IMPACT OF NUTRITIONAL STRATEGIES ON IMMUNOMETABOLIC BIOMARKERS, REPRODUCTIVE TRACT PHYSIOLOGY AND HEALTH OF HOLSTEIN COWS

BY

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DISSERTATION

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ABSTRACT

High-producing dairy cows are faced with a dysfunctional immune system and an increased inflammatory state during the peripartal period. Furthermore, the increase in the energy demands associated with a decrease in dry matter intake (DMI) results in a negative protein and energy balance. Physiological and metabolic changes occur to support lactation while in a nutrient deficit. A difficult or improper adaptation to this challenging period is associated with increased risk for developing disorders, including reproductive tract inflammatory disorders. This could negatively impact cows' reproductive performance, further increasing the economic losses caused by poor management of transition period.

To address this challenge, we evaluated the effects of feeding rumen-protected Lys (RPL) through the transition period on the reproductive physiology and health of dairy cows. Methionine and Lys are often the most limiting amino acids in dairy cattle diets, and it was previously determined that methionine has an impact on the uterine inflammation and immunity (Experiment 3), and metabolism of dairy cows. However, whether lysine plays a similar role is unknown. Experiments 1 and 2 were companion studies and were conducted to determine the effect of feeding RPL (AjiPro-L Generation 3, Ajinomoto Heartland Inc., Chicago, IL) prepartum, postpartum, or both on follicular dynamics, uterine health, and mRNA gene expression of the endometrium, and mRNA and protein expression of placental samples of Holstein cows. Seventy-five multiparous Holstein cows were assigned to 1 of 2 dietary treatments with or without RPL in a randomized, complete block design. Prepartum (–28 d to calving), animals were fed a diet (68% of dietary DM from forage) with RPL (PRE-L; 0.54 % RPL of dietary DMI) or without RPL (PRE-C). After calving, half of the cows from each prepartum treatment group were assigned to a diet (56% forage) with RPL (PRE-L POST-L;

PRE-C POST-L; 0.40 % RPL of dietary dry matter intake) or without RPL (PRE-C POST-C; PRE-L POST-C) until 28 days in milk (DIM). For Experiment 1, postpartum uterine health was assessed through evaluation of vaginal discharge, percentage of uterine polymorphonuclear cells (PMN) at 15 and 28 DIM, and histology and mRNA expression of uterine tissue harvested at 28 DIM. Additionally, the first postpartum follicular growth cycle was monitored via transrectal ultrasonography. Feeding RPL pre- and postpartum decreased the number of PMN cells in the uterus, while feeding RPL prepartum downregulated the expression of transcripts involved in inflammation process in endometrial samples collected at 28 DIM. Additionally, feeding RPL postpartum upregulated the expression of mucin transcripts and fibroblast growth factor 10, and downregulated the expression of superoxide dismutase 1 in the endometrium at 28 DIM. Finally, supplementation of RPL did not change days to first ovulation. For Experiment 2, placentas were collected after natural delivery (6.87 ± 3.32 h). One placentome from each placental region (cranial, central, and caudal) was collected, combined, and flash-frozen in liquid N to evaluate the expression of transcripts and proteins related to placental and protein metabolism and inflammation. Feeding rumen-protected Lys (RPL) during the last month of gestation resulted in increased uteroplacental expression of genes involved in placental glucose uptake and metabolism, such as glucose transporter 3 (*GLUT3*) and phosphoenolpyruvate carboxykinase 1 (*PCK1*), and increased protein abundance of LDL receptor-related protein 1 (LRP1). Moreover, increased expression of the transcript and protein fibroblast growth factor 2 (FGF2), and increased expression pf placental growth factor (*PGF*) indicate enhanced placental metabolic activity.

Previous data had established that feeding rumen-protected Met (RPM) during the transition period improved postpartum vaginal discharge and uterine immunity and health. Thus,

Experiment 3 aimed to evaluate the effect of feeding RPM during the peripartal period and early lactation on mRNA gene expression profiles of uterine cytological smear and endometrial samples of Holstein cows. Treatments consisted of a supplementation with RPM [MET; n = 11; RPM at a rate of 0.08% of DM: Lys:Met = 2.8:1, (Smartamine® M Adisseo, Alpharetta, GA, USA)] and no supplementation (CON; n = 9; Lys:Met = 3.5:1). Cows fed RPM had decreased expression of transcripts involved in inflammatory processes in cytological samples and increased expression of genes involved in overall tissue metabolism in the endometrium. Thus, feeding RPM during the transition period and early lactation modulates the uterine metabolism and immune defense system, which could decrease the susceptibility to reproductive tract inflammatory diseases.

Finally, Experiment 4 aimed to determine the association of prepartum and postpartum DMI, lactation performance, and days to first ovulation with measurements of uterine health in the early postpartum. Uterine health, which is crucial for the reproductive success of postpartum dairy cows, is affected by the impairment of PMN function faced by transitioning cows. The impairment in PMN function is attributed to increased exposure to metabolites of homeorhetic mechanisms to support lactation, such as NEFA, and to decreased availability of glucose. Thus, intake might be associated with cytological endometritis because it is closely related to nutrient availability, and it is one of the main determinants of NEB. We conducted a pooled statistical analysis of five studies, including data from 394 multiparous Holstein cows. Based on the cutoffs for the percentage of PMN, determined by taking the median value of the data set for 15 and 30 DIM, cows were categorized as follows: LOW15 (PMN % at 15 DIM ≤ 24%), HIGH15 (PMN % at 15 DIM > 24%), LOW30 (PMN % at 30 DIM ≤ 7%); and HIGH30 (PMN % at 30 DIM > 7%). Cytological endometritis at 15 DIM was associated with decreased DMI from 4 wk

prepartum (1.97 ± 0.5 kg of DM/d less) until 4 wk postpartum (3.01 ± 0.5 kg of DM/d less), and a tendency for decreased milk yield from 3-5 wk postpartum. Cytological endometritis at 30 DIM was only associated with DMI as a percentage of BW, but it was not associated with milk yield. Additionally, increase in the vaginal discharge score (evaluated using Metricheck) was associated to decrease in up to 2.26 kg/d in milk yield. Supporting the notion of ovarian function being associated with uterine inflammatory status, cows in HIGH15 and HIGH30 ovulated on average 3 days before cows in LOW15 and LOW30, respectively. Additionally, increased units of vaginal discharge score and increased percentage units of uterine PMN were linearly associated with decreased milk yield. Taken together, these results suggest that uterine health diagnostics at an earlier stage may demand nutritional adjustments to help prevent the negative impact of cytological endometritis on cows' performance.

In conclusion, feeding RPL and RPM during the transition period has beneficial impacts related to uterine health. Both RPM and RPL modulated postpartum uterine expression of genes involved with inflammatory and metabolic processes. Additionally, feeding RPL decreased the percentage of PMN in the uterus at 15 and 28 DIM, although not changing days to first ovulation. Moreover, increasing Lys supply prepartum increased placental expression of genes involved in glucose uptake and metabolism, and increased the expression of transcripts and proteins involved in placental metabolic activity. Lastly, DMI in the transition period is associated with cytological endometritis, and cytological endometritis at 15 DIM and increased vaginal discharge scores are associated with decreased milk yield.

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To my grandma, Iolanda (*in memoriam*). Thank you for being so present in my and my siblings' life. We will never forget your care and love towards us.

"*Dreams have no behavior.*
There would always be in that kid's dreams the primitivism of his existence.
And the images he organized with the help of his words were concrete.
He even got to pick up the wind's mane one day.
Was it a dream?"

Manoel de Barros, in Menino do Mato, 2010.

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À minha mãe, Ana Maria,

Ao meu afilhado, Arthur,

com amor.

"Outro saber de que não posso duvidar um momento sequer
na minha prática educativo-crítica é o de que, como
experiência especificamente humana, a educação é uma
forma de intervenção no mundo."
"Another knowledge that I cannot doubt for a
single moment in my educational-critical practice
is that, as a specifically human experience,
education is a form of intervention in the world."
Paulo Freire

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CHAPTER 1: LITERATURE REVIEW

OVERVIEW OF THE TRANSITION PERIOD

As Dr. Drackley stated in his distinguished paper about the biology of dairy cows during the transition period, published by the Journal of Dairy Science (1999):

> "*The transition period between late pregnancy and early lactation (also called the periparturient period) certainly is the most exciting stage of the lactation cycle. […] The success of the transition period effectively determines the profitability of the cow during that lactation*". (p. 2259-2260)

Indeed, it is critical, as the dairy cow undergoes several metabolic adaptations during the transition period to guarantee the nutrients required for maintenance, the conceptus, and colostrum and milk production. Furthermore, dairy cows experience reduced feed intake, negative energy and protein balance, higher circulating concentration of non-esterified fatty acids (NEFA) from lipid mobilization, metabolic inflammation, reduced immune function, and reduced availability of glucose, calcium, and phosphorus (Drackley, 1999; Larsen and Kristensen, 2009; Sordillo and Raphael, 2013; Grünberg, 2014; LeBlanc, 2014; Sheldon and Owens, 2017).

Upon decreasing intake, while the nutrient requirements increase, nutrient partitioning aims to maintain the cow's well-being (Baumgard et al., 2017). Thus, the challenges imposed on the animal - such as thermal environment, management, social interaction, nutritional deficiencies, and diseases (Collier et al., 2017) will reflect on its ability to regulate nutrient use for productive functions and, therefore, prevent it from having optimal performance.

Maintenance is a top priority for the animal, while milk synthesis or fetal development is secondary in using absorbed nutrients (Baumgard et al., 2017). Homeostasis and homeorhesis are the two main types of regulation of physiological processes to maintain the animal's well-being, and both relate to nutrient partitioning (Bauman and Currie, 1980). Homeostasis can be described as the capacity of an organism to "maintain functional variables within a range of values compatible with survival," as summarized by Damasio and Damasio (2016). Homeostatic mechanisms are primarily the short-term actions, such as the metabolic coordination process that ensures a steady-state concentration of glucose in circulation (Baumgard et al., 2017). Homeorhesis is a chronic regulation process of coordinated changes that prioritize a specific physiological state (Bauman and Currie, 1980). Insulin resistance (IR) in the adipose tissue and muscle is an example of a homeorrhetic mechanism, which prevents peripheral tissues' glucose utilization, stimulates lipolysis, and increases hepatic gluconeogenesis (De Koster and Opsomer, 2013). Fat mobilization also contributes to IR since it produces pro-inflammatory signals that impair the intracellular signaling of insulin (Tilg and Moschen, 2008).

Additionally, the hypoglycemic state during early lactation decreases the concentration of insulin-like growth factor-1 (IGF-1), which provides negative feedback on growth hormone (GH) secretion (White et al., 2015). Growth hormone (GH) is part of a metabolic pathway that influences the transition from late pregnancy to early gestation in dairy cows (Bell, 1995). Growth hormone release from the pituitary is controlled by GH releasing hormone and GH inhibiting hormone; GH will act on the liver upon binding to its receptor (GHR), and it will initiate the production of insulin-like growth factor I (IGF-I) (Lucy et al., 2001). Moreover, growth hormone acts on the adipose tissue to increase lipolysis, increasing blood concentrations of non-esterified fatty acids (NEFA). Additionally, GH increases gluconeogenesis in the liver

through direct effects on the gluconeogenesis pathways and indirect effects as an antagonist of insulin actions (Etherton and Bauman, 1998).

The hypoglycemic state at peripartum also leads to incomplete oxidation of NEFA, which results in the formation of ketone bodies such as beta-hydroxybutyrate (BHB) (White et al., 2015). Serum concentrations of BHB between ≥ 1.2 mM and < 3.0 mM characterize subclinical ketosis, while BHB ≥ 3.0 mM defines clinical ketosis (Du et al., 2018). However, the exact concentration at which the animal will present clinical signs varies according to the individual, making it essential to observe the cow closely and use the diagnostic tools to detect ketosis. High blood concentrations of NEFA and BHB are associated with decreased DMI and immunosuppression, which increase complications in the peripartum and decrease milk production (Wankhade et al., 2017).

NEGATIVE ENERGY BALANCE AND INFLAMMATION

Dairy cows with high body condition scores (BCS) are at high risk of infection during the transition from gestation to lactation due to intense fat mobilization with alterations of immune functions (Lacetera et al., 2004, 2005). High-producing dairy cows have the characteristics of high circulating NEFA, IR, and inflammation states, especially in the transition period (LeBlanc, 2014). As previously mentioned, IR is an adaptive homeorhetic mechanism to allow a negative energy balance to support lactation (De Koster and Opsomer, 2013). The resulting fat mobilization increases the circulating levels of NEFA, which have pro-inflammatory actions through the increase of cytokines from adipose tissue - tumor necrosis factor (TNF) and IL-6 - and through binding of NEFA to TLR4 (Toll-like receptors), initiating an inflammatory cascade through TNF (Moyes et al., 2013; Sordillo and Raphael, 2013). Non-esterified fatty acids also

impair polymorphonuclear cells (PMN) function (Scalia et al., 2006). Inactive PMN cells can incorporate and store large quantities of exogenous NEFA into triacylglycerol or oxidize them. However, the change in the fatty acid composition of the cell membrane can change membrane fluidity, altering some of the enzymes that are bound to the membrane (De Pablo Martinez and Álvarez De Cienfuegos, 2000), which could lead to an increase in the production of reactive oxygen species (ROS; Inoguchi et al., 1939). An in vitro study reported by Scalia et al. (2006) demonstrated this effect of increasing ROS production and reduced viability and an increase of necrosis in bovine PMN cells incubated with NEFA at a concentration of 2 mM. It was previously hypothesized that an increase in PMN necrosis associated with increased fat mobilization leads to a higher risk of metritis (Kaneene et al., 1997). Additionally, the most predominant saturated fatty acids in NEFA, stearate, and palmitate, also have pro-inflammatory effects through activation of nuclear factor kappa-B (NF-κB) and secretion of TNFα IL-1 and IL-8 (LeBlanc, 2014).

The mobilization of adipose tissue lipids during negative energy balance might also be linked to uterine diseases through cholesterol, abundant in endometrial stromal cells (Sheldon and Owens, 2017). In an immune response, tissue cells rely on fatty acid oxidation to supply energy. However, the cells that are particularly exposed to pathogens might increase fatty acid synthesis as part of their inflammatory response (Sheldon and Owens, 2017). The fatty acids mobilized from adipose tissue can be metabolized to acetyl-CoA for cholesterol synthesis through the mevalonate pathway (Brown and Goldstein, 2009). Lower cholesterol concentrations in postpartum dairy cows were previously associated with uterine disease (Sepúlveda-Varas et al., 2015). Most cholesterol in cells is located at the plasma membrane, partially responsible for its fluidity; TLRs are also located in the membrane of cells within cholesterol-rich rafts.

Therefore, changes in cellular cholesterol could affect innate immune response (Dykstra et al., 2003). Indeed, reducing cellular cholesterol may limit inflammation, but it could also increase the cell resilience to pore-forming toxins, such as pyolysin. Pyolysin is a toxin formed by *T. pyogenes* and binds to cholesterol-rich domains in the plasma membrane, forming pores that lead to osmotic cell death (Preta et al., 2015).

PLACENTA

The overall role of placenta is common to different species, in that it is a protective layer that also guarantees nutrient transfer to maintain fetal homeostasis (Peter, 2013). The bovine placenta presents some peculiarities that add to these already extraordinary characteristics. The feto-maternal contact is restricted to the placentomes, which are composed of fetal cotyledon and the maternal caruncle (Hradecky et al., 1988). Currently, the bovine placenta is referred to as cotyledonary synepitheliochorial; cotyledonary appoints to the fetal part of a placentome formed by trophectodermal proliferation (Peter, 2013). The description of synepitheliochorial refers to the persistence of the uterine epithelium modified into a hybrid fetomaternal syncytium formed by the migration and fusion of binucleate/giant cells with uterine epithelial cells (Wooding and Wathes, 1980; Peter, 2013). These cells are important for the endocrine function of the placenta and deliver compounds to the maternal tissue, such as AA (Ullmann et al., 1985; Nakano et al., 2002; Schuler et al., 2006; Ushizawa et al., 2006).

The placenta is a central programming agent of adult health and disease (Gabory et al., 2013). Environmental stimuli, such as maternal nutrition for example, can influence placental morphology and alter fetal nutrient supply (Tarrade et al., 2015). Therefore, the maternal diet is an important factor to guarantee offspring health and performance (Norouzitallab et al., 2019).

The fetus acquires AA through an asymmetric bidirectional transfer with net movement occurring in favor of the fetal circulation (Smith et al., 1992). For instance, there is evidence of the presence of saturable transport systems for Lys at both surfaces of the trophoblast with efflux occurring in favor of the fetal circulation (Wheeler and Yudilevich, 1989). Recently, Alharthi et al. (2017) reported that calves whose mothers received Met supplementation during late pregnancy had greater body weight, and hip and wither height, which persisted through 9 weeks of age. Moreover, these calves also tended to have lower fecal scores regardless of colostrum source (Alharthi et al., 2019). These effects are attributed to alterations in the uteroplacental transport of indispensable and dispensable (e.g., nonessential) AA, glucose, and mTOR (mechanistic target of rapamycin) signaling (Batistel et al., 2017). Mechanistic target of rapamycin is an intermediate in a key translational control pathway that regulates the cell cycle, proliferation, and growth (Kennedy and Lamming, 2017). Though a considerable amount of research on fetal programming with non-ruminants' species, mostly focusing on using animals that can model studies for humans, the importance of nutrient manipulations during late-pregnancy and their influence in fetal and postnatal development of the offspring is evident (Godfrey and Barker, 2001; Gao et al., 2012).

OVARIAN RESUMPTION AFTER CALVING

A follicle consists of an oocyte surrounded by supporting somatic cells (Fortune, 2003). The focus of this section is on the ovarian resumption postpartum; however, a brief illustration of the follicular development is presented in Figure 1.1. Briefly, follicular development can be divided into a pre-antral phase (gonadotrophin-independent) and an antral phase (increasing dependency on gonadotrophin) (Webb et al., 2004). Dairy cows have two or three waves of

follicle growth in a cycle (Thatcher, 2017). Each wave comprehends emergence, selection, and dominance of follicles and is followed by atresia or ovulation (Crowe et al., 2014), which is dependent on the LH pulse frequency in the dependence phase (Sheldon and Owens, 2017).

During the late prepartum period, the combined negative feedback of high concentrations of progesterone and estradiol on the hypothalamic-pituitary-axis results in suppression of follicular waves (Roche et al., 1998). Upon calving, progesterone and estradiol reduce to basal levels, which removes the negative feedback of estradiol on the hypothalamic-pituitary (HP) axis and allows for the secretion of GnRH secretion by the hypothalamus and the FSH and LH release into peripheral circulation (Duffy et al., 2000). This stimulates the first follicular growth wave in the early postpartum (Crowe et al., 2014). The selection of the first dominant follicle (> 8 mm) happens around 10 DIM, and its ovulation is dependent mainly on the GnRH/LH pulse in the postpartum (Crowe et al., 1998). As summarized by Crowe et al. (2014), dairy cows ovulate the first dominant follicle of the first follicular wave postpartum in 30% to 80% of the cases, while in 15% to 60% of the cases the dominant follicle undergoes atresia or becomes cystic in 1% to 5% of cows. Cyclic and lactating Holstein cows usually have two follicular waves per estrous cycle (Sartori et al., 2014).

The initiation of a follicle wave after parturition happens regardless of the energy balance status (Butler, 2003). However, NEB results in fewer follicles growing, and they develop to a smaller size at ovulation (Perry et al., 1991), which implies that there might be an effect in the pre-recruitment phase. Additionally, factors that influence NEB, such as energy intake, BCS, and milk yield, are linked to reduced LH frequency (Crowe et al., 2014). There is also evidence that cows in severe NEB have a lesser expression of IGF-binding protein (IGFBP)-1 in granulosa cells compared to cows in mild NEB, which could impact the bioavailability of IGF-1, needed to

modulate the pre-recruitment stages of follicles (Llewellyn et al., 2007). Other factors affecting resumption of ovarian activity in dairy cows are BCS, parity, season, and disease occurrence (Opsomer et al., 2000; Wathes et al., 2007). For instance, Opsomer et al. (2000) reported acute BCS loss in the first two months postpartum, clinical ketosis, and abnormal vaginal discharge as major risk factors for a prolonged interval from calving to first ovulation. Ensuring maximized DMI in the early postpartum period is a strategy to prevent acute BCS loss and prevent increased NEB metabolites in blood circulation, which are associated with increased risk for metabolic disorders (Overton and Waldron, 2004; Maizon et al., 2004). Dairy cows affected by metabolic disorders in the early postpartum are more prone to reduced conception rates (Lucy et al., 2001; Crowe et al., 2014).

