors declare no conflicts of interest.

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Chapter 5.

GENERAL DISCUSSION AND CONCLUSION

5.1.1. Scientific Perspective Driving the Research Approach

Pre-and post-parturient cows sustain significant changes along the whole genital tract including the perineal area, the vagina, the cervix, and the uterus. Therefore, as a whole, each segment needsto return to its normal physiological status post calving. All these changes represent for each segment of the

genital tract active anatomic, metabolic, microbiotic, and inflammatory processes necessary to reinstate a normal status and function, and eventually a potential pregnancy. All these processes are collectively and commonly described as uterine involution. This term is therefore very limitative in itself. The genital tract reinitialization would be more representative of all the changes occurring in all the segments of the reproductive genital tract. The genital tract reinitialization is the preparation (all aspects) of the genital tract in which necessary changes are made for a full operational status for the establishment of a new pregnancy. With that perspective in mind, epidemiological, microbial, and immunological tools have been used to explore the changes of the different segments of the genital tract of dairy cows in pre-and postpartum. The first specific aim was to assess the importance of the clinical cervicitis and its association with other common PUDs in dairy cows and the role of vaginitis. The second aim was to measure the immune response of the endometrial and vaginal (fornix) cells when clinical endometritis was present in the postpartum period. Finally, the third aim was to assess the microbiota in the vagina before calving and the uterus in the first 5 weeks postpartum. Each thesis chapter has been prepared for submission to a peer-reviewed scientific journal.

5.1.2. Importance of the cervix and the vagina in the PUD complex

The cervix is a self-contained organ of the reproductive tract that serves as an anatomical and functional barrier between the vagina and uterus. However, our understanding of cervical inflammation and bacterial contamination, and its subsequent influence on reproductive efficiency in dairy cows, is incomplete. A very small number of studies have suggested that cervicitis is related to other uterine diseases on the reproductive performance of dairy cows (Deguillaume et al., 2012; Hartmann et al., 2016). Cervicitis has a prevalence of 11 to 30% in

dairy cows, and about 75% of cows with cervicitis also exhibit clinical or subclinical endometritis (Hartmann et al., 2016). Similarly, in women of reproductive age diagnosing cervicitis is difficult partly because it is frequently asymptomatic, and so remains undiagnosed (Li et al., 2019; Srinivasan et al., 2015). The literature reports a prevalence of about 20 to 25% in women and an association with an increased risk of pelvic inflammatory disease and adverse pregnancy outcomes (Srinivasan et al., 2015). Nothing is really known about the immune response and the microbiota of the uterine cervix. The vagina is the female copulatory organ and extends from the caudal extent of the cervix to the border of the vestibule at the external urethral orifice. One important function of the vagina (like the cervix) is to serve as a line of defence against bacterial invasion, which it does by secreting fluids that inhibit the growth of undesirable bacteria. Very little information is available on vaginitis in postpartum dairy cows. The compartmentation of the reproductive genital tract makes the study of each segment of the genital tract and their relationship very difficult. The lack of a complete genital examination in postpartum dairy cows could explain the limited understanding of the general process involved in PUD.

5.1.3. Importance of the Project for the Cattle Industry

In dairy cows, the occurrence of postpartum uterine diseases (PUDs) is high: up to 40%, PUDs include clinical conditions like retained placenta, metritis, clinical endometritis and pyometra, as well as non-clinically evident conditions like subclinical endometritis. All of these conditions have a negative effect on fertility, causing a decrease in pregnancy rates and an increase in the number of services per pregnancy and days open (Williams et al., 2007). The infertility is directly linked to changes in the uterine environment and ovarian function. The uterine inflammatory response and the presence of bacterial toxins like lipopolysaccharides (Escherichia coli) and pyolysin (*Trueperella pyogenes*) have a negative effect on ovarian function, resulting in the establishment of anovulatory conditions and postpartum anestrus (Herath et al., 2009; Williams, 2013). Of course, the distinction between physiological and pathological uterine involution depends on the severity of the disorder, the postpartum stage, the duration of the inflammatory process, and, most importantly, whether it impairs cow fertility at the end of the voluntary waiting period.

The importance of the symbiotic relationship between microbes and their host has prompted a deeper investigation into the novel bacterial communities throughout the body, such as the reproductive tract microbiome. However, few studies have explored the bacterial communities in the uterus and vagina of dairy cows and their relationship to postpartum diseases such as metritis, clinical endometritis and subclinical endometritis. The data and literature presented in this thesis provide valuable novel information and draw conclusions that can provide direction for future studies in bovine reproductive tract microbiota research. It is important to recognize that previous literature in reproductive microbiota research has been predominately studied in humans. Literature in bovine reproductive tract microbiota research is sparse but growing. Additionally, the methodology in bovine reproductive microbiota studies has not been fully refined to meet the rigorous contamination requirements typical of human studies. Current literature in bovine reproductive tract microbiota research acknowledges the presence of vaginal, and uterine microbiota. However, in humans, there is evidence that refutes the existence of all reproductive tract microbiotas except for the vaginal microbiota.