INTERACTION BETWEEN UTERUS AND OVARIES

The uterus can affect ovarian function, mainly through the inflammation process disrupting endocrine functions. These processes help explain the negative impacts of postpartum uterine diseases on the fertility of dairy cows and are further explored in section 1.6 (page 21). Briefly, inflammatory mediators disrupt the release of gonadotrophin-releasing hormone (GnRH) and impact the pulsatile LH frequency from the anterior pituitary (Krause et al., 2014), delaying ovulation (Canfield and Butler, 1990; Cheong et al., 2016). Ovarian function is affected by the same factors that impact uterine health. Supporting this hypothesis, evidence in the literature demonstrate that uterine contamination with pathogenic bacteria results in slower growth of the dominant follicle, either through a direct impact of bacterial endotoxin decreasing estradiol production or an indirect impact of the inflammatory response to the infection (Sheldon et al., 2002; Herath et al., 2009a). The ratio of estradiol to progesterone influences the responsiveness

of the pituitary to GnRH, with high estradiol and low progesterone resulting in greater LH release in response to GnRH (Stevenson and Pulley, 2016).

An influx of PMN cells into the uterine tissue and lumen is a typical inflammatory response of innate uterine immunity (Murphy et al., 2012). Cytological endometritis after 35 days postpartum is associated with reduced fertility and delayed resumption of ovarian cyclicity (Galvão et al., 2009, 2010; Cheong et al., 2011; Vieira-Neto et al., 2014; Gobikrushanth et al., 2016). The uterus is connected to the ovaries anatomically through a uterine-ovarian vein and ovarian artery, which allows the transfer of bacterial and pro-inflammatory products from the uterus to the ovaries (Knickerbocker et al., 1988). Additionally, uterine pH is also associated with follicle development, as Cheong et al. (2017) reported, where cows with a pH < 8.5 in the uterus at calving were more likely to have ovulatory first dominant follicles. Elevated uterine pH is associated with prolonged inflammation (Cheong et al., 2012). Therefore, maintaining a healthy environment in the uterus is crucial to guarantee an appropriate ovarian function and fertility of dairy cows in the postpartum.

UTERINE INVOLUTION

Uterine involution returns the uterus to its non-pregnant size and function after parturition, which usually takes from 4 to 6 weeks (Hafez, 2000). Sheldon (2004) described that for the subsequent gestation to be successfully established, four concomitant events should be completed: “uterine involution, regeneration of the endometrium, return to ovarian cyclic activity, and elimination of bacterial contamination,” illustrated in Figure 1.2. Uterine involution is dependent on myometrial contractions, the elimination of bacterial infections, and histological regeneration of the endometrium. The shreds of fetal membranes, maternal tissue, fetal fluids,

mucus, and blood – known as lochia - are eliminated in the first 14 to 23 DIM (Palmer, 2014). Physical shrinkage of the uterus occurs on a decreasing logarithmic scale, with the uterine weight changing from approximately 9 kg at parturition to 1 kg after 30 days (Sheldon, 2004). Both processes are dependent on myometrial contractions, which could result from PGF-2α stimuli since the serum concentrations of metabolites of PGF-2α are usually elevated around the same time (Peter, 1987). The regeneration of the epithelium takes around 3 to 4 weeks to be completed, and it is crucial to guarantee an appropriate uterine environment for conception since the endometrium is usually damaged from parturition. The caruncular tissue is sloughed as part of the tissue remodeling process during postpartum (Sheldon and Owens, 2017).

UTERINE INNATE IMMUNITY

Nearly all cows experience bacterial contamination of the reproductive tract at parturition (Sheldon et al., 2006) and therefore rely on immune and inflammatory responses to clear these pathogens (LeBlanc, 2014). The host response to bacterial pathogens in the uterus is characterized by inflammation of the endometrium with neutrophil and macrophage infiltration and possible accumulation of pus in the uterine lumen (Sheldon and Owens, 2017). Inflammation is considered a mechanism of innate immunity, and, most frequently, microbial stimuli in the context of tissue injury initiate inflammation (Nathan, 2002). The result of inflammation should be protecting the tissue from the spread of infection, with affected tissues returning to their functional state (Nathan and Ding, 2010). The vascular component of the inflammatory process results in vasodilation, increased permeability, and increased blood flow at the site of the injury or infection (Murphy et al., 2012). The innate immune response will be discussed in more detail because the uterine tissue response to postpartum bacterial infection points to an essential role of

the innate immunity, with epithelial and stromal cells in the endometrium acting in the initial sensing and response to pathogens (Sheldon, 2015).

Innate immunity from polymorphonuclear (PMN) cells, which is an unspecific response, and the anatomical barrier formed by endometrial epithelial cells constitute the major players in the innate immune defense of the uterus (Sheldon et al., 2009, 2019). The pathogen recognition receptors, such as TLR, recognize pathogen-associated molecular patterns (PAMPs) (Mogensen, 2009) and trigger signaling pathways that control the induction of a pro-inflammatory response through the release of cytokines (IL1β, IL1A, IL6) and chemokines (IL8) (Sheldon et al., 2019). Indeed, uterine samples from diseased animals have a greater abundance of transcripts for *IL1-α*, *IL1-β*, *IL-6*, and *TNF*; chemokines *IL-8* and CCL5; and receptors *TLR4* and *IL1R* (Herath et al., 2009b; Fischer et al., 2010). The pro-inflammatory interleukins will stimulate the liver to produce acute-phase proteins (such as haptoglobin – Hp) and attract neutrophils and macrophages. The use of recombinant IL8 is being researched lately as a strategy in the early postpartum to decrease the incidence of reproductive inflammatory diseases in dairy cows (Bicalho et al., 2019; Zinicola et al., 2019), as it could potentially modulate innate immune function (Mitchell et al., 2003). In a study reported by Bicalho et al. (2019), recombinant bovine IL8 purified from bacterial cultures was administered in the uterus of dairy cows, which resulted in an increase of neutrophil proportion in the uterine lumen within one hour after administration. Upon removal of the PAMPs, neutrophils should undergo apoptosis, so the inflammation process resolves; however, in some cases, this inflammation shifts from physiological to pathological, as in the development of subclinical endometritis (Sheldon et al., 2019; Pascottini et al., 2020).

The endometrium also provides the initial response against microbes by producing antimicrobial peptides and Mucin-1 (MUC1) (Kasimanickam et al., 2014). However, the

presence of MUC1 in the endometrium at breeding time is detrimental to pregnancy (Johnson et al., 2001). Therefore, timely coordination of activation of the immune system is critical for warranting a smooth regression of the uterine environment. The resolution of the inflammation process is critical to achieving optimal reproductive performance. Mucin-1 is an inducible innate immune factor and an essential component of the first line of defense against bacterial invasion of epithelial surfaces (Lagow et al., 1999). Kasimanickam et al. (2014) collected endometrial samples from cows with diagnosed uterine diseases or a healthy uterine environment to evaluate the gene expression of *MUC1* and cytokines genes. The mRNA expression of *MUC1, ILβ1*, and *IL8* (and others) was greater for cows with uterine inflammatory conditions than cows considered normal (Kasimanickam et al., 2014). Mucin-1 is also present at the apical surface of the uterine epithelium at the time of implantation and may protect the mucosal surface from infection and the action of degradative enzymes (Carson et al., 1998; Aplin, 1999). Its antiadhesive properties represent a barrier to embryo attachment, specifically those with poor quality. Meseguer et al. (2001) reported that MUC1 was lost from epithelial cells beneath and near the attached embryo, whereas regular expression persisted in neighboring cells. The expression of *MUC1* in the endometrium is upregulated by P4 and downregulated by the embryo (Meseguer et al., 2001), and it could be associated with infertility if over-expressed at the time of implantation, decreasing endometrial receptivity.

POSTPARTUM REPRODUCTIVE TRACT INFLAMMATORY AND INFECTION DISORDERS

As previously stated, pathogenic organisms' contamination of the endometrium triggers an inflammatory response. When this inflammatory response is rapidly and robustly established

at the beginning of the infection, it results in a neutrophil influx and pathogen clearance, reducing the incidence of uterine disorders (LeBlanc, 2014; Pascottini et al., 2020). Once the infection is controlled, the inflammation should resolve, and the damaged tissue should repair, allowing the uterus to return to homeostasis (Nathan and Ding, 2010). However, poor clearance of pathogens associated with inadequate resolution of inflammation leads to clinical and subclinical uterine diseases. Therefore, the occurrence of postpartum reproductive inflammatory diseases is closely related to the immune capacity of the animal. It is noteworthy that the cow needs to move from a state of immune tolerance during gestation (crucial to maintain embryonic and posterior fetal survival) to an active immune state at calving (Bradford et al., 2015), which presents another challenge to the animal. Reproductive tract inflammatory diseases in the early postpartum of dairy cows include metritis, purulent vaginal discharge, cytological endometritis, cervicitis, and vaginitis (Dubuc et al., 2010; Pascottini & LeBlanc, 2020). Due to the scope of this dissertation, only retained placenta and cytological endometritis will be reviewed.

Retained placenta (RP) is the failure to expel the placenta, or fetal membranes, within 12 h to 24 h postpartum (Kelton et al., 1998), resulting in retained fetal membranes for an average of 7 days (Eiler and Hopkins, 1992). Retained placenta is usually not classified as a reproductive tract inflammatory disease but is one of the risk factors more often associated with the development of uterine infection (Potter et al., 2010) and consequent decrease in fertility (Coleman et al., 1985). The pro-inflammatory environment in the uterus after parturition is responsible for the expulsion of the placenta through rupture of the fetal membranes, contraction of the uterus, and dilation of the cervix (Van Engelen et al., 2009). A lack in uterine motility to expel the fetal membranes has little to no effect on the pathogenesis of retained placenta (Laven and Peters, 1996), which seems to be more related to a failure of the breakdown of the

cotyledon-caruncle attachment (LeBlanc, 2008). Kimura et al. (2002) reported an impaired neutrophil function – assessed through chemotaxis and oxidative burst activity – and lesser plasma concentrations of interleukin-8 (IL8) in cows developing RP than cows that expelled their placenta normally. There might be an association between hypocalcemia and retained placenta since Ca plays an essential role in immune function in dairy cows (Kimura et al., 2006). The impact of RP is variable because it depends on the subsequent development of uterine diseases, such as metritis or endometritis (LeBlanc, 2008).

The disruption of the endometrium with presence of inflammatory cells (Bondurant, 1999), resulting in increased proportion of PMN in uterine cytology characterizes cytological endometritis (Kasimanickam et al., 2004; Gilbert e al., 2005; Dubuc et al., 2010). The diagnostic of cytological endometritis is based on the PMN proportion in endometrial cytology samples collected by uterine lavage or endometrial cytobrush (Dubuc et al., 2010) and both techniques were proven to provide similar results (Barlund et al., 2008). According to Sheldon et al. (2019) and Dubuc et al. (2010), between 10 to more than 50% of cows experience cytological endometritis at 4-8 weeks postpartum, which is associated with a decrease in reproductive performance. The herd prevalence of cytological endometritis varies due to the use of different techniques, cutoff values, and DIM for the evaluation of the uterine cytology (McDougall et al., 2020). For example, studies report cutoff points varying from ≥ 5% to ≥ 10 % for cows examined at 35 DIM (Bicalho et al., 2016; Dubuc et al., 2010; Kasimanickam et al., 2004a).

Although the cutoff points and the time point for the diagnostic of cytological endometritis vary among the studies, a common item among them is to use reproductive performance measurements to optimize the cut-points, such as pregnancy status after first service (Denis-Robichaud and Dubuc, 2015), hazard of pregnancy or time to first pregnancy after

calving (Bicalho et al., 2016; Galvão et al., 2009; Gilbert et al., 2005). Using reproductive performance measurements as a main outcome for the association with uterine cytology has its merit, since the physiological status of the uterus is crucial for the establishment and maintenance of gestation (Fonseca et al., 1983; Sheldon, 2004), directly impacting the reproductive success of dairy cows. The serum concentration of haptoglobin (Hp) and serum amyloid A (SAA), as well as TNF-α, IL-6, IL-10, are higher in cows with cytological endometritis in early postpartum (Brodzki et al., 2015). The cause of cytological endometritis is unknown, but it could involve the process of resolving bacterial infections, immunopathology without pathogenic bacteria, or abnormalities of postpartum tissue regeneration and repair (Sheldon and Owens, 2017).

Could subclinical endometritis result from failure of macrophages to clear apoptotic neutrophils or even a failure of the neutrophils themselves to undergo timely apoptosis? In the process of resolving the inflammation, viable cells (such as macrophages) engulf apoptotic cells, which triggers the release of inflammation-resolving cytokines such as transforming growth factor-β (TGF-β) and interleukin-10 (IL-10) (Kennedy and Deleo, 2009). However, when the apoptotic cells are not cleared out rapidly, they proceed to necrosis, generating pro-inflammatory signals (Nathan and Ding, 2010). Subclinical endometritis is defined as an inflammation of the uterine endometrium in the absence of purulent material in the vagina and usually without pathogens (Sheldon et al., 2006). The occurrence of subclinical endometritis has been previously associated with genes linked to the regulation of apoptotic signaling, alongside genes linked to the immune system, cell adhesion, G-protein coupled receptors signaling pathways, and chemotaxis (Salilew-Wondim et al., 2016). Certain inflammatory states inhibit neutrophil apoptosis (Mevorach et al., 1998), as do specific pathogens, such as the respiratory syncytial

virus (Elbim et al., 2009). Likewise, inflammation can be prolonged by a deficiency of factors that promote the ingestion of apoptotic cells (Nathan and Ding, 2010). In dairy cows, information about endometrial neutrophil counting is abundant, but data on their functionality is still lacking. Recently, Lietaer et al. (2021) evaluated the counts, viability, and functionality of endometrial neutrophils isolated from clinically and metabolically healthy Holstein cows at different time points around calving. The greater percentage of apoptotic neutrophils at 9 DIM than at 21 and 37 DIM, and the lower percentage of necrotic endometrial neutrophils at 9 DIM indicate a possible resolution of inflammation in these healthy cows. Therefore, it would be necessary to retrospectively evaluate the viability and the functionality of isolated endometrial neutrophils of cows developing subclinical endometritis, to assess whether SCE results from a failure of macrophages to clear apoptotic PMN cells or even maybe a failure of PMN cells to undergo timely apoptosis. Subclinical endometritis could also be a function of the systemic environment, not only the endometrium, but the exact cause of subclinical endometritis remains unclear and consequently is an exciting research point.

NUTRITION AND THE TRANSITION PERIOD: FOCUS ON LYSINE AND METHIONINE

Protein utilization is a complex process in ruminants. Previously, dairy cows' diets were formulated to meet crude protein requirements, defined as the nitrogen content multiplied by a factor of 6.25, based on the nitrogen content of most proteins – 16% (NRC, 2001). Although predominantly used while the understanding of amino acids (AA) requirements was not available, this approach is not the more accurate to meet the animal's demands due to the complexity of nitrogen metabolism in the rumen. Later, the identification of different fractions of protein – one

fraction that is rapidly degraded by the ruminal microorganism (rumen degraded protein, RDP) and one fraction that escapes through the rumen undegraded (rumen undegraded protein, RUP) – was a significant step forward in the understanding of ruminant nitrogen metabolism (Schwab and Broderick, 2017). Around 10% of DM as RDP will be used for microbial growth as long as sufficient energy is available for the process (Weiss, 2002).

Nonetheless, if an excess of RDP is fed, it will result in excess nitrogen that cannot be used by rumen microbes and therefore is absorbed into the bloodstream as ammonia (Weiss, 2002). Ammonia is then converted into urea and recycled into the saliva, reabsorbed by the rumen, or excreted in the urine. High concentrations of blood urea nitrogen (BUN) are associated with decreased reproductive efficiency. High BUN or milk urea nitrogen (MUN) might be associated with a decrease in uterine pH (Elrod et al., 1993), which alters the uterine environment, decreasing pregnancy per artificial insemination (PAI) and probability of conception at first service (Ferguson et al., 1993; Butler et al., 1996). However, previous research also reports that no such effects are seen at second or third service (Ferguson et al., 1993; Guo et al., 2004). Moreover, Santos et al. (1998) reported that increasing RUP in dairy cows' diets does not consistently improve lactation performance (Santos et al., 1998). These illustrate some of the reasons why diet formulations and delivery need to provide the appropriate amount of nutrients and exceeding the requirements might not be beneficial. Conversely, the production responses of lactating dairy cows to increase post-ruminal supply of IAA are usually greater, especially when the crude protein approximates 14 to 18% of dry matter (DM) in the diets and increases the milk protein content, which can be independent of the milk yield (Schwab, 1996). Additionally, the milk yield responses to supplementation of rumen-protected

Lys or Met are more commonly seen in early lactation than mid to late lactation cows (Schwab, 1996; Socha et al., 2005).

Indispensable AA are not synthesized by the animal or are synthesized at rates insufficient to meet requirements, and therefore they need to be supplied through the diet (NRC, 2001). Dispensable AA are readily synthesized within the body from metabolites of intermediary metabolism and amino groups from extra AA (Schwab and Broderick, 2017). Lysine and Met are the most limiting AA in dairy cows' diets due to the low content in feedstuffs fed to cows compared to their concentration in ruminal bacteria and milk (NRC, 2001; Schwab and Broderick, 2017). Methionine is usually the first limiting AA in high forage diets containing low concentrations of corn and high concentration of soybeans or animal-derived protein sources (NRC, 2001). In contrast, Lys is typically the first limiting AA in high corn-based diets (NRC, 2001).

The most efficient method to avoid ruminal degradation and increase the bioavailability of intestinal Met and Lys is through amino acid encapsulation with ruminal-inert, pH-sensitive polymers (rumen-protected AA; Schwab, 1996). The use of rumen-protected AA technologies allows for the supply to the small intestine with proper AA profile (Schwab and Broderick, 2017). Rumen-protected AA technologies include encapsulation with fatty acids or saturated fatty acids and minerals (i.e. Ca-salts), and encapsulation with fatty acids or pH sensitive polymers, among others (Schwab, 1996; Schwab and Broderick, 2017). Lys is water soluble and the most common method to protect is through lipid coating, which also helps to increase the shelf life through increasing the melting temperature and preventing auto-oxidation (Watanabe et al., 2006). However, it is quite challenging to protect Lys from ruminal degradation while maintaining its bioavailability. Improving the intestinal digestibility of Lys remains a challenge.

Ultimately, the nutritional requirements of a dairy cow are for specific AA, as the digestibility and AA profile of microbial protein and RUP can vary largely (Santos et al., 1998). Evidence in literature point to a better relationship between intake of the most limiting AA and milk protein yield than the relationship between CP or MP intake and milk protein yield (VandeHaar and St-Pierre, 2006; Vyas and Erdman, 2009; Moraes et al., 2018). Additionally, the efficiency of use of MP for protein synthesis depends on the AA profile in that MP, particularly how well the profile and quantity of IAA matches the AA profile required by the animal (Schwab and Broderick, 2017). Although the number of studies exploring the effects of feeding rumen-protected indispensable AA to dairy cows only increased in the past decades (Schwab and Broderick, 2017), there are still some inconsistencies in recommendations and expression of indispensable AA content in the diet that make it challenging to determine the actual requirement during the periparturient period for dairy cows. The recommendations of the NRC (2001) are for supplementation of Met at 2.4% of metabolizable protein and Lys at 7.2% of metabolizable protein for optimized milk production. However, mammary gland net removal of individual IAA varies (Hanigan et al., 2000) and, consequently, the mammary metabolism of AA as well, since it is regulated by intracellular concentration of AA, among other factors (Cant et al., 2018; Huang et al., 2021).

EFFECTS OF MET AND LYS SUPPLEMENTATION ON IMMUNITY AND REPRODUCTIVE OUTCOMES

Methionine is an important methyl donor involved in protein synthesis and methylation processes (Obeid, 2013). Methyl donors are important for the synthesis of key compounds, such as phosphatidylcholine (Obeid, 2013). However, a negative methyl donor balance as a

consequence of a nutritional imbalance may result in increased mobilization of body protein in dairy cows, since there is a daily methyl group requirement to fulfill the needs of mammary gland and liver (Loor, 2010). Additionally, DNA methylation is one of the main epigenetic mechanisms for regulation of gene expression (Han et al., 2007) and depends on the availability of methyl donors supplied by AA such as Met (Niculescu and Zeisel, 2002). Furthermore, Met possesses high nutritional value and important physiological functions, such as growth promotion (Avila et al., 2000). Methionine is also closely related to the immune function of livestock and poultry, which not only has effects on the growth and development of immune organs, but also on the specific and nonspecific immune function of the organism (Konashi et al., 2000; Zhang and Guo, 2008; Wu et al., 2018). In dairy cows, RPM was proven to benefit neutrophil function (Osorio et al., 2013), to decrease the purulent vaginal discharge score postpartum, increasing overall cow performance and health (Osorio et al., 2013; Stella et al., 2018). Moreover, RPM also improved the uterine neutrophil infiltration and glandular morphology (Stella et al., 2018). Previous research conducted by the Dairy Focus Lab at the University of Illinois demonstrated a that RPM fed from three weeks prepartum until 73 DIM helped to enhance cows' resilience mechanisms in the uterus and therefore improved their capacity to prevent uterine diseases from happening (Guadagnin et al., 2021). Additionally, in the same study, the higher mRNA expression of *FGF7*, *MAT1A*, *LCAT*, and *SAAH* in the uterine tissue demonstrates that RPM can also be involved in cells nutrient metabolism and proliferation processes that are crucial for uterine regeneration and preparation for conception (*unpublished,* Chapter 2).

Although most of the studies with dairy cattle evaluate the effects of Met on immune function (Osorio et al., 2013; Batistel et al., 2017; Skenandore et al., 2017; Stella et al., 2018),

Lys may also be a key player in improving innate immunity. Literature in poultry estimated that the amount of the body's Lys contained in the immune system increased by 10-fold upon a lipopolysaccharide (LPS) challenge (Chen et al., 2003). Furthermore, it appears that additional nutritional resources are needed for an acute phase response; however, upon clearance of pathogen infection, amino acids (AA) such as Lys would become available for the subsequent adaptive response (Iseri and Klasing, 2014). Subsequent work from our group (*unpublished,* described in Chapter 2) detected that feeding rumen-protected Lys pre- and postpartum improved uterine immunity as evidenced by the number of cells per gland and modulation of genes that are involved in the inflammation process, decreasing the number of polymorphonuclear (PMN) cells in the uterus. Cows that received RPL pre- and postpartum tended to have less chances (OR = 0.89, 95CI = 0.04 – 1.37) to develop subclinical endometritis at 28 DIM than cows that did not receive RPL. Additionally, there was greater mRNA expression of α-aminoadipate $_{\delta}$ semialdehyde synthase (AASS) in uterine tissue samples of cows supplemented with RPL during pre- and postpartum (*unpublished,* described in Chapter 2), evidencing catabolism of Lys also in the reproductive tract. In an innovative study trial, our group also followed up the offspring from these same cows from calving through 56 days of age. Calves from cows that received RPL prepartum had a tendency for greater DMI and crude protein intake (Thomas et al., 2021). Interestingly, there was also an interaction of treatment and sex, with male calves from cows that were not fed RPL being more likely (OR = 2.80, 95CI = 1.27 – 6.19) to be medicated than males from cows that were fed RPL prepartum (Thomas et al., 2021). Female calves from cows that were fed RPL tended (OR = 1.75, 95CI = 0.80 – 3.83) to me more medicated than male calves from cows that received RPL prepartum (Thomas et al., 2021). Moreover, calves from cows that were not fed

RPL had more days on antibiotics (5.6 days) than calves from cows that were fed RPL prepartum (4.2 days) (Thomas et al., 2021). These changes resulted from *in utero* effects of supplementation with RPL, since all calves did not receive maternal colostrum but a colostrum replacer and then fed the same milk replacer and starter as well. Additionally, there is increasing evidence of the placental contribution to sexual dimorphism in health and diseases (Gabory et al., 2013), as well as maternal nutrition during late pregnancy being directly implicated in the triggering processes for this placental contribution (Batistel et al., 2019).

Previous literature has disclosed that the supplementation of rumen-protected Lys (RPL) and/or Met increased milk yield, milk protein yield, and DMI (Xu et al., 1998; Socha et al., 2005; Lee et al., 2012; Zhou et al., 2016; Batistel et al., 2017; Fehlberg, 2020). Greater metabolizable protein and Lys intake during the pre-calving period contribute to increased DMI postpartum (Girma et al., 2019; Fehlberg, 2020) by alleviating the deficiency of those components in the transition diets. Innate immunity is particularly important for the dairy cow following parturition; therefore, a transition cow diet providing an adequate profile of AA (notably Lys and Met), could alleviate body mobilization (Carder and Weiss, 2017; Lee et al., 2019) and meet the AA requirements of the immune system.

Lysine and Arg are the two major moieties of natural antimicrobial peptides (Guaní-Guerra et al., 2010). Lysine has a positively charged side chain at a physiological pH (Sokalingam et al., 2012), which is believed to confer antimicrobial activity as the AA residue binds to the negatively charged acidic phospholipids of bacterial cell membrane, disrupting it and causing bacterial cell death (Guaní-Guerra et al., 2010). In a study conducted to evaluate Lys-derived and Arg-derived carbon quantum dots (CQD), Li et al. (2020) reported that these nanomaterials presented distinctive antibacterial activity against both gram-negative and gram-

positive bacteria, inhibiting their growth *in vivo* and promoting growth of typical mammalian cells, accelerating wound healing (Li et al., 2020). The authors suggest that Lys-CQD and Arg-CQD may have signaling agents that promote cell proliferation. Examples of these signaling agents could be related to intracellular oxidative damage. Moderate levels of reactive oxygen species (ROS) promote cell proliferation, but higher levels of ROS can lead to cell death (Panieri et al., 2013). Lys-CQD inhibited the activities of the antioxidant enzyme superoxide dismutase in bacteria, but no inhibitory effect was found in fibroblasts or red blood cells of mice (Li et al., 2020).

RESEARCH OBJECTIVES

The specific research objectives of this dissertation are:

1) To evaluate the effects of feeding rumen-protected Met (RPM) on mRNA expression profiles of cytological and endometrial samples of Holstein cows at 15, 30, and 73 days in milk.
2) To evaluate the effects of feeding rumen-protected Lys (RPL; AjiPro-L Generation 3, Ajinomoto Heartland Inc., Chicago, IL) prepartum [0.54 % RPL of dietary dry matter intake (DMI)], postpartum (0.40 % RPL of dietary DMI), or both on follicular dynamics, uterine health, endometrial morphology, and transcriptional expression of genes related to endometrial metabolism and immunity of multiparous Holstein cows.
3) To determine the effects of the maternal supplementation with rumen-protected Lys (0.54 % RPL of dietary DMI) during late-pregnancy on protein abundance and transcriptional expression of genes involved in placental amino acid transport system, protein metabolism, energy metabolism, and immune metabolism.

4) To determine the association of dry matter intake (DMI), lactation performance, days to first ovulation, and vaginal discharge with cytological endometritis at 15 DIM (CYT15) and at 30 DIM (CYT30). Additionally, to determine the association of vaginal discharge with CYT15 and CYT30 and productive parameters.

FIGURES

Figure 1.1 Summary of folliculogenesis, adapted from Dunlop and Anderson (2014). The regulation of primordial follicle pool development into primary follicles might be upregulated by mechanistic target of rapamycin (**mTOR**) complex, stem cell factor (**SCF)**, bone morphogenetic proteins 4 and 7 (**BMP4** and **BMP7**), leukemia inhibitory factor (**LIF**), and fibroblastic growth factor (**bFGF**); and downregulated by phosphatase and tensin homologue (**PTEN**), transcription factor fork-head box O3a (**FOXO3a**), and anti-Müllerian hormone (**AMH**). The regulation of development of secondary follicles from primary follicles might be upregulated by activin and downregulated by AMH. The growth from secondary follicles to antral follicle is increasingly gonadotrophin-dependent and ovulation is entirely gonadotrophin-dependent.

Figure 1.1 (cont.).

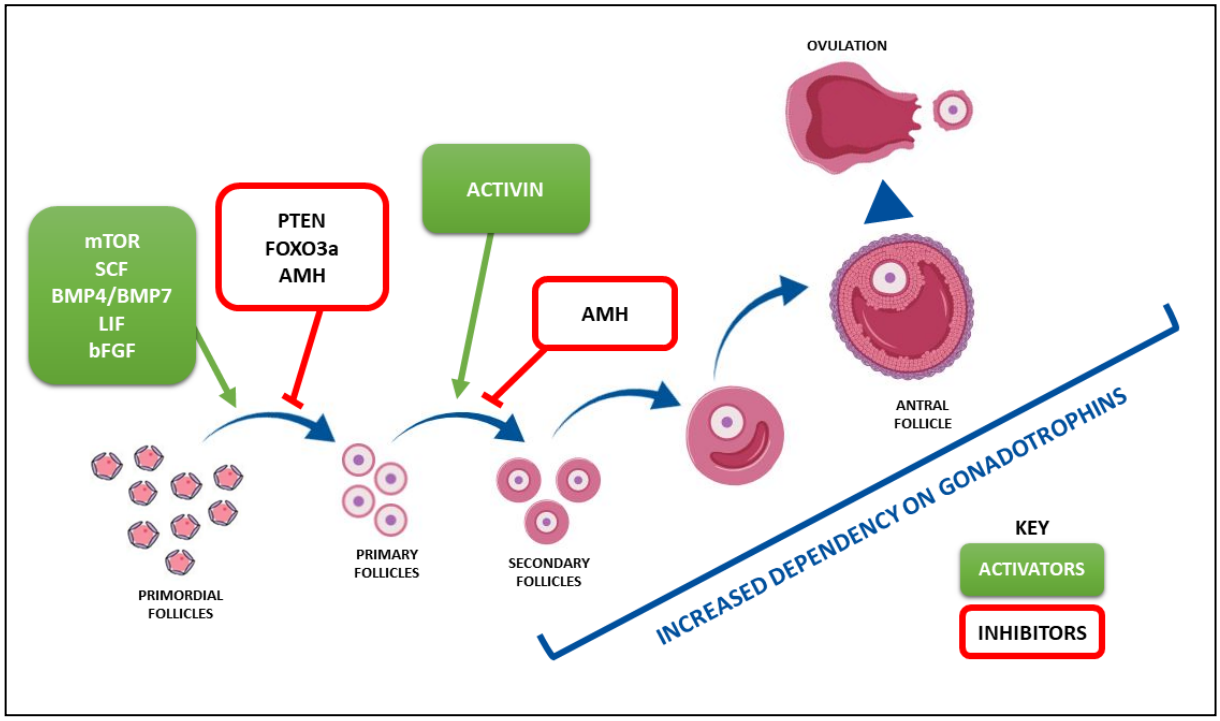
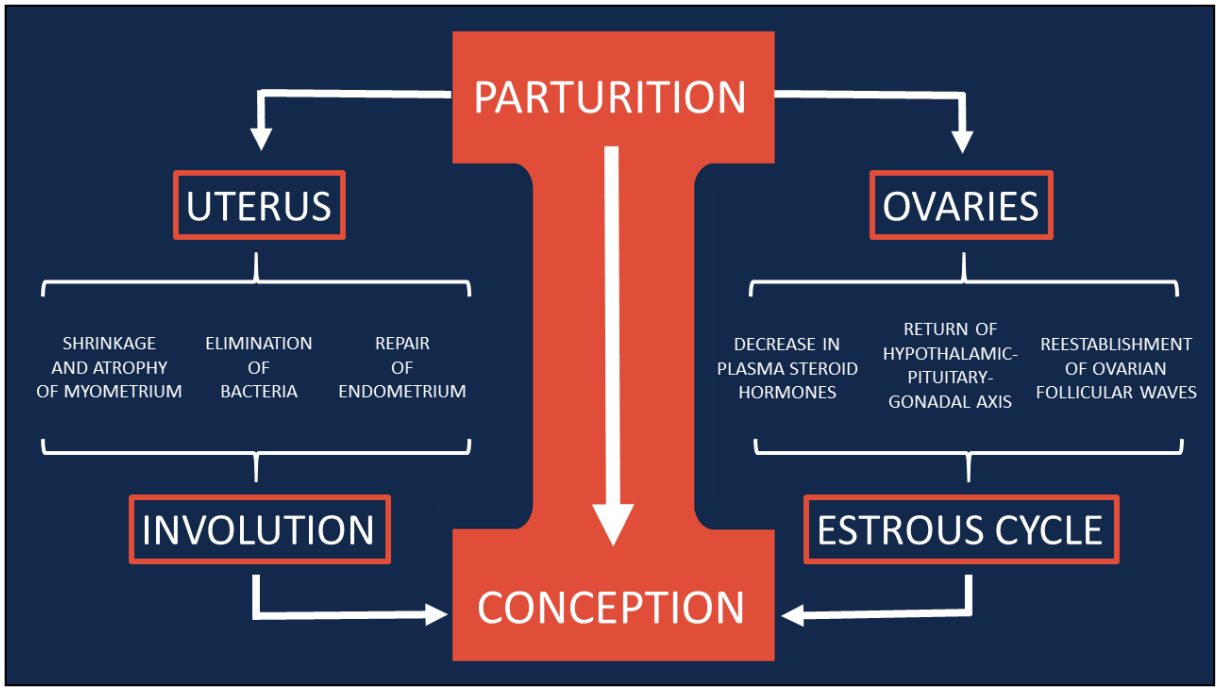


Figure 1.2 Adapted from Hafez (2000) and Sheldon (2004). Processes required in the uterus and ovaries during postpartum for the next pregnancy to be established.



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CHAPTER 2: METHIONINE SUPPLY DURING THE PERIPARTUM PERIOD AND EARLY LACTATION ALTER IMMUNOMETABOLIC GENE EXPRESSION IN CYTOLOGICAL SMEAR AND ENDOMETRIAL TISSUE OF HOLSTEIN COWS[1]

ABSTRACT

The objective of the present study was to evaluate the effect of feeding rumen-protected methionine (**RPM**) during the peripartal period and early lactation on mRNA gene expression profiles of uterine cytological smear and endometrial samples of Holstein cows (n = 20). Treatments consisted of a supplementation with RPM [MET; n = 11; RPM at a rate of 0.08% of DM: Lys:Met = 2.8:1, (Smartamine® M Adisseo, Alpharetta, GA, USA)] and no supplementation (CON; n = 9; Lys:Met = 3.5:1). Uterine cytology smears and endometrial samples were collected at 15, 30, and 73 days in milk (**DIM**) and analyzed for expression of genes related with metabolism, inflammation, and methionine metabolism. Regarding the cytological smear samples, RPM supplementation tended to increase mRNA expression of methionine adenosyltransferase 1 alpha (***MAT1A***) and increased the mRNA expression of fibroblast growth factor 7 (***FGF7***), with an effect of time for the latter. On the other hand, RPM decreased mRNA expression for glucose transporter 4 (***GLUT4***), interleukin 1 beta (***IL-1β***), interleukin 6 (***IL-6***), interleukin 8 (***IL-8***), prostaglandin E synthase 3 (***PTGES3***), translocator protein 18 kDa (***TSPO***), mucin 1 (***MUC1***) and superoxide dismutase (***SOD1***) in cytological smear samples. There was an effect of time for all variables except *MAT*1*A*, with decreasing expression over time. There was a TRT × TIME interaction for GLUT4 mRNA expression, with higher GLUT4 mRNA expression for cows fed CON than for cows fed RPM at time 15 and a tendency to higher expression for

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cows fed CON on time 30 when compared with cows fed RPM. For uterine tissue samples, feeding RPM increased the mRNA expression of lecithin-cholesterol acyltransferase (***LCAT***), S-adenosyl-L-homocysteine hydrolase (***SAAH***), *FGF7*, *GLUT4*, and apolipoproteins 3 (***APOL3***), with an effect of time for APOL3 where its expression increased over time. There was a tendency for cows fed RPM to have decreased *IL1β* mRNA expression. In conclusion, feeding RPM during transition period and early lactation is beneficial for uterine immune response and metabolism in early lactation as indicated by the favorable expressions of genes affecting the uterine immunometabolism during such a challenging period.

Keywords: cytology, gene expression, immune function, uterus.

INTRODUCTION

As already well established, the periparturient period is the most critical phase in a high-producing dairy cows' life (Drackley, 1999). They undergo substantial metabolic and physiological adaptations during the transition from pregnancy to lactation and coordinated shifts in nutrient partitioning must occur to meet the increase demand for energy, protein, and other nutrients necessary for fetal growth and lactation (Drackley, 1999; Loor, 2010). Additionally, a metabolic homeostatic balance is needed to favor innate immunity, which is crucial for the uterine involution and pathogen evasion in the early postpartum (LeBlanc, 2014). Dairy cows are susceptible to develop uterine diseases, as 80-90% of them have their reproductive tract contaminated with bacteria after calving (Dohmen et al., 1995; Ghanem et al., 2014). What is determinant in the evolution from a contamination to an infection or disease establishment is the immune response, which needs to be robust and effective in the pathogen evasion, but without exacerbating to evolve to a disease status (LeBlanc, 2014; Pascottini and LeBlanc, 2020).

Uterine infection is one of the most common problems in the postpartum period of cows and can cause several economic losses (LeBlanc, 2014; Sheldon et al., 2019; Sheldon et al., 2020). Poor clearance of pathogens and/or inadequate resolution of inflammation leads to the development of clinical and subclinical diseases (Ghanem et al., 2014; Sheldon et al., 2019; Sheldon et al., 2020). During such infectious or inflammatory processes, the epithelial cells from the endometrium produce multiple inflammatory cytokines, chemokines, and defense substances, such as interleukins and mucins. In cows with clinical endometritis, there is a higher gene expression of interleukin-8 (*IL8*), interleukin-6 (*IL6*), and interleukin-1β (*IL1β*), compared with healthy cows (Pascottini and LeBlanc, 2020; Kasimanickam et al., 2013), because immune cells release interleukins, alongside with tumor necrosis factor α (TNFα), after pathogen recognition (Bondurant, 1999). The endometrium also provides the initial response against microbes by producing antimicrobial peptides and Mucin-1 (MUC1) (Kasimanickam et al., 2014), however, the presence of MUC1 in the endometrium at breeding time is detrimental to pregnancy (Bazer et al., 2020). Therefore, a timely coordination of activation of the immune system is critical for warranting a smooth regression of the uterine environment, as well as the resolution of the inflammation process is critical to achieve an optimal reproductive performance.

Methionine is one of the most limiting amino acids (AA) in lactating cows (NRC, 2001) and it is an important methyl donor involved in protein synthesis and methylation processes. Methyl donors are important for the synthesis of key compounds, such as phosphatidylcholine (Obeid, 2013). However, nutritional imbalance may result in a negative methyl-donor balance, which leads to increase mobilization of body protein to fulfill the requirements of mammary gland and liver of dairy cows (Loor, 2010). Additionally, DNA methylation is one of the main epigenetic mechanisms for regulation of gene expression (Liangfeng et al., 2007) and depends on

the availability of methyl donors supplied by amino acids such as methionine (Avila et al., 2000). Furthermore, methionine possesses high nutritional value and important physiological functions (Avila et al., 2000), such as growth promotion (Mirzaaghatabar et al., 2011). Moreover, methionine is closely related to the immune function of livestock and poultry, which not only has effects on the growth and development of immune organs, but also on their specific and nonspecific immune function (Konashi et al., 2000; Zhang et al., 2008; Stella et al., 2018). Also, rumen-protected methionine (**RPM**) is effective in providing extra metabolizable methionine to balance peripartal diets, which in turn helps to increase dry matter intake (**DMI**) after parturition (Zhou et al., 2016) and improve the postpartum lactation performance as well as the health (whole blood phagocytosis capacity, antioxidant status and immune function (Cerri et al., 2012).