Hence, until more bovine studies with rigorous contamination checks are published, it is difficult to conclude the value of studying other reproductive tract microbiotas besides the vaginal microbiota. Little is known about the role of the vaginal microbiota in bovine reproductive performance, specifically regarding microbial composition changes due to treatments, pregnancy status, days of gestation, or endogenous hormones. Therefore, the research presented in this thesis had contamination checks in place and was purposefully designed to address gaps within the current literature.

The work described in this thesis provides an evaluation of the effects of the diversity of the microbiome and innate immune factors on infectious reproductive disease and fertility. The overall purpose of the study was to assess the importance of the cervicitis, to identify and characterize the microbiome before and after calving, and to assess the immune response (IL-1, IL-8 and AGP) in postpartum cows in commercial Canadian dairy herds.

Inflammation is present in the uterus of all cows after calving and this is part of normal uterine involution. Upon completion of uterine involution, the uterine inflammation is cleared and the uterus is ready to support the next pregnancy. Many cows fail to complete this physiological process efficiently and have delayed clearance of uterine inflammation. The primary factors

associated with delayed clearance of uterine inflammation were: uterine health-related (metritis).

5.1.4. Pathogens or not Pathogens

These findings suggest that *E. coli* is important for uterine disease in early lactation, while a subsequent *T. pyogenes* infection after the second week postpartum plays a role in subsequent uterine infection and disease. These findings are supported by others (Bicalho et al., 2012). The vast majority of the studies investigating the bacterial etiology of uterine diseases used traditional culture methods, but less than 1% of the microorganisms in an environment are cultured under laboratory condition. Although their importance is unquestionable, culture-based studies might have underestimated the microbial complexity of the intrauterine environment. The postpartum dairy cows uterine bacterial diversity was investigated using the metagenomic pyrosequencing of the 16S rRNA gene. With this technique, this limitation was surpassed, and the microbial population of the postpartum intrauterine environment of dairy cows was described. In general, the results highlight the importance of known pathogens already associated in previous studies with uterine health and reproductive performance. *Fusobacterium spp*. and *Trueperella spp*. were detected more frequently in cows affected by clinical endometritis than in healthy cows, and their relative abundance was also increased in cows diagnosed with clinical endometritis. Nevertheless, associations between uterine health and other pathogens, such as *Ureaplasma spp., Prevotella spp., Bacteroids spp.,* and *Helcococcus spp*, among others, were observed. Although this study reinforces the importance of traditional intrauterine pathogens, it also shows the complexity of the postpartum intrauterine environment and raises the questions: how many other microbes are playing a role in the pathogenesis of uterine diseases? And what is their functional profile? To add clarity to those questions, future research should exploit the microbial diversity differences between cows affected by post uterine diseases and healthy cows by using the Metatranscriptomics technique. Different from the metagenomics sequencing of the 16S rRNA gene, with this technique, we sequence random fragments of DNA from microbes; whole genome metagenomics provides an alternative, more global way of assessing biological functions in microbial communities, generating data regarding the abundance of genes related to, for instance, virulence factors, metabolism, and antibiotic resistance. This type of study would generate novel data regarding the importance of different virulence factors and metabolic pathways. Perhaps alternative treatments or preventive methods could be conceived with the use

of this information. Controversy still remains regarding the importance of specific bacteria, particularly E. coli and *T. pyogenes*, on the occurrence of bovine reproductive tract disease. A current lack of consensus amongst published authors indicates that no single bacterium may be a necessary cause of an inflammatory response in the reproductive tract. Although T. pyogenes has been consistently shown to be associated with the presence of reproductive tract disease and decreased reproductive performance in high-producing cows in confined systems (Sens and Heuwieser, 2013) some recent studies did not find a similar association (Bicalho et al. (2012) and Sens and Heuwieser (2013) found an association between *Fusobacterium necrophorum* and A-haemolytic streptococci with reduced reproductive performance. It has also been hypothesized that bacterial endotoxins in addition to the commonly researched lipopolysaccharides as mentioned previously, such as lipoteichoic acid (LTA; from Gram-positive bacteria), maybe more involved in reproductive tract disease than indicated in the current literature (LeBlanc, 2014). Hence, the mere presence of a specific bacterial species in the uterus may not be as significant as previously assumed. Sheldon (Sheldon et al., 2009) have postulated that bovine herpesvirus type 4 may have a synergistic effect on bacteria in the pathogenesis of reproductive tract disease. Moreover, pathogens that have not been a focus of previous studies (e.g. fungi) could also have a potential effect, although, similar to anaerobic bacteria, these pathogens may be difficult to isolate. It is evident that more research is required on the causality of the different pathogens on bovine reproductive tract diseases.