Our hypothesis is that the supplementation of RPM during the transition period and early lactation will modulate the uterine metabolism and immune defense system, which could decrease the susceptibility to reproductive tract inflammatory diseases. An integrated process of events needs to take place synchronously in the postpartum period to secure a proper uterine health and involution, consequently improving conception. Therefore, we aimed to evaluate the effects of RPM on mRNA gene expression profiles of cytological and endometrial samples in Holstein cows at 15, 30, and 73 days in milk (**DIM**). Our goal with these time points was to have a way of assessing the periods that comprehend uterine clearance and involution, regeneration of endometrium, and the breeding period at the end of a typical voluntary waiting period.

MATERIALS AND METHODS

Experimental Design and Dietary Treatments

All experimental procedures performed were approved by the University of Illinois (Urbana-Champaign) Institutional Animal Care and Use Committee (#13023). Details of the project management were reported elsewhere (Stella et al., 2018; Zhou et al., 2016). Briefly, a total of 20 multiparous Holstein cows were included in the experiment from 21± 1 days before calving date until 73 ± 1 DIM. All cows were fed a total mixed ration (TMR) diet that met the energy requirements of the cows according to the NRC (2001). At 21 ± 1 days before expected calving date, cows were randomly assigned to one of two treatments consisting of supplementation with RPM [**MET**; n = 11; RPM at a rate of 0.08% of dry matter (DM): Lys:Met = 2.8:1, (Smartamine ® M Adisseo, Alpharetta, GA, USA)] or no supplementation (**CON**; n = 9; Lys:Met = 3.5:1). Supplementation of Smartamine® M (0.08% DM) was calculated using the data of TMR offered on a DM basis and was provided until 73 ± 1 DIM. The dosage of RPM was established based on Zhou et al (2016).

Endometrial cells at cytological smear

Endometrial cell samples were collected at 15, 30, and 73 ± 1 DIM using a sterile cytology brush (Andwin Scientific, CA, USA). The sterile cytology brush was inserted into a sterile stainless-steel rod (SSR) and then placed into a stainless-steel tube for passage through the cervix. The tube was placed in a sanitary plastic sleeve to prevent contamination of the vagina and the cervix. The vulva was washed with warm water and dried with paper towels before being sprayed and wiped with iodine scrub solution and 70% ethanol. The cytology rod was passed through the first ring of the cervix, where the plastic sleeve was punctured, and the SSR was advanced into the body of the uterus. Once in the uterine body, the stainless-steel tube was pulled back to expose the cytology brush, which was rotated three times while in contact with the

endometrium to collect the samples. The cytology brush was then retracted back into the SSR prior to removal from the cow. The instrument was sanitized with disinfectant or autoclaved between uses. The cytology brush was placed into sterile DNA/RNA free Cryovial tubes (Simport, Beloeil, Quebec, Canada), and frozen in liquid nitrogen.

Endometrial Biopsy

Uterine biopsies of the endometrial lining were performed at 15, 30, and 73 ± 1 DIM using the Eppendorf Biopsy Forceps (Aries Surgical, Davis, CA, USA). Once the cow was restrained, the hair above the intercoccygeal space was trimmed and disinfected with iodine scrub solution and 70% ethanol before lidocaine (5 mL, 2% lidocaine hydrochloride solution, Hospira Inc., Lake Forest, IL, USA) administration into the intercoccygeal space. The vulva and perineal area were cleaned with water, dried, and finally cleaned with iodine scrub solution and 70% ethanol. The biopsy gun was placed in a sanitary plastic sleeve to prevent contamination of the vagina and the cervix and then introduced into the reproductive tract through transrectal palpation and manipulation of the cervix. Once inside the uterine body, the biopsy gun was opened, and a piece of endometrial tissue was positioned inside the forceps of the gun via transrectal palpation. The tissue was removed from the biopsy gun with sterile forceps, placed into sterile DNA/RNA free Cryovial tubes (Simport, Beloeil, Quebec, Canada), and frozen in liquid nitrogen.

RNA extraction, target gene cDNA synthesis and qPCR

The RNA was extracted from both endometrial and cytological smear samples, using the miRNAeasy kit (Quiagen, Hilden, Germany) and following the manufacturer's protocols. Samples were treated on-column with DNaseI (Qiagen), and quantification was accessed using the NanoDrop ND-1000 (NanoDrop Technologies, Rockland, DE, USA). Complementary DNA

was synthesized using 100 ng RNA. Firstly, random primers (10 m*M*) (Invitrogen Corp. CA, USA) and DNase/RNase free water were mixed and incubated at 65ºC for 5 min and kept on ice for 3 min. Then a second mix containing DNase/RNase free water, first strand buffer (5%), oligo dT18 (Operon Biotechnologies, AL, USA), dNTP mix (10 m*M*) (Invitrogen Corp.), RevertAid Reverse Transcriptase (200 U/mL) (Fermentas Inc., MD, USA) and RNase Inhibitor (20 U/mL) (Promega, WI, USA) was added. The reaction was performed in an Eppendorf Mastercycler (Eppendorf North America, Hauppauge, NY, USA) using the following temperature program: 25ºC for 5 min, 42ºC for 60 min, and 70ºC for 5 min cDNA was then diluted 1:3 with DNase/RNase free water.

Primers were designed using Primer Express 2.0 with minimum amplicon size of 80 bp (when possible, amplicons of 100e120 bp were chosen) and limited 30 G þ C (Applied Biosystems). When possible, primer sets were designed to fall across exon-exon junctions. Primers were aligned against publicly available databases using BLASTN at NCBI (Nucleotide BLAST, 2008) and UCSC's Cow (*Bos taurus*) Genome Browser Gateway. Prior to qPCR, primers were tested in a 20 µL PCR reaction using the same protocol described for qPCR except for the final dissociation protocol. For primer testing we used a universal reference cDNA (RNA mixture from different bovine samples) to ensure identification of desired genes. Five µL of the PCR product were run in a 2% agarose gel stained with SYBR safe. Only those primers that did not present primer-dimers and a single band at the expected size in the gel and had the right amplification product (verified by sequencing) were used for qPCR. The accuracy of a primer pair also was evaluated by the presence of a unique peak during the dissociation step at the end of qPCR. Primers are shown in Table 2.1 and the final data were normalized using the geometric

mean of *GAPHD*, *ACTB*, and *H2AFZ*, which were validated as suitable internal control genes in bovine uterine tissue (Cerri et al., 2012; Gomez et al., 2017).

Quantitative PCR was performed using 4 µL diluted cDNA combined with 6 µL of a mixture composed of 5 mL of SYBR Green master mix (Quanta Biosciences, Gaithersburg, MD, USA), 0.4 mL each of 10 m*M* forward and reverse primers, and 0.2 mL DNase/ RNase free water in a MicroAmpTM Optical 384-Well Reaction Plate (Applied Biosystems, CA, USA). Each sample was run in triplicate. A non-template control plus a 6- point relative standard curve were used, according to Taylor et al. (2019). The reactions were performed in an ABI Prism 7900 HT SDS instrument (Applied Biosystems) using the following conditions: 5 min at 95ºC, 40 cycles of 1 s at 95ºC (denaturation) and 1 min at 60ºC (annealing þ extension). The presence of a single PCR product was verified by the dissociation protocol using incremental temperatures to 95ºC for 15 s, 65ºC for 15 s plus 95ºC for 15 s. Data were calculated with the 7900 HT Sequence Detection Systems Software (version 2.2.1, Applied Biosystems). The protocols used for the extraction and qPCR analysis were previously established at Dr. Loor's Lab at the University of Illinois (Zhou et al., 2016; Osorio et al., 2013).

Statistical Analysis

All statistical analyses were performed using SAS 9.4 (SAS Institute Inc. Cary, NC, USA). Cow was considered as the experimental unit. Analyses involving repeated measures over time (e.g., 15, 30 and 73 DIM) were compared among treatments by analysis of variance for repeated measures using the MIXED procedure for the fixed effects treatment (TRT), time (TIME) and their interaction (TRT × TIME). Cow was included as a random effect. The following model was used:

$$Y_{jkl} = \mu + T_j + W_k + (TW)_{jk} + \varepsilon_{jkl}$$

where Y_{jk}= the observations for dependent variables; μ = the overall mean; T_j= the fixed effect of treatment; W_k= the repeated effect of time (DIM); $(TW)_{jk}$ = the interaction between treatment and time; and ε_{jkl}= the random residual error. The estimation method was restrictive maximum likelihood (REML) and the degrees of freedom method was Kenward-Rogers. Variables were subjected to 5 covariance structures: compound symmetry, autoregressive order 1, autoregressive heterogeneous order 1, unstructured, and toeplitz. The covariance structure that yielded the lowest corrected Akaike information criterion was compound symmetry and used in the model. Residual distribution was evaluated for normality and homoscedasticity. Gene expression results were log2-scale transformed if needed to comply with normal distribution of residuals, and subsequently back-transformed. Statistical significance was declared as $P \leq 0.05$, and tendency was declared as $0.05 > P \leq 0.10$.

RESULTS

Cow performance data were described elsewhere (Zhou et al., 2016), as well as uterine health status data (Stella et al., 2018). Briefly, cows that were fed RPM tended to have a lesser incidence of retained placenta and clinical ketosis; additionally, feeding RPM lead to greater DMI in close-up and first 30 DIM (Zhou et al., 2016). Therefore, cows fed RPM had greater milk yield, energy-corrected milk, and fat-corrected milk (Zhou et al., 2016). Regarding the uterine health parameters, cows that were fed RPM had greater percentage of polymorphonuclear (PMN) cells at 15 DIM, but lower at 30 DIM and at 73 DIM, when compared with cows that were not fed RPM (Stella et al., 2018). Moreover, cows that were fed RPM tended to be classified as not having subclinical endometritis at 30 DIM, in comparison with cows that were not fed RPM (Stella et al., 2018).

Results for mRNA expression in cytological smear

Results for mRNA expression in cytological smear samples are in Figure 2.1 and Table 2.2. Methionine supply during the transition period and early lactation increased mRNA expression in cytological smear samples for *FGF7* ($P < 0.01$) and had a tendency for increased *MAT1A* ($P = 0.07$), when compare with cows fed CON (Figure 1). We did not observe any interaction between treatment and time for these two genes ($P > 0.05$), but there was an effect of time ($P < 0.01$) for *FGF7*, with a greater expression of this transcript at 15 and 30 DIM when compared with 73 DIM for cows that received RPM. This was not observed in *MAT1A* ($P = 0.36$). On the other hand, cows that were fed RPM decreased mRNA expression in cytological smear samples for *GLUT4, IL-1β, IL-6, IL-8, PTGES3, TSPO, MUC1* and *SOD1* when compared with cows fed CON ($P < 0.05$). There was an effect of the time ($P < 0.05$) for mRNA expression in cytological smear samples, with decreasing expression over time for all variables except for *MAT1A*. There was a TRT × TIME interaction for *GLUT4* mRNA expression ($P < 0.01$), with higher *GLUT4* mRNA expression for cows fed CON than for cows that were fed RPM at 15 DIM ($P < 0.01$) and a tendency ($P = 0.08$) to greater expression for cows fed CON at 30 DIM when compare with cows that were fed RPM (Figure 1). There was a TRT × TIME interaction for *TNFα* and *P450scc* mRNA expression ($P < 0.05$) and a tendency ($P = 0.10$) for *MTHFR* mRNA expression. There was an effect of time for the expression of *CD45, SAAH, APOE, LIPA, and SOD2* transcripts and a tendency for the expression of *APOL3* and *LCAT* transcripts.

Results for mRNA expression in endometrial biopsy samples

Results for endometrial biopsy samples are in Figure 2.2 and Table 2.3. The expression of *LCAT* transcripts tended to be greater in the endometrium of cows that received RPM ($P = 0.08$). The expression of *FGF7*, *APOL3*, *SAAH*, and *GLUT4* was greater ($P \leq 0.05$) for cows

receiving RPM than for cows fed CON. There was an effect of time ($P = 0.03$) for the expression of *APOL3* transcript in cows that were fed RPM, with greater expression at 73 DIM when compared with 15 DIM and 30 DIM. However, RPM tended to decrease ($P = 0.09$) *IL1B* expression in endometrial biopsy samples. No effect of TIME ($P = 0.57$) or a TRT × TIME interaction ($P = 0.46$) fo*r LCAT, FGF7, SAAH, IL1B* and *GLUT4* genes was observed. There was an effect of time ($P < 0.05$) for the expression of *MTHFR* and *MAT1A* transcripts and a tendency for the expression of *TLR4* ($P = 0.08$) and *EDN2* ($P = 0.09$) transcripts.

DISCUSSION

We aimed to evaluate the effects of feeding RPM on gene expression of immunometabolic compounds found in the endometrium and cytological smear of Holstein cows. Methionine has several biological functions, including antioxidants synthesis and the synthesis of immune-related proteins because supports adequate balance of indispensable amino acids (Osorio et al., 2013; Zhou et al., 2016). As previously reported, these cows that were fed RPM during the transition period had lesser PMN percentage at 30 and 73 DIM, as well as tended be classified as not having subclinical endometritis when comparing with cows not fed RPM (Stella et al., 2018). Our hypothesis is that the supplementation of methionine during the transition period and early lactation modulated the uterine metabolism and immune defense system, which decreased the susceptibility to reproductive tract inflammatory diseases and helped to provide a better uterine environment to favor conception.

Cows fed RPM had increased *FGF7* endometrial gene expression throughout the early postpartum period, which is a crucial period due to the process of uterine involution and regeneration (Gier and Marion, 1968; Wagner and Hansel, 1969; Bazer et al., 2009). These

complex series of events and the epithelial regeneration at caruncular regions are critical components of maintaining the integrity of epithelial barrier (Gier and Marion, 1968). Fibroblast growth factors are also important mediators of intercellular communication, being required alongside hepatocyte growth factor to maintain uterine secretions that are essential for growth and development of the conceptus (Bazer et al., 2020). This is part of a cascade of events that start even before the implantation events, with a downregulation of receptors for progesterone (PGR) and estrogen in the uterine epithelia (Bazer et al., 2009). This loss of PGR expression makes the action of progesterone to be restricted to PGR-positive uterine stromal cells, which express prostamedins such as *FGF7* and *FGF10* (Bazer et al., 2020). Then, the prostamedins act on uterine epithelia and trophectoderm to regulate expression of interferons-stimulated genes, with a following process that is important for establishing uterine receptivity to implantation and maintenance of conception in mammals (Bazer et al., 2020; Spencer et al., 2007). Fibroblast growth factor-7 is a paracrine-acting mitogen and stimulates endometrial stromal cells' proliferation through extracellular signal-regulated kinases (*ERK*) and c-Jun N-terminal kinase (*JNK*) (Subramaniam et al., 2013; Zhou et al., 2017). The *ERK* cascade is thought to regulate proliferation, differentiation, and cytokine production (Dunn e al., 2003) and the *JNK* pathway plays a role in cell growth (Spencer et al., 2007). Endometrial decidualization is a crucial process for embryo implantation and maintenance, with the formation of a decidual lining into which the blastocyst implants; and is also influenced by fibroblast growth factors (Dunn et al., 2003; Zhou et al., 2017). The FGF7 gene has a methionine residue at its amino terminal (Igarashi et al., 1998(, thus an increase in methionine availability could be leading to an increase in the *FGF7* transcript abundance. Zhou et al (2017) suggested that one of the roles of *FGF7* production in stromal/decidual cells is stimulating the expression of insulin-like growth factor-binding protein

I and prolactin, by activating *ERK* and *JNK* in an autocrine manner. Furthermore, the authors also suggested that *FGF7* may be involved in endometrial cells decidualization via other mechanisms besides promoting proliferation, such as through immunomodulatory effects and angiogenesis. One could hypothesize that the greater values of *FGF7* mRNA expression in endometrial samples at 73 DIM for cows fed RPM could lead to increased conception rates and pregnancy maintenance (Zhou et al., 2017).

The immune system responds against bacteria that cause uterine diseases through the stimulation of TLR4-dependent inflammatory responses by endometrial cells (Ju et al., 2014). Then, upon ligands of TLR4, the canonical nuclear factor-kappa B (NF-κB) pathway is activated, which leads to the secretion of proinflammatory cytokines and chemokines, such as IL-1β and IL-8 (Schaefer et al., 2004). Recent review papers have been suggesting that the development of uterine diseases is dependent on the ability of the cow to avoid, tolerate and resist infections with pathogenic bacteria, which characterize this animal as resilient (Sheldon et al., 2019; Sheldon et al., 2020). Avoidance, tolerance, and resistance are complimentary strategies, and preventing uterine diseases from happening could rely on a resilience mechanism. One of the uterine diseases that happen in the early postpartum, endometritis, is believed to be caused by failure to clear bacterial contamination (Gilbert et al., 2007; Sheldon and Dobson, 2004). Galvão et al. (2011) reported an increase in *IL-1β* and *IL-6* in cytological smear and uterine tissue samples of cows with endometritis. Interleukin-1β, along TNF-α, stimulates the expression of *IL-8* and adhesion molecules on vascular endothelial cells (Ghasemi et al., 2012). This leads to chemo-attraction and activation of neutrophils and monocytes, promoting increased phagocytosis and bacterial killing (Kolaczkowska and Kubes, 2013). Ghasemi et al. (2012) reported a greater cytokine expression in cytological smear samples of cows classified as having subclinical

endometritis versus disease-negative cows, with higher *IL-6* and *IL-8* mRNA expression. In the present study, the greater expression of *IL-1β* could be upregulating the expression of *IL-6* and *IL-8* in CON cows. A proper and well-balanced nutrition during the transition period, including the right amount of indispensable amino acids such as RPM, could be one of the best ways to enhance cows' resilience and prevent uterine diseases to happen.

Mucin-1 cell surface associated is an inducible innate immune factor and an important component of the first line of defense against bacterial invasion of epithelial surfaces (Kasimanickam et al., 2014; Gilbert et al., 2007). Therefore, if there is a high expression of *MUC1* we can presume that an inflammation is occurring at that site. In their study, Kasimanickam et al. (2013) reported a greater endometrial expression of *MUC1, IL-β1, IL-8* but not *IL-6* in cows with uterine inflammatory conditions compared with normal cows, which endorses our findings. Lipopolysaccharides increase the mRNA expression of *IL-6* and *IL-8* through DNA methylation and induces greater mRNA gene expression of *MUC1* [42]. Additionally, *MUC1* can have a detrimental effect on pregnancy rates as it can impact pregnancy recognition if not downregulated prior to implantation (Spencer et al., 2007). Uterine receptivity is dependent on progesterone, which in turn is influenced by interferon τ, the main actor in the pregnancy recognition process of ruminants. A downregulation of expression of genes such as *MUC1* need to happen to favor implantation (Bondurant, 1999; Kasimanickam et al., 2014). Therefore, it is possible that the time effect detected in the present study where cows fed CON decreased *MUC1* mRNA expression around 73 DIM is a consequence of the uterine receptivity to implantation process.

Bovine endometrium responds to inflammation by increasing the production of pro-inflammatory mediators, such as prostanoids (Sheldon and Dobson, 2004). Also, IL-1β has been

shown to be a stimulator of prostaglandin production in bovine endometrium (Galvão et al., 2011). When evaluating the eicosanoid pathway expression in bovine endometrial epithelial and stromal cells, Almughlliq et al. (2018) reported upregulation of *PTGES3* mRNA expression when the cells were treated with LPS, *IL1-β* and *TNF-α*. Furthermore, Chapwanya et al. (2009) reported that endometrial epithelial cells exposed to infectious (LPS) and pro-inflammatory agents (*IL-β1* and *TNF-α*) stimulated the production of *PTGES3*. Cows that did not receive methionine in the prepartum period had greater mRNA expression of *PTGES3* in cytological smear samples, another suggestion that they were facing an inflammatory process. Translocator protein 18 kDa has been considered important for cholesterol import to the inner mitochondrial membrane. The clear function of *TSPO* remains unclear, though there is evidence indicating that it plays a role in apoptosis and that the induction of *TSPO* occurs under stress situations, such as inflammation (Chang et al., 2014), as it is seen in the present study of cows fed CON. Another indication of a possible inflammation process occurring in the uterine environment of cows fed CON is the lesser mRNA expression of *APOL3*. Israa et al (2017) reported a downregulation of *APOL3* in ill individuals compared with healthy ones, and in fact the degree of inflammation faced by the ill patients was negatively correlated with the mRNA expression of apolipoproteins. It has been proposed that the apolipoprotein L family expression has a negative correlation with inflammation, since they are involved in the apoptotic process (Israa et al., 2017). Upon activation, neutrophils tend to increase the life span from the onset of inflammation (Israa et al., 2017), therefore a decrease in *APOL3* expression under inflammatory circumstances is not surprising.

Methionine is converted to S-adenosylmethionine, the major methyl donor, in the initial step of the transmethylation pathway (Stipanuk and Ueki, 2011). S-adenosylmethionine is an

important substrate for hepatic synthesis of S-adenosyl homocysteine (Taysi et al., 2015). This reaction is catalyzed by MAT enzymes, which are encoded by *MAT1A* and *MAT2A* (Kotb et al., 1997). Methionine adenosyltransferase 1A is expressed mostly in hepatic tissue (Israa et al., 2017), and this might be the first time the mRNA expression of *MAT1A* was identified in bovine cytological smear samples. Jacometo et al. (2016) reported that maternal supplementation of rumen-protected methionine resulted in upregulation of metabolites of methionine pathway (such as *MAT1A*) in calves. The altered mRNA abundance of methionine and transsulfuration pathways in the cytological smear and uterine samples could be related to the epigenetic changes elicited by essential nutrients in the maternal diet. The greater mRNA of *MAT1A* suggests an elevated concentration of S-adenosylmethionine in response to RPM supply during the prepartum period. In the methionine metabolism, S-adenosyl-homocysteine is converted to homocysteine by S-adenosyl-L-homocysteine hydrolase (*SAAH*). The greater mRNA of *SAAH* reported in this study is probably due to an upregulation following the supply of RPM, as was already reported in liver tissues by Zhou et al (2016) and Osorio et al (2013). The following degradation of homocysteine through the transsulfuration pathway results in the formation of cysteine (Stipanuk and Ueki, 2011). Furthermore, the increase in either cysteine or methionine result in increase of the major metabolites of cysteine (Stipanuk and Ueki, 2011), such as the intracellular antioxidants taurine and glutathione (*GSH*) (Taysi et al., 2015; Kotb et al., 1997). An increase in GSH synthesis can occur because of a depletion in the GSH pool as a response to accumulation of reactive oxygen species (Shih et al., 2003), such as hydrogen peroxide. Hydrogen peroxide formation is a result of dismutation of superoxide into oxygen and hydrogen peroxide by superoxide dismutase enzymes, such as SOD1 (Afonso et al., 2007), which was decreased in cows fed RPM compared to CON. These well-known cellular adaptations were

already described in ruminants supplemented with RPM (Jacometo et al., 2016; Vailati-Riboni et al., 2017). Here, we suggest that the greater abundance of mRNA expression for metabolites of the transsulfuration pathway in conjunction with the lesser mRNA expression of *SOD1* and proinflammatory cytokines and chemokines for RPM fed cows reflect a controlled response to proinflammatory stimuli and resolution of inflammation.

Mounting an effective immune response involves large increase in cellular proliferative and secretory activities, intensifying the rates of gene expression and macromolecular synthesis that also place large bioenergetics demands on cells involved in the process (Kvidera et al., 2017; Plank et al., 1998). The gene expression of *GLUT4*, an important insulin-stimulated glucose transporter (Plank et al., 1998), was greater in cytological smear samples of CON cows compared to cows that received RPM. There is an increase in glucose utilization to initiate the leucocyte activation during an immune response, therefore we expected the greater expression of *GLUT4* for cows fed CON. Glucose is the primary fuel of activated immune cells (Calder et al., 2007) and the cytological smear sample is composed mainly by epithelial cells and polymorphonuclear leukocytes, such as neutrophils (LeBlanc, 2014). Glucose transporter-4 has higher affinity for glucose; thus, it allows for immune cells to compete for glucose in case of a low concentration in the microenvironment where they are present (Thong et al., 2005). Lymphocytes depend on a constant flux of glucose, especially under circumstances where their energy demands increase such as in an activated immune response, since they have low energy-store capacity (Calder et al., 2007). It is important to notice that although there are differences in the gene expression of *GLUT4* observed in the cytological smear and tissue samples, the magnitude of expression of *GLUT4* mRNA was similar in both kinds of samples for cows fed RPM. Additionally, there is a range of glucose-transporter isoforms which are responsive to both

insulin and immune stimulation, and these isoforms differ among different types of cells (Calder et al., 2007). Therefore, it makes sense to have a higher expression of *GLUT4* in samples composed by what it could be a majority of activated immune cells (cytological smear samples) then in uterine epithelial cells that are probably relying more on glucose-transport 2 for basal metabolism.

Pregnancy rates can be impacted by energy availability to the ruminant reproductive tract, particularly if we are talking about energy in the form of lipids (Mattos, 2000). To illustrate, cholesterol is a precursor for steroids such as progesterone, and increased concentrations of cholesterol can improve survival of the embryo through increasing the lifespan of the corpus luteum (Staples et al., 1998). Additionally, Acosta et al (2016) reported an increase in the endogenous lipid reserves of embryos from cows that were fed RPM, which could enhance their survivability. Later, Toledo et al (2017) reported that cows supplemented with RPM had reduced embryo mortality because they had increased capacity to maintain pregnancy and embryonic size. It was also previously hypothesized by Thatcher et al (1994) that the endometrial lipid status could directly impact in the corpus luteum maintenance and embryonic survival through a synergistic action with interferon τ. Lecithin-cholesterol acyltransferase is involved in the cholesterol efflux from cells, since it converts cholesterol into cholesterol ester (Rousset et al., 2009). The higher *LCAT* mRNA expression in uterine tissues from cows receiving RPM is indicative of a greater fatty acid and cholesterol metabolism for these animals, and consequently could be involved in a cholesterol utilization for hormone signaling. Although the lipids profile of the endometrium was not evaluated in the present study, Stella (2018) reported an increase in lipids in uterine tissue at 30 and 73 DIM for cows fed RPM in a similar way. Also, Acosta et al (2016) reported a higher lipid deposition in embryos for cows fed RPM

during the pre and post-partum period. These results taken together shed a light on the mechanisms that are important for maintaining viability of the embryo and, consequently, of the pregnancy.

In conclusion, the lesser mRNA expression of genes involved in inflammatory processes such *as IL6, IL8, IL-1β, PTGES3, TSPO, SOD1,* and *MUC1* are indicatives that cows that are fed RPM throughout the transition period are having a less inflammatory uterine environment after 15 DIM, likely reflecting an improvement in their uterine resilience mechanism and capacity to prevent uterine diseases from happening. Furthermore, the greater mRNA expression of *FGF7, MAT1A, LCAT*, and *SAAH* demonstrate that RPM can also be involved in cells nutrient metabolism and proliferation processes that are crucial for uterine regeneration and preparation for conception. Thus, the authors support increasing intestinal availability of Met during the transition period as its importance and beneficial effects over reproductive immunity, and consequently performance, are highlighted here.

FIGURES AND TABLES

Figure 2.1. Least squares mean and associated standard errors for mRNA expression in cytological smear samples for cows receiving no supplementation (Control -○-) and rumen-protected methionine (RPM -♦-) from 21 days before calving until 73 days after calving. A) *FGF7* = Fibroblast growth factor 7; B) *MAT1A* = Methionine adenosyltransferase 1 alpha; C) *GLUT4* = Glucose transporter 4; D) *IL1β* = Interleukin 1β; E) *IL6* = interleukin 6; F) *IL8* = Interleukin 8; G) *PTGES3* = Prostaglandin E synthase 3; H) *TSPO* = Translocator protein; I) *MUC1* = Mucin 1; J) *SOD1* = Superoxide dismutase 1.

Figure 2.1 (cont.).

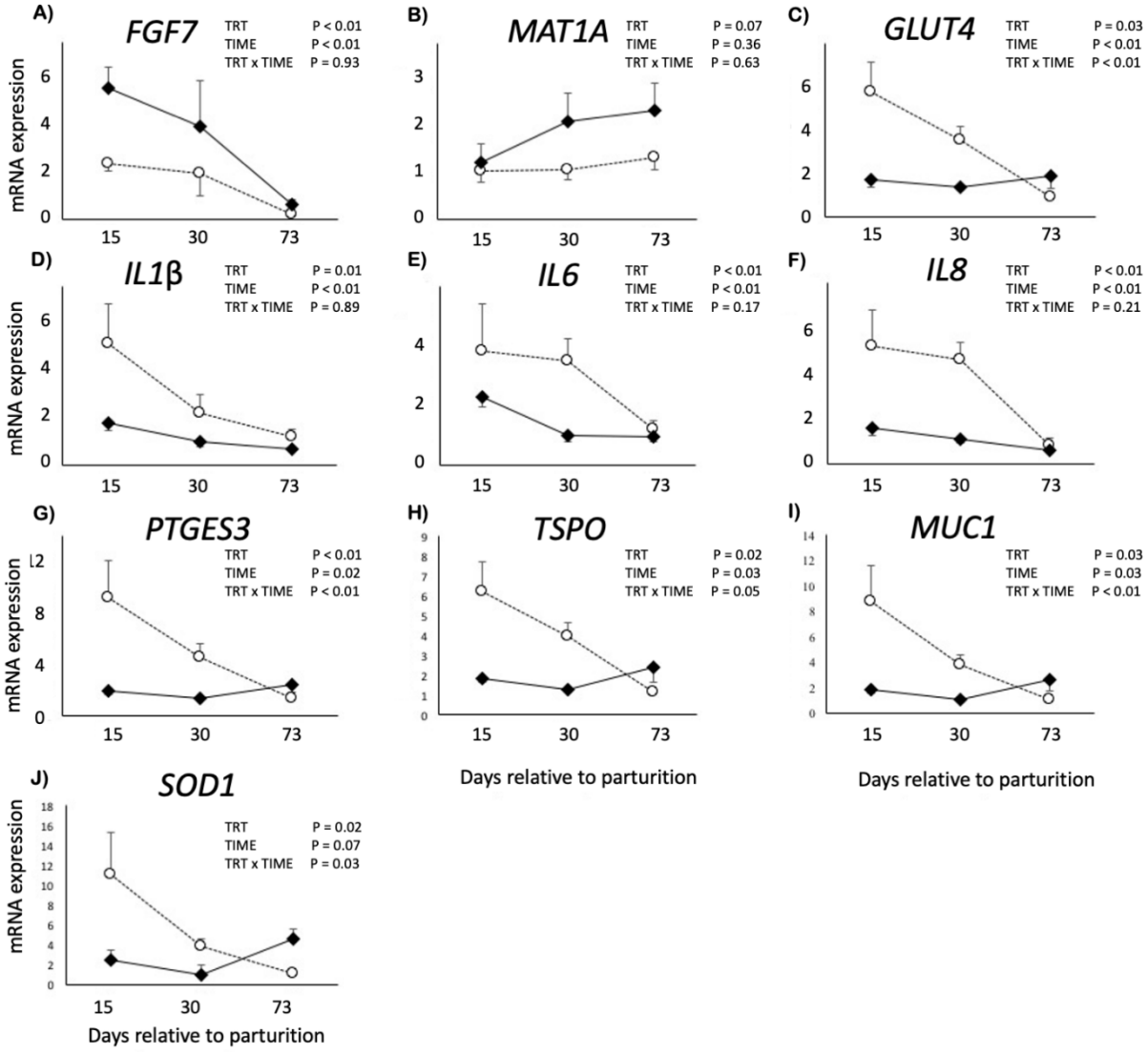

Figure 2.2. Least squares means and associated standard errors for mRNA expression in biopsy endometrial samples for cows receiving no supplementation (Control -○-) and rumen-protected methionine (RPM -♦-) from 21 days before calving until 73 days after calving. A) *LCAT* = Lecithin-cholesterol acyltransferase; B) *FGF7* = Fibroblast growth factor 7; C) *APOL3* = Apolipoprotein 3; D) *SAAH* = S-adenosyl-L-homocysteine hydrolase; E) *IL1β* = Interleukin 1β; F) *GLUT4* = Glucose transporter 4.

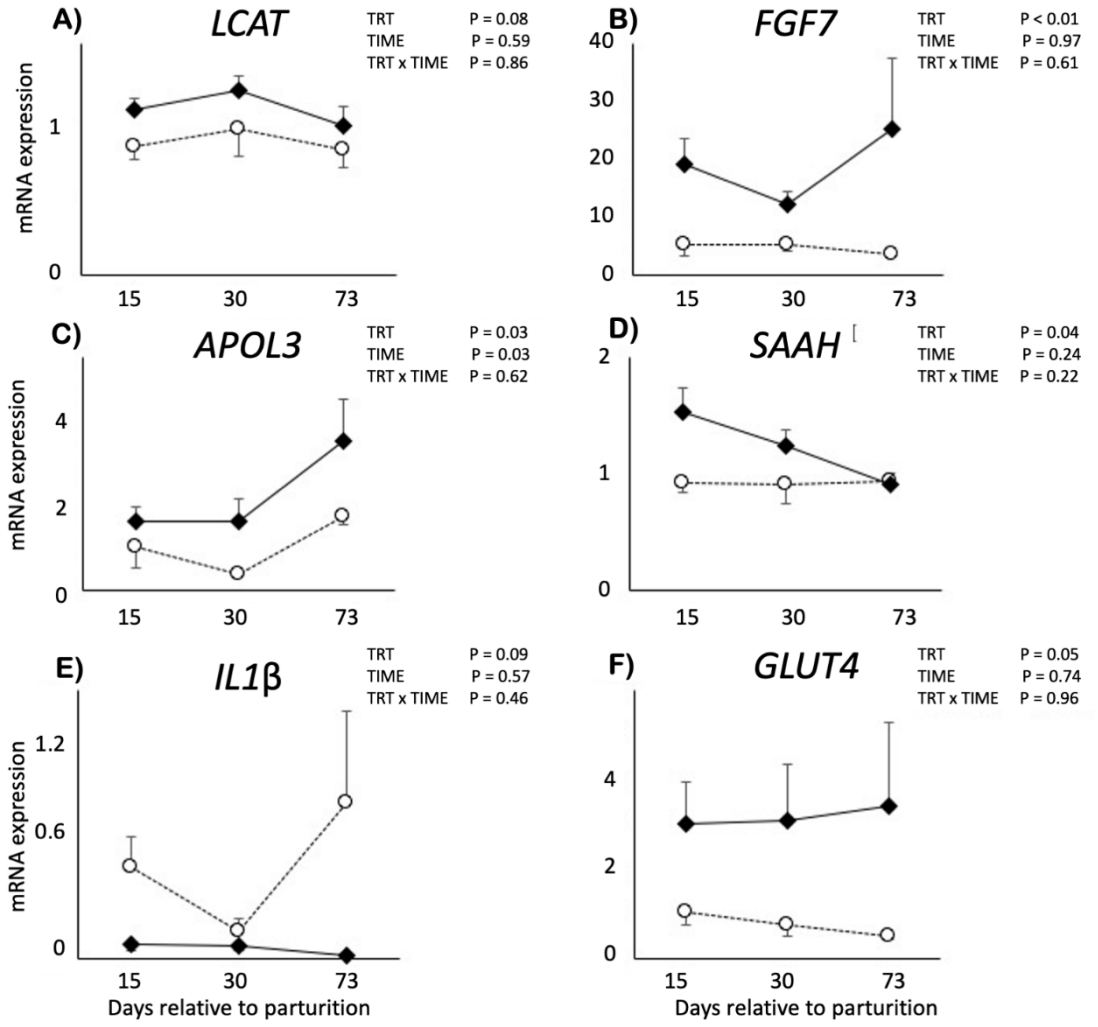

Table 2.1. Hybridization position, sequence, and GeneBank accession number of primers for *Bos Taurus* used to analyze gene expression.

Gene	Gene title	Primer sequence (5' - 3')	Accession number
IL-1β	Interleukin 1 Beta	F: ATTCTCTCCAGCCAACCTTCATT R: TTCTCGTCACTGTAGTAAGCCATCA	NM_174093.1
IL-6	Interleukin 6	F: CCAGAGAAAACCGAAGCTCTCAT R: CCTTGCTGCTTTCACACTCATC	NM_173923.2
IL-8	Interleukin 8	F: GACAGCAGAGCTCACAAGCATCT R: AAGCTGCCAAGAGAGCAACAG	NM_173925.2
TLR4	Toll-like recepter 4	F:TGCGTACAGGTTGTTCCTAACATT R: TAGTTAAAGCTCAGGTCCAGCATCT	NM_174198.6
TNFα	Tumor Necrosis Factor α	F: CCAGAGGGAAGAGCAGTCCC R: TCGGCTACAACGTGGGCTAC	NM_173966.3
CD45	Protein tyrosine phosphatase receptor type C	F:GCAGCAAGTGGTTTGCTCTC R: GCCGAGACTGGGATTGTCAG	NM_001206523.1
CD14	Myeloid Cell-Specific Leucine-Rich glycoprotein	F:TGAACATTGCCCAAGCACAC R:GCCGAGACTGGGATTGTCAG	NM_174008.1
MAT1A	Methionine Adenosyltransferase 1 Alpha	F: GGCACTGTCTATTTCCATCTTTACCTA R: AGTCCAAGTCCCTGACGATAACA	NM_001046497.1
SAAH	S-Adenosyl-L-Homocysteine Hydrolase	F: TGTCAGGAGGGCAACATCTTT R: AGTGCCCAATGTTACACACAATG	NM_001034315.1
MTHFR	Methylenetetrahydrofolate reductase	F: TGAGGGGAGACCCCATAGGT R: TCAGGTGCTTCAGATCAGCC	NM_001011685.1
APOE	Apolipoprotein E	F: CGGTTTCTGGAGGCGAAGAA R: ATATCCGCCTGGCATCCTGC	NM_173991.2

Table 2.1 (cont.)

APOL3	Apolipoprotein L,3	F: GGCGACTCTTCACCAAGGAA R: CTTCACCCCATGTTGGCATC	NM_001100351.1
FGF7	Fibroblast growth factor 7	F: ATTCTCTCCAGCCAACCTTCATT R: TCTCCTCCACTGTGTGTCCA	NM_001193131.1
LCAT	Lecithin-cholesterol acyltransferase	F: CCGTCATCCTCGTGCCC R: TTGCGGTAGCACATCCAGTT	NM_001046069.2
LIPA	Lipase A, lysosomal acid type	F: AGGACGGCTGCAGAATGAAA R: CATCCAGATGCCTGGGAAGG	NM_001103323.1
TSPO	Translocator protein	F: GACAGCAGAGCTCACAAGCATCT R: GAACCATACCCCATGGCCG	NM_175776.2
GLUT4	Glucose Transporter Type 4	F: AGGACGGCTGCAGAATGAAA R: CCAGGCCCAGGAGATGGA	BC114082.1
SOD1	Superoxide Dismutase 1 Both	F: GGCTGTACCAGTGCAGGTCC R: GCTGTCACATTGCCCAGGT	XM_005201085.1
SOD2	Superoxide Dismutase 2	F: TGTGGGAGCATGCTTATTACCTT R: TGCAGTTACATTCTCCCAGTTGA	NM_201527.2
EDN2	Endothelin 2	F: GAGCGTGCCTCACCACTG R: CTCTTGCCTTCGTGCAGGG	NM_175714.2
MUC1	Mucin 1, Cell Surface Associated	F: ACCATTGCCTGCAGAAACCT R: CATTGCCCTGGTTGTGTGTC	NM_174115.2
PTGES3	prostaglandin E synthase 3	F: AAGGGCAAAGCTTAATTGGC R: CCACCCATGTTGTTCATCATCT	NM_001007806.2

Table 2.1 (cont.)