5.1.5. Immune response

Innate immunity plays an important role in keeping postpartum reproductive tract microbiota balanced; where the cervix acts as an anatomical barrier protecting the uterus from external pathogens producing cervical mucus. During postpartum, puerperal physiological modifications occurring in the reproductive tract, changes in the local microbiota and arisen of potential pathogens, and physical traumas associated with calving or obstetrical manipulations may trigger cervical inflammation. Regulation of immune system responses and the shift between an overall state of anti-inflammatory and pro-inflammatory environments has been previously determined. In the present study, we measured an increase of neutrophils in the reproductive tract (uterus and vagina), a major production of pro-inflammatory cytokines (IL-1) and chemokines (IL-8), and the recruitment of circulating neutrophils and monocytes meaning that postpartum cervicitis has

been reported in tissues like uteri and ovaries (Fournier et al., 2000; Singh et al., 2008a). The protein AGP possesses an immunomodulatory activity and it is believed to play an important role in the regulation of local inflammation by reducing the tissue damage caused by excessive activation of the complement. In our study, AGP concentrations increased during cervicitis, mainly at 3 weeks postpartum ($P < 0.05$) however, when cervicitis and subclinical endometritis were both present AGP concentration in the vagina was higher at 5 weeks postpartum ($P < 0.05$). The present results agree with previous work that demonstrated a higher level of plasma AGP in cows developing a uterine infection in comparison with cows without endometritis (Lecchi et al., 2013). AGP can modulate locally the uterine innate immune response in different cases such as normal uterine involution, cervicitis or endometritis without the presence of a systemic inflammatory process. In cows, AGP is contained in neutrophil granules and released when activated. The present results agree with the increase in expression of IL-8 enabling the influx of neutrophils in the cervical tissue that excretes the matrix metalloproteinase which contributes to the softening of the cervix (van Engelen et al., 2009; van Engelen et al., 2007). Moreover, parturition is associated with the local accumulation of IL8, which acts synergistically with PGE2 to attract polymorphonuclear neutrophils. Hence, activation of the placental leukocyte population releases proteolytic enzymes that degrade the caruncles, thus facilitating fetal membrane separation and advancing the progression of labour (Attupuram et al., 2016; van Engelen et al., 2007). This is an expected finding considering that tissue macrophages release IL1 and IL8 acting as a pro-inflammatory and chemo-attracting process coordinating the recruitment of neutrophils to the site of infection enhancing an inflammatory repairing response. The increase of blood monocytes at 1 week postpartum may result in more differentiation of monocyte in macrophages in the reproductive tissues for better clearance of pathogens and more effective control of inflammation (Sheldon et al., 2018; Singh et al., 2008b).

5.2. Limitations

Sources of bias may be present in this thesis. For example, the herds enrolled in studies of this thesis were selected on the basis of a willingness to participate; thus selection was non-random. However, even selected for convenience, they were commercially managed herds with similar feeding and reproductive management during the same seasons in the same area of Saint-Hyacinthe, Québec. This would reduce the variability in the results of the microbiota study. A second deficient point of the study is the number of animals per group. This was more

specifically, a serious concern on the epidemiological study looking at the relationship between the clinical cervicitis and the other PUDs reducing the power of the study and the reliability of the results. This is also true for the microbiota study. Although independent-culture techniques are very powerful, they still have their limitations: 1) bacterial viability cannot be assessed, 2) clone library sequencing has a low sequencing depth, 3) the taxonomic classification may change based on the 16 rRNA gene regions studied, 4) classification at the species level may not be reliable, and 5) studies with composite and cross-sectional sampling limit interpretation (Galväo et al., 2019).

For the immune response study, a better chemokine and cytokine profiling (ex. IL-17, IL-10) would have given a better assessment of the clinical cervicitis and endometritis

5.3. Topics for Further Research

Good reproductive performance of cows is vital for the dairy industry and novel data have been presented in this thesis that fills gaps existing in current knowledge of bovine reproductive tract disease. However, several areas that would benefit from further research became clear, some of which have been discussed in the limitations section. To determine the causality of the different bovine PUDs is far from reach but bacterial markers could potentially be used during the prepartum period to predict cows that will develop endometritis. Results of this study add to the current knowledge on the pathophysiology of endometritis in cows.

5.4. Conclusion

In conclusion, the prevalence of clinical cervicitis in postpartum dairy cows is as high as the one of endometritis with about 31% of the cows affected. On the other hand, cytological endometritis is not as important to a much lower prevalence of 6%. In about 70% of the cases, clinical cervicitis occurred with other uterine diseases like metritis and endometritis. This highlights the point of an existing association between PUDs and the need of a complete genital examination when a specific condition is studied. In addition, signs of inflammation or infection of the vaginal vault is rare and not involved with other PUDs. In the second study, the increase of IL1, IL8, and AGP in endometrial cells strengths the biological relationship between both conditions. As in other studies, the 3 weeks postpartum was found to be a critical point with the increase of all inflammatory markers and neutrophils in the uterus. Interestingly, even though no

significant difference was observed in the alpha and b diversity of the uterine microbiota, a differential clustering was observed between diseased and health animals for 3 weeks after calving. With culture-dependent or not, *T. pyogenes* and *F. necrophorum* are still major pathogens of the reproductive tract in postpartum dairy cows and their presence in the vagina before calving raises several questions and further studies as etiological agents of PUDs.

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