P450scc	Cytochrome P450 family 11, subfamily A, member 1	F:CGTCAGCCTCCTGCACAAG R:GGTGATGGACTCAAAGGCAAA	NM_174093.1
IGF-I	Insulin-Like Growth Factor 1	F: CCTGGATTTCTTTTTGCCTCAT R: GGTGAAGGCGAGCAAGCA	NM_001077828.1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	F: TTGTCTCCTGCGACTTCAACA R: TCGTACCAGGAAATGAGCTTGAC	NM_001034034.2
ACTB	Actin Beta	F: ACCAACTGGGACGACATGGA R: GTCTCGAACATGATCTGGGTCAT	NM_173979.3
H2AFZ	H2A histone family, member Z	F: GAGGAGCTGAACAAGCTGTTG R: TTGTGGTGGCTCTCAGTCTTC	NM_174809.2

Table 2.2. Least squares means and associated standard errors for mRNA expression in cytological samples for cows receiving no supplementation (CON) and rumen-protected methionine (RPM) from 21 days before calving until 73 days after calving.

		Day[a]				P-value		
Gene	**Treatment**	**15**	**30**	**73**	**SEM**	**TRT[b]**	**TIME[c]**	**TRTxTIME[d]**
TLR4	CON	2.76	1.41	1.23	0.60	0.34	0.16	0.81
	RPM	3.06	1.91	1.26				
TNFα	CON	4.42^{a}	3.77^{a}	1.30^{b}	0.79	0.31	0.16	0.02
	RPM	2.21^{b}	1.47^{b}	2.93^{b}				
CD45	CON	2.87^{a}	2.23^{a}	6.99^{b}	1.04	0.49	0.02	0.53
	RPM	1.79^{a}	4.07^{b}	8.31^{c}				
CD14	CON	1.67	1.14	1.18	0.50	0.15	0.71	0.89
	RPM	2.40	1.87	1.51				
SAAH	CON	2.70^{a}	1.27^{b}	0.45^{c}	0.29	0.66	<0.01	0.94
	RPM	2.01^{a}	0.63^{b}	0.42^{b}				
MTHFR	CON	2.96^{A}	5.52^{A}	1.03^{AB}	3.34	0.78	0.32	0.10
	RPM	2.07^{A}	10.4^{C}	5.54^{A}				
APOE	CON	6.50^{a}	1.53^{b}	0.50^{c}	0.89	0.38	<0.01	0.99
	RPM	4.78^{a}	0.91^{b}	0.36^{b}				
APOL3	CON	20.1^{A}	2.32^{B}	3.24^{B}	7.3	0.77	0.07	0.41
	RPM	9.36^{A}	11.6^{A}	20.8^{B}				
LCAT	CON	1.33^{A}	2.89^{B}	1.62^{A}	0.90	0.20	0.08	0.11
	RPM	0.62^{A}	0.32^{A}	1.51^{B}				
LIPA	CON	6.48^{a}	2.14^{b}	0.60^{c}	1.11	0.63	<0.01	0.51
	RPM	4.16^{a}	1.15^{b}	0.81^{b}				
SOD2	CON	9.10^{a}	2.51^{b}	0.71^{b}	2.29	0.51	0.01	0.13
	RPM	6.03^{a}	2.59^{b}	3.00^{b}				
EDN2	CON	0.75	0.56	0.12	0.20	0.97	0.17	0.21
	RPM	0.26	0.49	0.26				

Table 2.2 (cont.)

P450scc	CON	3.92[a]	3.73[a]	1.22[b]	0.50	0.22	0.17	<0.01
	RPM	2.20[a]	1.41[b]	2.77[a]				
IGF-I	CON	0.89	1.29	1.45	0.27	0.48	0.28	0.79
	RPM	0.93	1.09	1.53				

[a]Days relative to the moment of sampling at 15, 30, and 73 days after calving using a cytology brush.
[b]TRT: Treatments, a close-up and a fresh cow diet from 21 days before calving until 73 d after calving.
[c]TIME: 15, 30 and 73 days relative to the calving.
[d]TRT × TIME: Treatment by time interaction.
Means with different superscripts differ (lowercase superscripts $P \leq 0.05$; uppercase superscripts $0.05 > P \leq 0.10$).

Table 2.3. Least squares means and associated standard errors for mRNA expression in endometrial biopsy samples for cows receiving no supplementation (CON) and rumen-protected methionine (RPM) from 21 days before calving until 73 days after calving.

		Day[a]				P-value		
Gene	Treatment	15	30	73	SEM	TRT[b]	TIME[c]	TRTx TIME[d]
IL-6	CON	1.25	1.85	1.29	0.60	0.93	0.85	0.84
	RPM	1.51	1.04	1.14				
TLR4	CON	3.70^{A}	2.76^{A}	0.65^{B}	0.95	0.92	0.08	0.95
	RPM	1.83^{A}	1.45^{A}	0.64^{B}				
TNFα	CON	0.29	0.72	0.96	0.21	0.62	0.41	0.46
	RPM	0.61	0.15	0.02				
CD45	CON	1.59	2.18	1.28	0.35	0.46	0.86	0.90
	RPM	0.84	1.11	0.70				
CD14	CON	1.87	1.84	1.02	0.39	0.89	0.33	0.65
	RPM	2.70	1.39	0.86				
MTHFR	CON	0.54^{a}	0.55^{a}	3.1^{b}	0.27	0.73	<0.01	0.12
	RPM	1.03^{a}	0.98^{a}	1.78^{b}				
APOE	CON	1.61	2.29	1.41	0.31	0.50	0.58	0.68
	RPM	1.59	1.36	0.98				
SOD1	CON	0.97	0.92	1.10	0.12	0.44	0.42	0.22
	RPM	1.67	1.33	1.12				
SOD2	CON	0.64	0.72	1.00	0.13	0.36	0.45	0.29
	RPM	0.97	0.58	0.38				
IL-8	CON	0.70	3.17	2.61	0.54	0.78	0.97	0.86
	RPM	1.25	0.96	1.08				
MAT1A	CON	0.53^{a}	0.50^{a}	3.45^{b}	0.39	0.23	<0.01	0.82
	RPM	0.79^{a}	0.59^{a}	3.48^{b}				
EDN2	CON	1.75^{A}	2.92^{B}	1.34^{A}	0.52	0.72	0.09	0.35
	RPM	0.61^{A}	2.37^{B}	0.63^{A}				
LIPA	CON	0.69	0.62	0.66	0.11	0.87	0.57	0.38
	RPM	1.06	0.50	0.61				
TSPO	CON	1.67	1.49	0.67	0.39	0.60	0.57	0.69
	RPM	1.19	0.85	0.68				
P450scc	CON	2.53	1.11	1.78	0.28	0.47	0.44	0.14
	RPM	1.76	2.51	1.47				

Table 2.3 (cont.)

IGF-I	CON	0.54[a]	0.77[b]	1.31[c]	0.19	0.95	0.04	0.86
	RPM	0.37[a]	0.49[a]	1.51[b]				
PTGES3	CON	1.25[a]	0.82[a]	2.29[b]	0.66	0.79	0.04	0.19
	RPM	0.66[a]	3.86[b]	1.94[c]				

[a]Days relative to the moment of sampling at 15, 30, and 73 days after calving using a cytology brush.
[b]TRT: Treatments, a close-up, and a fresh cow diet from 21 days before calving until 73 d after calving.
[c]TIME: 15, 30 and 73 days relative to the calving.
[d]TRT × TIME: Treatment by time interaction.
Means with different superscripts differ (lowercase superscripts $P \leq 0.05$; uppercase superscripts $0.05 > P \leq 0.10$).

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CHAPTER 3: EFFECT OF FEEDING RUMEN-PROTECTED LYSINE THROUGH THE TRANSITION PERIOD ON POSTPARTUM UTERINE HEALTH OF DAIRY COWS[2]

ABSTRACT

Feeding rumen-protected methionine as an indispensable amino acid (IAA) source has been shown to improve reproductive performance in dairy cows, but the effect of feeding rumen-protected lysine (RPL) during the peripartum period on reproductive performance is not well explored. Therefore, we aimed to determine the effects of feeding RPL (AjiPro-L Generation 3, Ajinomoto Heartland Inc., Chicago, IL) prepartum, postpartum, or both on follicular dynamics, uterine health, and mRNA gene expression of the endometrium. Seventy-five multiparous Holstein cows were assigned to 1 of 2 dietary treatments with or without RPL in a randomized, complete block design. A 2 × 2 factorial arrangement of treatments was used. Prepartum (–28 d to calving), animals were fed a diet (68% of dietary DM from forage) with RPL [PRE-L; 0.54 % RPL of dietary dry matter intake (DMI)] or without RPL (PRE-C). After calving, half of the cows from each prepartum treatment group were assigned to a diet (56% forage) with RPL (PRE-L POST-L; PRE-C POST-L; 0.40 % RPL of dietary dry matter intake) or without RPL (PRE-C POST-C; PRE-L POST-C) until 28 days in milk (DIM). Vaginal discharge was detected with a Metricheck® device (MC, Simcro, New Zealand) to detect metritis, and at 28 DIM polymorphonuclear leukocytes (PMN) were evaluated as a percentage of the epithelial cells using a cytology brush (Andwin Scientific, CA) and an endometrial tissue biopsy was collected for mRNA expression and histology. The first postpartum follicular growth cycle was monitored

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at 7, 10, 11, 13, 15, 17, 19, 21, 23, 25, 27, and 28 DIM via transrectal ultrasonography. Time to first ovulation did not differ between treatments and averaged 18 ± 1.6 DIM. Follicular diameter at first ovulation was not affected by the treatments ($P = 0.13$), but the growth rate of dominant follicle before first ovulation tended to be lower for cows in POST-L in comparison with cows in POST-C ($P = 0.10$). Prevalence of fetid vaginal discharge and metritis did not differ between treatments. Cows in PRE-L POST-L had lower PMN percentage at 15 and 28 DIM than cows in PRE-L POST-C, PRE-C POST-L, and PRE-C POST-C. Feeding RPL prepartum downregulates the expression of *TLR4*, *SLC7A6*, *EHMT2*, and tends to downregulate the expression of *PTGES3* in uterine tissues at 28 DIM. Additionally, it upregulates the expression of *APOL3* and *NFKB1*, and tends to upregulate the expression of *AHCY* and *MAT2A*. In conclusion, feeding RPL pre- and postpartum improved indicators of uterine immune status, but did not change days to first ovulation postpartum.

Key words: amino acid, inflammation, uterus, subclinical endometritis

INTRODUCTION

Nearly all dairy cows experience at least some degree of negative energy (Drackley, 1999) and protein (Larsen et al., 2014) balance during early postpartum. Coupled with an immunosuppression state (Pascottini and LeBlanc, 2020), this metabolic status is associated with increased risk of uterine diseases among other metabolic disorders (Velazquez et al., 2019). This is partly a result of impaired endometrial function, as a decrease in the energy supply can alter the inflammatory response and increase the risk of uterine diseases (Sheldon et al., 2017). Additionally, the mechanisms for adaptation to negative energy balance such as mobilization of fatty acids from adipose tissue may contribute to reduced innate immune function, which increases the risk of reproductive diseases (LeBlanc, 2020). Thus, in this critical period for the dairy cows' productive life, there might be competing demands for nutrients for lactation and for immune response, including AA (Iseri and Klasing, 2014). Lysine (Lys) and methionine (Met) are considered the most limiting AA in dairy cows' diets, particularly in the US, and research indicates that it should be fed at a ratio of 3:1 to optimize milk production (NRC, 2001). Although focusing on the ratio of Lys to Met could be of practical use when formulating diets, it could lead to deficiencies of these AA when actual DMI does not meet the predicted, such as during the transition period (Vyas and Erdman, 2009). Therefore, quantifying the indispensable AA (IAA) is a more accurate approach, and providing these IAA as a ruminal-protected source improves the duodenal flow of AA (Patton, 2010; Robinson, 2010). For instance, reports indicate increased milk yield, milk protein, and DMI upon supplementation of rumen-protected methionine (RPM) and rumen-protected lysine (RPL) on Holstein cows' diets (Xu et al., 1998; Socha et al., 2005; Zhou et al., 2016; Batistel et al., 2017). Additionally, greater MP and Lys intake during the pre-calving period increased DMI postpartum (Girma et al., 2011; Fehlberg et al., 2020).

The reproductive success of postpartum dairy cows is associated with multiple factors, such as uterine health, involution and regeneration, and ovarian resumption (Galvao et al., 2004; Chebel et al., 2006; Santos et al., 2009; LeBlanc, 2014; McCoy, 2006). Innate immunity is crucial for the health of the reproductive tract of dairy cows following parturition and is affected by AA supply (Batistel et al., 2018; Zhou et al., 2016). Uterine infection is common in the postpartum period and can have a detrimental effect on ovarian and uterine function (Bromfield and Sheldon, 2013). Therefore, improving immune function and reducing the risk of reproductive tract inflammatory diseases could lead to better reproductive outcomes. Uterine infections can also be detrimental to ovarian resumption, since inflammation can impact the first dominant follicle (DF) growth and function through neuroendocrine mechanisms of inhibition of hypothalamic GnRH release and pituitary LH secretion (Williams et al., 2001). Moreover, there is also evidence of direct localized inflammatory mediators, resulting from uterine bacterial contamination after calving, affecting the ovary by suppressing estradiol secretion and decreasing the growth rate of follicles (Sheldon et al., 2002). Additionally, chronic inflammation can result in the disruption of uterine regeneration processes in the early postpartum period (LeBlanc, 2014; Lucy et al., 2003), which can potentially alter the functional capacity of the uterus (Gray et al., 2001a) and future reproductive efficiency (Gray et al., 2001b). Therefore, ovarian resumption could benefit from modulation of the uterine immune response through nutritional strategies. However, the effects of feeding RPL on the reproductive tract physiology and immune response are still lacking.

Research conducted mainly in monogastric animals provided evidence of the immune system requirements for Lys; for example, Lys consumption by the immune system increased 10-fold in an LPS challenge in poultry (Klasing and Calvert, 1999). Lysine can also play a role

in biosynthesis processes, such as the synthesis of acute-phase proteins in response to an increase in circulating cytokines (Iseri and Klasing, 2014) or the synthesis of non-essential amino acids (Lapierre et al., 2009). These processes are pertinent to and activated during the transition period when the immune response of the high-producing dairy cow is activated and the animal is under a state of systemic inflammation (Bradford et al., 2015; Pascottini et al., 2020). Though there is limited research in dairy cows relating Lys supply to immune response and inflammatory status, there is evidence of decreased inflammatory response upon supplementation of RPL through the transition period (Fehlberg et al., 2021(Abstr.)). The decreased inflammatory response is demonstrated by and increased in negative acute-phase proteins, a decrease in positive acute-phase proteins, and downregulation of interleukin-1β prepartum and interleukin-8 and serum amyloid A3 (Fehlberg et al., 2021(Abstr.)).

Therefore, our objective was to determine the effects of feeding RPL prepartum, postpartum, or both on uterine health, endometrial morphology, and transcriptional expression of genes related to endometrial metabolism and immunity of multiparous Holstein cows. Additionally, we aimed to evaluate whether supplementation with RPL could impact follicular dynamics of the first follicular wave postpartum. Our experimental design is unique in allowing for the evaluation of the effects of prepartum and postpartum supplementation of RPL separately or the effect of prepartum supply of RPL on postpartum outcomes. We hypothesized that supplementing RPL would improve markers of uterine health due to modulation of uterine metabolism and immune defense system, leading to earlier ovarian resumption.

MATERIAL AND METHODS

Animal Care and Housing, and Experimental Design

All experimental procedures were approved by the University of Illinois (Urbana-Champaign) Institutional Animal Care and Use Committee (#18157). Animal handling, experimental design, and diets have been previously described in depth by Fehlberg et al. (2020). Based on the tertiles for ME305, cows were categorized into low, intermediate, or high ME305, and a similar concept was used for BCS. Eighty-nine multiparous Holstein cows were blocked by lactation number (3.3 ± 1.1), previous 305-d mature-equivalent milk production (11,363 ± 1,860 kg), expected calving date, and body condition score (BCS) during the far-off period (3.76 ± 0.84). Each block had 4 cows in it, with exception one block that contained 6 cows. Cows were then assigned to 1 of 2 dietary treatments [TMR with or without RPL (AjiPro-L Generation 3; 42% L-Lys-HCl; Ajinomoto Heartland Inc., Chicago, IL)] for the prepartum period. Prepartum (-28 d to calving), animals were fed a diet with RPL [**PRE-L** (n = 38); 0.54 % RPL of dietary DMI] or without RPL [**PRE-C** (n = 37)] top dressed in a carrier of 300 g of dried sugarcane molasses. After calving, half of the cows from each prepartum treatment group were assigned to a diet with RPL [**PRE-L POST-L** (n = 18); **PRE-C POST-L** (n = 19); 0.40 % RPL of dietary DMI] or a diet without RPL [**PRE-C POST-C** (n = 18); **PRE-L POST-C** (n = 20)] until 28 DIM, in a randomized, complete block design, using a 2 × 2 factorial arrangement of treatments. Cows not fed with RPL received 300 g of dried sugarcane molasses only. The number of cows per treatment was calculated for the study reported by Fehlberg et al. (2020) to detect a minimum of 1.1 ± 0.75 kg/d difference in postpartum DMI between groups, assuming a power of 0.9 and a two-tailed α of 0.05. Additional calculations were made to ensure that the experimental design had the power to detect a minimum of 4 ± 0.5 % in endometrial cytology from different independent groups, assuming a power of 0.8 and a two-tailed α of 0.05. This

additional power analysis determined that, to detect such a difference among groups for PMN % evaluations, it would be required a minimum of 16 cows per treatment group, which was met by the experimental design. The exclusion criteria included calving with twins or not having consumed the treatment for at least 16 d during the prepartum period. Four cows were excluded due to twins (PRE-C POST-C, n = 1; PRE-C POST-L, n = 1, PRE-L POST-C, n = 1; PRE-L POST-L, n = 1) and 1 cow was excluded for calving too early (PRE-L POST-C, n = 1). Nine cows were excluded postpartum due to health problems (PRE-C POST-C, n = 1; PRE-C POST-L n = 1; PRE-L POST-C n = 2; and PRE-L POST-L, n = 5). A total of 75 cows concluded the study.

According to the manufacturer, there is 80% rumen bypass and 80% intestinal digestibility to result in 64% bioavailability of this encapsulated RPL product. This would provide 1.4 g of intestinally available Lys prepartum and 1.0 g of intestinally available Lys postpartum per kg of DMI (Miura et al., 2017). Top dress was applied to the TMR once daily following morning feeding. The amount of RPL top-dressed was adjusted daily and for each cow based on their individual DMI of the previous day. Cows that were not receiving RPL were top-dressed with 300g of dried sugarcane molasses. Daily DMI was determined for each cow by weighing refusals and total amount fed and determining the difference. Cows were fed for 10 % refusals to allow for ad libitum feed intake. All cows had free access to water. Diets (TMR) were formulated using AMTS.Cattle.Pro version 4.7 (2017, AMTS, LLC, Groton, NY) to meet or exceed recommendations. Prepartum diet was formulated for dry cows at 694 kg of BW and a predicted DMI of 13 kg/d. Postpartum diet was formulated for cows at 14 DIM, 733 kg of BW, producing 39 kg of milk/d with a target of 3.7% milk fat and 3.2% milk protein, and a predicted DMI of 19 kg/d. According to AMTS.Cattle.Pro prediction, cows in PRE-C received 1.17 kg/d

of MP, resulting in 6.86% MP as Lys and 2.98% MP as Met with a Lys:Met ratio of 2.30, while cows in PRE-L received 1.19 kg/d of MP, resulting in 8.24% MP as Lys and 2.94% MP as Met with a Lys:Met ratio of 2.80. Cows in PRE-L POST-C and PRE-C POST-C consuming the postpartum diet received 2.28 kg/d of MP, resulting in 6.27% MP as Lys and 2.54% MP as Met, with a Lys:Met of 2.46; whereas cows in PRE-L POST-L and PRE-C POST-L received 2.22 kg/d of MP, resulting in 7.15% MP as Lys and 2.55% MP as Met, with a Lys:Met of 2.80.

Ultrasonography of Ovarian Structures

The first postpartum follicular growth cycle was monitored at 7, 10, 11, 13, 15, 17, 19, 21, 23, 25, 27, and 28 DIM via transrectal ultrasonography (IBEX Pro, 7.5-MHz linear array probe, E.I. Medical Imaging, Loveland, Colorado). Patterns of development of the follicles from the first follicular wave postpartum were mapped and evaluated following descriptions adapted from Sirois and Fortune (1988) and reported in Ryan et al. (2020). For every cow, each ovary was scanned several times and in at least more than one plane or direction (lateral to medial direction, medial to lateral direction, dorso-ventral, intermediate oblique, and cranio-caudal). All measurements were performed by the same trained person. Ultrasound videos of ovarian structures were recorded to allow measurement of the follicles. Follicular structures from both ovaries were and patterns of development were characterized by follicular mapping. Diameter of follicles were measured through ImageJ software (version 1.47, National Institutes of Health, MD), using a software tool calibrated against a scale provided by the ultrasound unit. Measurements of all follicles ≥ 5 mm in diameter were recorded and a dominant follicle was defined as a follicle > 10 mm in diameter in the absence of other follicles (Beam and Butler, 1995; Savio et al. 1990). From there, only the DF was mapped until its disappearance or until 28 DIM, the last day for ultrasound measurements. Ovulation was classified as the disappearance of

the previously identified DF and the appearance of a *corpus luteum* (CL) in the subsequent examinations. Growth rate was calculated as the difference in diameter from the last measurement by the first measurement, divided by the difference in days from those two measurements. A cow was considered to have a follicular cyst if the ovarian structure had a thin walled (≤ 3 mm) round, anechoic density consistent with the presence of an antrum > 25 mm in diameter, in the absence of a corpus luteum (Hamilton et al., 1995; Garverick, 1997). By the end of the experiment, a CL was not detected in 25 cows. From these, 14 cows developed follicular cysts (PRE-L POST-L = 4; PRE-L POST-C = 6; PRE-C POST-L = 2; PRE-C POST C = 2) and 11 cows had just not ovulated by 28 DIM (PRE-L POST-L = 1; PRE-L POST-C = 5; PRE-C POST-L = 1; PRE-C POST-C = 4).

Vaginal Discharge Evaluation

Evaluations of vaginal discharge were performed at 4, 7, 10, 13, 15, and 17 DIM. These days were chosen because although technically described as occurring at any time within 21 DIM (Sheldon et al., 2006; LeBlanc et al., 2011), the majority of metritis cases occur in the first 14 DIM, peaking at around 5 to 7 DIM (Galvão et al., 2011). The evaluation was performed using the Metricheck® device (MC, Simcro, New Zealand) following guidelines presented in LeBlanc and Bicalho (2017). The device was composed of a 50 cm long stainless-steel rod with a 4 cm rubber hemisphere to collect vaginal contents. The MC device was disinfected with chlorhexidine diacetate (Nolvasan Solution, Zoetis Animal Health, Florham Park, NJ) before and after each use in a single cow. The evaluation began with cleaning the perineal region of the cow with a paper towel and disinfectant solution. The tail was moved to the side and the MC was inserted into the vaginal canal until the cervix was reached. The device was then retracted and removed from the reproductive tract with the vaginal contents remaining in the rubber

hemisphere. With the vaginal content in the rubber hemisphere, evaluation of smell was scored (smell 0 = no odor or smell 3 = fetid odor). The vaginal content was then poured onto a paper towel for examination and scored on a scale of 0 to 3: score 0 = clear or translucent mucus; score 1 = mucus containing flecks of white or off-white pus; score 2 = discharge containing ≤ 50% white or off-white mucopurulent material; and score 3 = discharge containing ≥ 50% purulent material, which may be white, yellow, or sanguineous (Sheldon et al., 2006). Cows were classified as having metritis if the MC score plus smell was equal or exceeded 3, meaning a fetid odor was detected along mucus- or mucopurulent characteristic of the discharge.

Cytology of the Uterine Endometrium

Cytology of the endometrium was performed using a cytology brush (Andwin Scientific, CA) at 28 DIM. The sterile cytology brush was mounted to a sterile stainless-steel rod and inserted into a larger sterile stainless-steel rod (SSR) covered with a plastic sleeve for passage through the cervix and into the uterine body without contamination. Prior to the procedure, the cow was restrained, and the vulva was cleaned with water and 70% ethanol. After passage of the cytology rod through the first ring of the cervix, the SSR was exposed through the plastic sleeve and was advanced into the uterine body. Once inside the uterine body, the outer SSR was pulled back to expose the cytology brush. The SSR that was mounted to the cytology brush was then rotated three times while the cytology brush remained in contact with the endometrium. Finally, the cytology brush was retracted back into the outer SSR and removed from the reproductive tract. The SSRs were washed and autoclaved between each day of use. If multiple samples were being taken within a single day, the SSRs were sanitized in a chlorhexidine diacetate disinfectant solution between each animal. Cytology slides were prepared immediately by rolling the cytology brush onto a clean glass microscope slide and fixed using a cytology fixative

(Cytoprep, Fisher Scientific, Pittsburg, PA). Once the fixative was dry, the samples were transported to the laboratory where they were stained (Camco Quik Stain 2 – Self Buffered Differential Wright-Giemsa Stain, Cambridge Diagnostic Products, FL). After being allowed to dry for 24 h, the slides were covered using mounting medium (Permount, Fisher Scientific, Pittsburg, PA) and dried for at least 48 h before being scanned. Following guidelines described in Stella et al. (2018), all slides were scanned at the Institute for Genomic Biology at the University of Illinois with 20× magnification using whole slide imaging (Nanozoomer Digital Pathology System, Hamamatsu Photonics, Japan). Five areas were captured at 20× magnification from five separate locations, one image from each corner of the sample area of the slide and one image from the center, to represent the entire the slide (NDP.view software, Hamamatsu Photonics). A minimum of 100 cells were manually counted using the software ImageJ (National Institutes of Health, MD) to determine the percentage of PMN (PMN/ (PMN + epithelial cells)). Cell counting was performed by the same technician for all samples. Seven samples were excluded due to staining issues (PRE-L POST-L n = 1; PRE-C POST-L n = 2; PRE-C POST-C n = 4).

Endometrial Biopsy

Endometrial tissue samples were collected transcervically from the body of the uterus at 28 ± 2 DIM. Biopsy samples were harvested for histological evaluation of uterine glands and expression of transcripts in the endometrial tissue. Uterine biopsy was not performed on 3 cows (PRE-L POST-C = 1, PRE-C POST-L = 1, and PRE-C POST-C = 1) due to MC score plus smell > 3. The biopsy instrument (48 cm in length; 2 cm diameter; Aries Surgical, Davis, CA) was covered with a sanitary disposable sleeve and inserted into the vagina. The biopsy forceps were positioned at the cervical opening, and the sleeve was retracted over the instrument. The exposed

biopsy forceps were then threaded through the cervix and into the uterine body. Endometrial tissue was collected at a location approximately 1 cm beyond the end of the cervix. A subset of the sample was flash-frozen in liquid nitrogen for gene expression analysis. The other subset of the sample was placed into phosphate buffered saline (PBS) containing 4% paraformaldehyde for 24 h. The samples were then set in a block of paraffin wax at the University of Illinois Veterinary Diagnostic Lab for hematoxylin and eosin staining.

Hematoxylin and eosin stain for endometrial gland analysis. After whole slide scanning, individual gland structures were labeled and images were captured (NDP.view software, Hamamatsu Photonics, Japan). Total glandular area and perimeter, glandular epithelial height, number of glandular epithelial cells, and number of glands per tissue sample were manually measured through ImageJ software (version 1.47, National Institutes of Health, MD). The same trained technician obtained all glandular measurements. Twenty-two samples were excluded due to incorrect processing (PRE-L POST-L n = 5; PRE-L POST-L n = 6; PRE-C POST-L n = 5; PRE-C POST-C n = 8).

RNA Extraction and Real Time Quantitative PCR

The extraction and quantitative PCR analysis were performed using previously established protocols from the Mammalian NutriPhysioGenomics Laboratory at the University of Illinois (Vailati-Riboni et al., 2015). All evaluated transcripts and primer information are reported in the supplemental materials (Supplemental Tables A.1-A.2). Primers were chosen based on previous research demonstrating the expression of such targets involved in inflammatory and metabolic processes in the bovine reproductive tract (Brewer et al., 2020; Guadagnin et al., 2021). To control analytical and tissue sampling variation, the final data were normalized to expression of the geometric mean of *GAPHD*, *ACTB*, and *H2AFZ*, which were

validated as suitable internal control transcripts in bovine uterine tissue (Cerri et al., 2012; Gómez et al., 2017). The delta CT (ΔCT, cycle threshold) for each gene was calculated following guidelines reported by Schmittgen and Livak (2008), by subtracting the geometric mean of the CT of *GAPDH*, *ACTB*, and *H2AFZ* from the CT of the gene of interest. Fold change was calculated using the $2^{-\Delta\Delta CT}$ method, as described by Schmittgen and Livak (2008).

For RNA extraction from endometrial cells, the Direct-zol® RNA Miniprep system (Zymo Research, CA, USA) was used following the manufacturer's protocols. To start, 600µl of TRI-Reagent® was added to uterine tissue and homogenized completely. 600µl of 100% ethanol was directly added to the solution and homogenized. The sample was added to a Zymo-Spin IIC® column with the collection tube and centrifuged. The column was moved to a new collection tube and the collection tube containing the filtrate was discarded. The RNA samples were treated with DNase. Next, 400µl Direct-zol® RNA, a prewash was added to the column, and centrifuged. Subsequently, the filtrate was discarded, and this step was repeated. 700µl RNA wash buffer was added to the column, and it was centrifuged. The column was carefully transferred from the collection tube into the RNase-free tube, 20µl DEPC-treated nuclease-free water was added to it (Life Technologies, Madrid, Spain) and it was centrifuged. One and a half microliters of solution were used to measure the concentration of RNA in NanoDrop ND-1000 (NanoDrop Technologies, Rockland, DE). Complementary DNA was synthesized using 100 ng RNA. First, random primers (10 mM) (Invitrogen Corp. CA) and DNase/RNase free water were mixed and incubated at 65 °C for 5 min and kept on ice for 3 min. Then a second mix containing DNase/RNase free water, first strand buffer (5%), oligo dT18 (Operon Biotechnologies, AL), dNTP mix (10 mM) (Invitrogen Corp.), RevertAid Reverse Transcriptase (200 U/mL) (Fermentas Inc., MD) and RNase Inhibitor (20 U/mL) (Promega, WI) was added. The reaction

was performed in an Eppendorf Mastercycler (Eppendorf North America, Hauppauge, NY) using the following temperature program: 25 °C for 5 min, 42 °C for 60 min, and 70 °C for 5 min. cDNA was then diluted 1:3 with DNase/RNase free water. Sample concentration was measured using the NanoDrop ND-1000 (NanoDrop Technologies, Rockland, DE), and RNA quality was measured using an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA). Samples used in the analysis had a mean RNA integrity number of 7.1 ± 0.4.

Messenger RNA expression was analyzed on QuantStudio 7 Flex PCR system (Applied Biosystem, Foster city, CA) using primers designed using Primer Express 2.0 with minimum amplicon size of 80 bp (when possible, amplicons of 100 to 120 bp were chosen) and limited 30 G þ C (Applied Biosystems). When possible, primer sets were designed to fall across exon-exon junctions. Primers were aligned against publicly available databases using BLASTN at NCBI (Nucleotide BLAST, 2008) and UCSC's Cow (*Bos taurus*) Genome Browser Gateway.

Statistical Analyses

Statistical analysis was performed using SAS 9.4 (SAS Institute Inc. Cary, NC, USA). The MIXED procedure of SAS was used to conduct statistical analysis. The model included treatment with RPL prepartum (PRE) or not, RPL postpartum (POST) or not, and PRE × POST interaction. The following mixed linear regression model was used:

$$Y_{jklm} = \mu + B_j + C_k + D_l + (CD)_{kl} + A_m + \varepsilon_{jklm}$$

where; Y_{jklm} = the observations for dependent variables; μ = the overall mean; B_j = the fixed effect of block; C_k = the fixed effect of PRE; D_l = the fixed effect of POST; $(CD)_{kl}$ = the interaction of PRE and POST; A_m= the random effect of cow; and ε_{jklm}= the random residual error. A log transformation for the variables glandular area, number of cells per gland, and diameter of dominant follicle at first measurement was performed since residuals were not

normally distributed and with homogenous variance. Data presented for these variables were back transformed. The Kenward-Roger degrees of freedom approximation was used to determine the denominator degrees of freedom for tests of fixed effects (Littell, 2002). Statistical analysis of transcript expression was performed after the $2^{-\Delta CT}$ transformation was calculated to obtain the mean ± SD, following guidelines reported by Schmittgen and Livak (2008).

Endometritis was classified if the proportion of PMN at 15 DIM was greater than 40% (Ryan et al., 2020) and at 28 DIM if the proportion was greater than 18% (Sheldon et al., 2006). Metritis was classified as MC score plus the MC smell ≥ 3 (Sheldon et al., 2006; Skenandore et al., 2017). Fetid vaginal discharge was classified as MC smell = 3, independently of the MC score. Endometritis at 28 DIM, metritis, and fetid vaginal discharge were analyzed as binary traits for the determination of their prevalence. A model for development of CL by 28 DIM was evaluated by logistic regression using a binomial distribution in the GLIMMIX procedure of SAS. Odds ratio was used to compare the likelihood of 2 treatments to incur in the event (development of a CL by 28 DIM). Association between treatments and time to first ovulation was assessed using Kaplan Meier curves and Cox's proportional hazard regression. Treatments and block were forced into models, with cow was considered as a random effect. Statistical difference was considered at $P \leq 0.05$ and trends at $0.05 > P \leq 0.10$.

RESULTS

Cow performance data were described elsewhere (Fehlberg et al., 2020). Briefly, cows did not differ in dry matter intake (DMI, PRE-L 12.1 ± 0.21 kg; PRE-C 11.8 ± 0.21 kg; $P = 0.80$) or body weight (BW, PRE-L 808 ± 2 kg; PRE-C 803 ± 2 kg; $P = 0.12$) prepartum. There was a tendency for cows that received PRE-L to have greater DMI postpartum (18.1 ± 0.74 kg)

compared to those that received PRE-C (16.8 ± 0.74 kg; $P = 0.08$). Cows in PRE-L had greater BW postpartum (717 ± 6 kg) compared to cows that received PRE-C (706 ± 6 kg; $P = 0.05$). Energy-corrected milk (48.8 ± 1.9 kg/d), 3.5 % fat-corrected milk (50.1 ± 2.1 kg/d), milk fat (1.9 ± 0.1 kg/d), milk true protein (1.4 ± 0.1 kg/d), milk casein (0.6 ± 0.1 kg/d), and milk lactose yields (2.1 ± 0.1 kg/d) were greater for cows fed RPL prepartum compared to those that were not (44.2 ± 1.9, 45.3 ± 2.1, 1.7 ± 0.1, 1.3 ± 0.1, 0.5 ± 01, 1.9 ± 0.1 kg/d, respectively). Plasma concentrations of Lys prepartum (69.75 ± 1.83 μ*M*) increased for cows consuming RPL compared to those that did not (62.46 ± 1.83 μ*M*) but did not differ postpartum. Plasma concentrations of total AA, total dispensable AA, total branched-chain AA, total sulfur AA, and total urea cycle AA decreased, and total indispensable AA tended to decrease prepartum when RPL was consumed by cows. Postpartum concentrations of Lys in plasma as a percentage of indispensable AA increased when RPL was consumed by cows postpartum.

Follicular Dynamics

Follicular dynamics data are in Table 3.1. Cows that received RPL postpartum tended to have smaller growth rate of the first DF than cows that did not receive RPL postpartum. Treatments did not differ in the likelihood of observing a follicular cyst at 28 DIM ($P = 0.23$). Cows in PRE-C POST-L were more likely ($P = 0.04$, OR = 5.016, 95% CI = 1.06 to 23.83) to have a CL at 28 DIM (84%; 16/19) than cows in PRE-L POST-C (50%; 11/22). Days to first ovulation were 18.5 ± 1.64 for PRE-L POST-L, 16.8 ± 1.09 for PRE-L POST-C, 17.9 ± 1.51 for PRE-C POST-L, and 17.7 ± 1.26 for PRE-C POST-C ($P = 0.39$). A total of 27.8% (5/18) of PRE-L POST-L, 55.0% (11/20) of the PRE-L POST-C, 15.8% (3/19) of the PRE-C POST-L, and 33.3% (6/18) of the PRE-C POST-C were treated as right censored because they had not

ovulated by or had a follicular cyst at 28 DIM. Kaplan-Meier survival curves showed no difference ($P = 0.28$) between treatments in time to first ovulation (Figure 3.1).

Vaginal Discharge, Cytology of the Uterine Endometrium and Morphology of Uterine Glands

The prevalence of fetid vaginal discharge (MC smell = 3) did not differ and were 11.1% for cows in PRE-L POST-L (2/18 cows), 15.8% for cows in PRE-L POST-C (3/19 cows), 10.0% for cows in PRE-C POST-L (2/20 cows), and 22.2% for cows in PRE-C POST-C (4/18 cows). Prevalence of metritis (MC score plus smell ≥ 3) did not differ and were 11.1% for cows in PRE-L POST-L (2/18 cows), 15.8% for cows in PRE-L POST-C (3/19 cows), 10.0% for cows in PRE-C POST-L (2/20 cows), and 16.7 % for cows in PRE-C POST-C (3/18 cows). Prevalence of cytological endometritis at 15 DIM also did not differ [PRE-L POST-L = 5.9% (1/17); PRE-L POST-C = 10% (2/20); PRE-C POST-L = 17.6% (3/17); and PRE-C POST-C = 21.4% (3/14)]. Prevalence of endometritis at 28 DIM was similar for cows among treatments [PRE-L POST-L = 17.6% (3/17); PRE-L POST-C = 30% (6/20); PRE-C POST-L = 23.5% (4/17); and PRE-C POST-C = 42.8% (6/14)]. Cows in PRE-L POST-L (9.15 ± 4.10%) had lesser PMN % at 15 DIM ($P < 0.01$) than cows in PRE-L POST-C (23.2 ± 4.10%), PRE-C POST-L (27.8 ± 4.10%), and PRE-C POST-C (23.3 ± 4.10%). Similarly, cows in PRE-L POST-L (10.4 ± 3.68%) had lesser PMN % at 28 DIM ($P < 0.01$) than cows in PRE-L POST-C (15.8 ± 3.68%) and PRE-C POST-C (17.2 ± 3.68%), while cows in PRE-C POST-L did not statistically differ from the rest (12.9 ± 3.68%; Table 3.2).

The morphology of the uterine glands was assessed through histology and is described in Table 3.2. Treatments did not affect the number of uterine glands, the glandular area or perimeter ($P = 0.20$). However, there was a tendency for a treatment effect of POST ($P = 0.09$) on the number of cells per gland, with cows that were fed RPL postpartum having more cells per gland

(68.4 ± 7.68 cells) than cows that did not receive RPL postpartum (55.1 ± 7.68 cells). The same tendency for an effect of POST ($P = 0.06$) was observed when the glandular epithelial height was evaluated, but in this case cows that did not receive RPL postpartum tended to have greater epithelial cell height (8.44 ± 0.19 μm) than cows that received RPL postpartum (7.90 ± 0.18 μm).

Expression of mRNA Transcripts from Uterine Samples

The mRNA expression of measured transcripts is reported in Figure 3.2. Feeding RPL prepartum downregulated the expression of *TLR4*, *SLC7A6*, *EHMT2* ($P \leq 0.05$), and tended to downregulate the expression of *PTGES3* ($P = 0.06$) in uterine tissues at 28 DIM; additionally, it upregulated the expression of *APOL3* and *NFKB1* ($P = 0.04$), and tended to upregulate the expression of *AHCY* ($P = 0.08$) and *MAT2A* ($P = 0.07$). When fed postpartum, RPL upregulated the mRNA expression of *MUC1* ($P = 0.04$), tended to upregulate *MUC4* ($P = 0.06$), and tended to downregulate *SOD1* ($P = 0.07$) mRNA transcript. There was a PRE × POST interaction effect for the *FGF10* mRNA transcript, with a downregulation for cows in PRE-C POST-L, PRE-L POST-C; and POST-L PRE-L. Additionally, there was a tendency for a PRE × POST interaction effect for the expression of *HGF*, where cows in PRE-C POST-L, PRE-L POST-C, and PRE-L POST-L had a downregulation of *HGF* mRNA transcript in comparison with cows in PRE-C POST-C.

DISCUSSION

Follicular Dynamics

The first ovulation following parturition is associated with fertility, as multiple estrus cycles before the first artificial insemination result in a greater probability of pregnancy (Butler, 2003). While the importance of the DF size at ovulation and its impact on fertility are well-

understood (Vasconcellos et al., 2001), the growth rate of the DF is less studied. For example, in one of few studies relating the growth rate of DF in dairy cows with their nutrition, the supplementation of Met down-regulated pro-inflammatory transcripts in follicular cells of the first DF postpartum but did not affect time to first of ovulation or growth rate of the first DF (Acosta et al., 2017). In our study, cows that did not receive RPL postpartum tended to have a greater growth rate of DF than cows that received RPL postpartum. The growth of the DF depends on endocrine stimuli and after deviation, the DF relies more on LH than on FSH for growth (Aerts and Bols, 2010). Changes in the endometrium because of infection and inflammation disturb this endocrine function (Zerbe et al., 2003; Sheldon et al., 2006), as GnRH and LH release are impaired because of uterine inflammation (Sheldon, 2009). Nevertheless, because no difference was evident regarding the follicle size at ovulation time when RPL was fed or not, we suggest that this difference in growth rate might be of limited biological significance.

Vaginal Discharge, Cytology of the Uterine Endometrium and Morphology of Uterine Glands

Cows in PRE-L POST-L had lesser PMN percentage at 28 DIM, however, there was no difference in the mRNA expression of *IL1β, IL6, and IL8*. Furthermore, a lesser PMN percentage in the uterus at the fourth week postpartum was previously associated with improved uterine immunity (Stella et al., 2018). A possible factor contributing to the lesser PMN % in the uterus of cows in PRE-L POST-L could be the contribution of Lys to production of natural antimicrobial peptides (Li et al., 2020). Antimicrobial peptides have a cationic characteristic which is conferred by amino acid residues, particularly Lys and Arg (Guaní-Guerra et al., 2010). In the present study, feeding RPL prepartum tended to increase DMI postpartum (Fehlberg et al., 2020). Thus, we suggest that feeding RPL through the transition period had a positive but

indirect effect on the immune response in the early postpartum period, through the increase of DMI.

Uterine glands are crucial for fertility, as demonstrates in Gray et al. (2001b), which reported failure in the conceptus development at early stages of pregnancy in uterine gland knockout sheep. Adenogenesis, or the process of uterine gland development, can be impacted by nutritional factors, such as insufficient colostrum ingestion (Bartol et al., 2013; Vallet et al., 2013). Huang et al. (2012) investigated the cellular mechanisms of regeneration of the endometrium at different time points of the postpartum period of wild-type mice and detected cell proliferation occurring in the glandular epithelium. However, cell death signals were only identified in luminal epithelium and stroma (Huang et al., 2012). This implies regional differences in cell proliferation and death during the postpartum period in mice. Safeguarding the obvious limitations in drawing a comparison between studies using different species, it is possible for this mechanism to be true for the bovine uterine epithelium as well. Thus, a greater number of cells per uterine gland as a response to RPL feeding could be indicative of greater proliferation of the glandular epithelia. This is endorsed by the modulation in the expression of uterine transcripts, such as *HGF* and *FGF10*, which will be discussed subsequently. Changes in uterine glandular epithelia occur through the estrus cycle but also with inflammation, which results in glandular atrophy (Ohtani et al., 1993). Cows that received RPL postpartum had greater numbers of cells per uterine gland, which indicates cell proliferation. Cell proliferation is part of the tissue healing process. Uterine involution can be similar to wound healing, as it also comprises a dynamic process of inflammation, cell proliferation, and tissue remodeling (Eming et al., 2007). Li et al. (2020) reported that Lys-derived carbon quantum dots (CDQ) promoted growth of typical mammalian cells [NIH 3T3 (highly contact-inhibited cell line developed from

NIH Swiss mouse embryo cultures) and red blood cells] and when injected into infected wounds in mice, they accelerated wound healing. The authors suggested that Lys-CQD may have signaling agents that promote cell proliferation, which could be related to intracellular oxidative damage (Li et al., 2020). One possible explanation is that Lys may contribute to mitogenic signaling activity, promoting cell proliferation. However, further research is needed to elucidate this action.

Expression of mRNA Transcripts from Uterine Samples

Overall, our results indicate downregulation of transcripts involved in inflammatory processes at 28 DIM for cows that were fed RPL in comparison with those that were not. Cows with uterine inflammation have greater expression of toll-like receptor (*TLR*) transcripts (Herath et al., 2006; Gabler et al., 2010; Kasimanickam et al., 2014), which corroborates our findings. Furthermore, the upregulation of *AHCY* transcript, along downregulation of *SOD1*, indicates increased supply of homocysteine that is probably being used for antioxidant synthesis. This was also previously reported by Osorio et al (2014), which observed increased hepatic mRNA abundance of *AHCY* and decreased abundance of *SOD1* in dairy cows' liver upon supplementation with Met. The upregulation of *MAT2A* would support the supply of S-adenosylmethionine, which links methionine and one-carbon metabolism to mechanistic target of rapamycin complex 1 (mTORC1) through SAMTOR protein (Gu et al., 2017; Coleman et al., 2020). Thus, the upregulation of *AHCY* and *MAT2A* transcripts suggest that feeding RPL also affects Met metabolism in uterine tissue. The mechanism (or mechanisms) responsible for the effect on increased supply of RPL on methionine metabolism is not clear. One possible explanation is the theory behind limiting AA, where the protein synthesis depends on the supply of the most limiting AA and the efficiency of its use (Wolfe, 2017). Thus, increasing the supply

of intestinally available Lys could indirectly impact Met metabolism and protein synthesis. However, further research is needed to confirm this hypothesis.

Chapwanya et al (2009), when evaluating gene expression of uterine tissue from cows at 2 weeks postpartum, reported an increase in the expression of *NFKB1* and *TLR4* in comparison with cows in late postpartum (9 weeks). Additionally, the expression of *NFKB1* was related to the level of inflammation of the uterine tissue at 2 weeks postpartum, which was classified as mild, moderate, or severe according to uterine cytology and histological characteristics. With this, the authors proposed that the predominant uterine immune response in the early postpartum period in dairy cows was mediated by TLR/NFκB. However, recent studies in mice suggest a different role and function for *NFKB1*. Best et al (2019) reported that the *NFKB1* deletion in mice increased NFκB activation and signaling during tendon healing, leading to increased macrophage recruitment and general inflammation. The transcription factor NFκB is involved in innate and adaptive immune responses and is a mediator of inflammation (Liu et al., 2017). The canonical pathway is one of the activation routes of the NFκB pathway and is activated upon cellular exposure to inflammatory cytokines (such as IL-1β) or in response to LPS, upon its binding to Toll-like receptors (Perkins, 2007; Didonato et al., 2012). The *NFKB1*, on the other hand, encodes a protein that is a transcription inhibitor, being a suppressor of inflammation (Cartwright et al., 2016). Thus, the upregulation of *NFKB1* in cows that received RPL prepartum is consistent with the downregulation of *TLR4* for the same animals.

Moreover, the upregulation of *MUC1* in the uteri of cows that received RPL postpartum is consistent with the upregulation of *NFKB1*, as it was previously reported that *MUC1* increases the phosphorylation and degradation of IκBα (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha), thus blocking apoptosis process (Ahmad et al., 2007). Both

MUC1 and *MUC4* contribute to cell proliferation, through activation of extracellular signal-regulated kinases 1 and 2 in the case of *MUC1* (Schroeder et al., 2001); and activation of growth factor receptors in the case of *MUC4* (Bafna et al., 2010). Both these transcripts were upregulated for cows that were fed RPL postpartum, which reflected in greater cell proliferation, as exemplified by the tendency for greater number of cells per uterine gland in cows that received RPL postpartum. The upregulation of *APOL3* in dairy cows fed RPL prepartum can also point out to a lesser inflammatory environment in their uteri, since it was proposed that the expression of apolipoprotein L family transcripts are negatively correlated with inflammation (Israa et al, 2017). This would be due to the involvement of these apolipoproteins in apoptotic processes, but neutrophils tend to increase their life span from the onset of inflammation (Kolaczkowska and Kubes, 2013). Moreover, cows in PRE-L POST-L had lesser percentage of PMN in the uterus at 28 DIM, which support this premise.

Euchromatic histone Lysine methyltransferase 2 is a gene that encodes the histone methyltransferase enzyme, also known as G9a (Lee et al., 2006). Its role and function, although still not completely understood, has been more studied in mice models than in ruminants, thus we cautiously tried to find a possible explanation for the downregulation of *EHMT2* transcript at 28 DIM in the uterus of dairy cows fed RPL during prepartum. Antignano et al. (2014), when studying murine intestinal inflammation, reported that the expression of *EHMT2* is necessary for development of intestinal inflammation. This happens through regulation of T helper-17 (Th17) and regulatory T cells responses, by an increase differentiation towards Th17 response, which promotes inflammation through production of cytokines and recruitment of neutrophils (Littman and Rudensky, 2010). This immune response begins with the recognition of pathogen-associated molecular patterns by receptors, such as TLRs (Janeway and Medzhitov, 2002). Thus, it is

possible that the downregulation of *EHMT2* is following the downregulation of *TLR4* in the uterus of dairy cows that were fed RPL prepartum.

The *HGF* transcripts were downregulated in the uterus of cows in PRE-L POST-L and PRE-L POST-C, but upregulated in those of cows in PRE-C POST-L, when comparing with cows in PRE-C POST-C. This growth factor is involved in cell proliferation and morphogenic activity of several epithelial cells, including endometrial cells (Barros et al., 1995). Yoshida et al. (2004) reported a modest reduction in growth of endometrial stromal cells cultured in serum-free medium with supplementation of anti-HGF antibody, suggesting a role of endogenous HGF in stimulating endometrial stromal cell proliferation. Additionally, HGF stimulated epithelial morphogenesis in the ovine uterus (Chen et al., 2000a). Thus, and if the same stimulation also happens in glandular cells, it is possible that *HGF* was involved in the morphological alterations of cells from uterine glands observed here. Fibroblast growth factor 10, as well as HGF, are considered stromal-derived growth factors and their expression were reported in the female ovine reproductive tract (Chen et al., 2000a and 2000b). The expression of *FGF10* transcript in our study follows the expression of HGF transcript, which corroborate previous studies that reported a role of these growth factors in epithelial growth and differentiation of endometrium (Rubin et al., 1995; Chen et al., 2000a and 2000b).

CONCLUSIONS

Feeding RPL around parturition altered the expression of transcripts involved in inflammatory and immune responses. The downregulation of *TLR4*, *PTGES3*, *SOD1*, and *EHMT2*; and the upregulation of *APOL3*, *NFKB1*, *MUC1*, and *MUC4*, in conjunction with the lesser uterine PMN percentage, are indicatives of a potentially less severe inflammatory process

by week 4 postpartum. Additionally, a stimulus of cell proliferation is suggested by the tendency of RPL to increase the number of glandular epithelial cells. There was no effect of feeding RPL on the size of the first ovulatory follicle nor days to first ovulation. Increasing intestinal availability of Lys throughout the transition period improved several indicators of uterine health.

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FIGURES AND TABLES

Figure 3.1. Survival curves for dietary treatments included as top-dress with rumen-protected Lys (RPL) prior to calving (PRE) and after calving (POST) across days to first ovulation. Treatments consisted of a crossover design by RPL inclusion PRE or POST in which cows consumed a top dress either with (L) or without (C) RPL for 4 wk prepartum and for 4 wk postpartum [with RPL prepartum and postpartum (PRE-L POST-L), with RPL prepartum and without RPL postpartum (PRE-L POST-C), without RPL prepartum and with RPL postpartum (PRE-C POST-L), and without RPL prepartum and postpartum (PRE-C POST-C)] in a carrier of 300 g of dried molasses. The y-axis represents the overall probabilities. The x-axis represents the

number of days post-calving. Log-rank test indicates no differences in the survival experiences observed between treatments ($P = 0.28$).

Figure 3.1 (cont.).

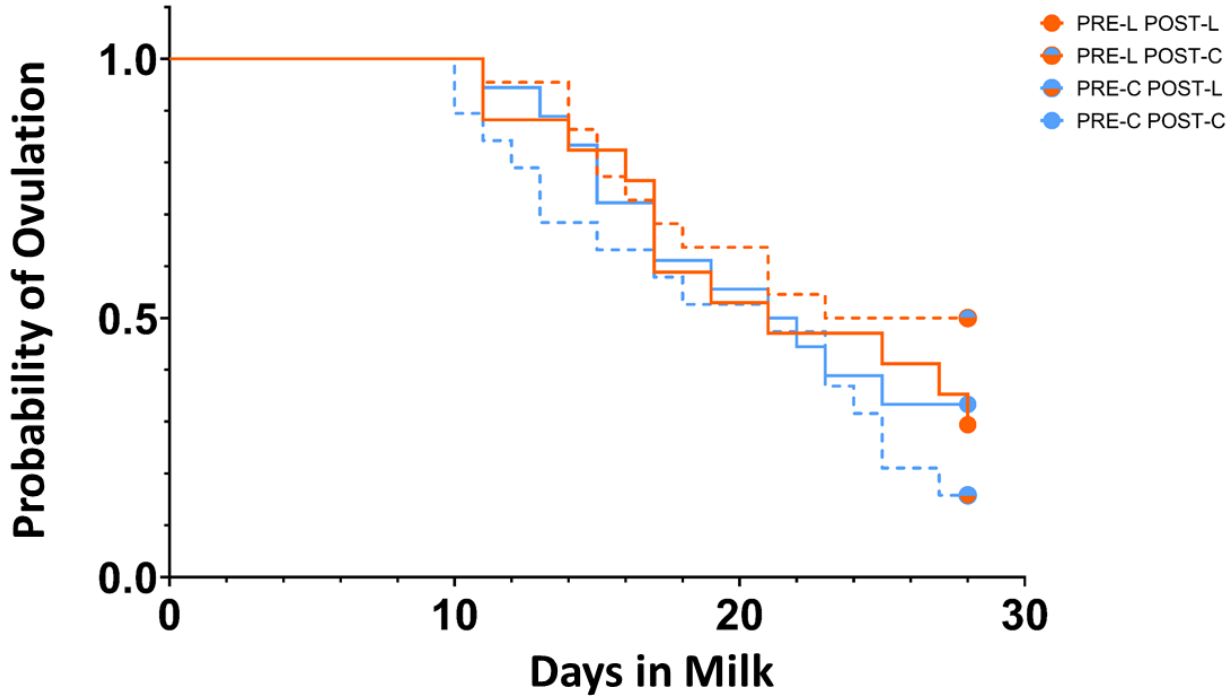

Figure 3.2. Effects of feeding rumen-protected lysine (RPL) in pre- (PRE) and/or postpartum (POST) diets of dairy cows on the relative mRNA expression in uterine tissue. Samples were taken at 28 days postpartum. Treatments consisted of RPL inclusion PRE or POST in which cows consumed a top dress either with (L) or without (C) RPL for 4 wk prepartum until 4 wk postpartum [with RPL prepartum and postpartum (PRE-L POST-L), with RPL prepartum and without RPL postpartum (PRE-L POST-C), without RPL prepartum and with RPL postpartum (PRE-C POST-L), and without RPL prepartum and postpartum (PRE-C POST-C)]. (a) Postpartum effect of feeding RPL in prepartum (PRE) diets on mRNA expression of Toll-like receptor-4 (*TLR4*), Prostaglandin E synthase 3 (*PTGES3*), Apolipoprotein 3 (*APOL3*), Adenosylhomocisteinase (*AHCY*), Methionine adenosyltransferase 2 α (*MAT2A*), Light chain of the second Na+ dependent lysine transporter (*SLC7A6*), Histone-lysine 9 N-trimethyltransferase (*EHMT2*), and nuclear factor kappa B1 (*NFKB1*); (b) Postpartum effect of feeding RPL in postpartum (POST) diets on mRNA expression of Mucin 1 (*MUC1*), Mucin 4 (*MUC4*), and Superoxide dismutase 1 (*SOD1*); (c) Postpartum effect of feeding RPL in pre- and/or postpartum diets on mRNA expression of Hepatocyte growth factor (*HGF*) and Fibroblast growth factor 10 (*FGF10*).

Figure 3.2 (cont)

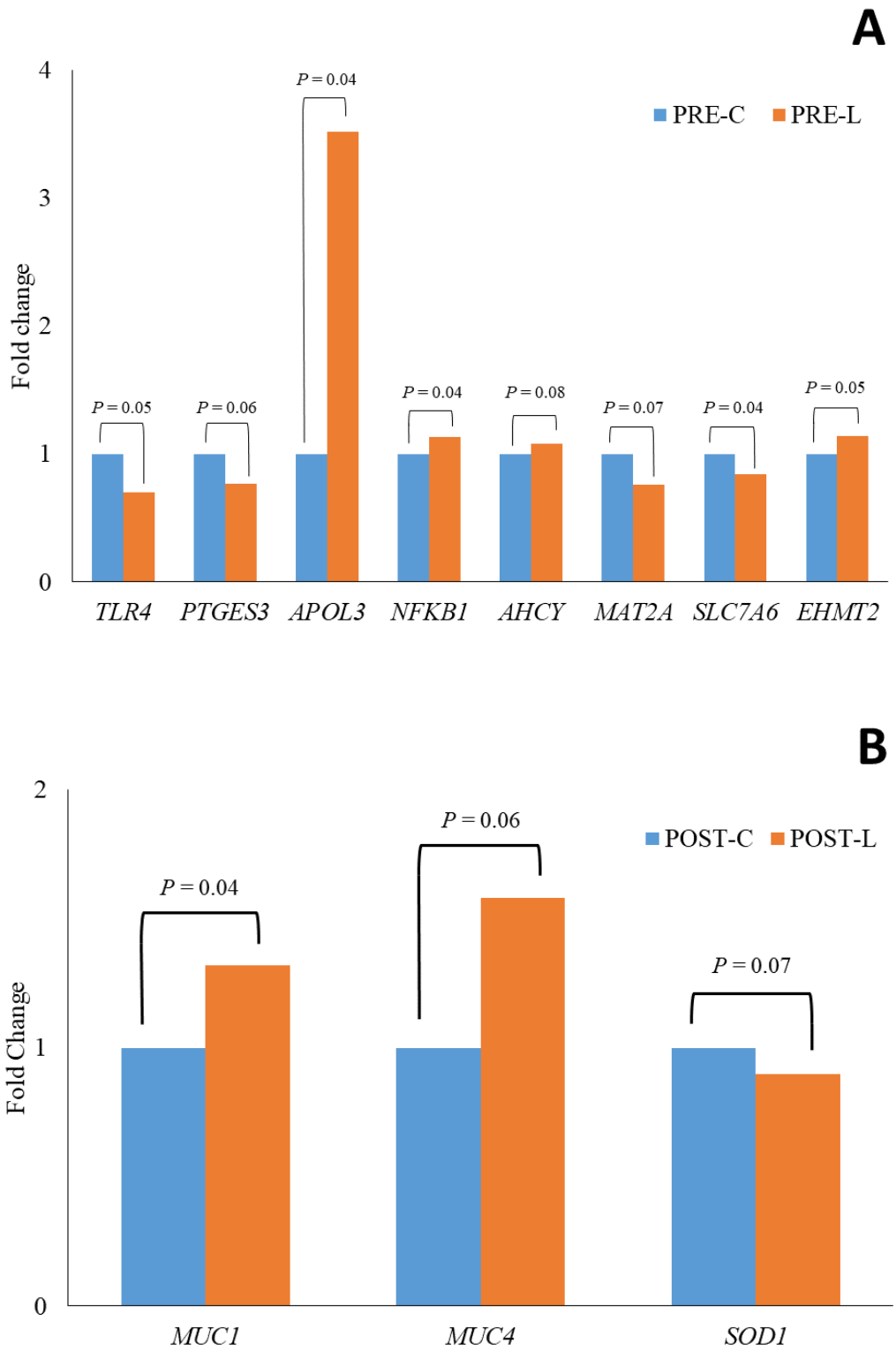

Figure 3.2 (cont.)

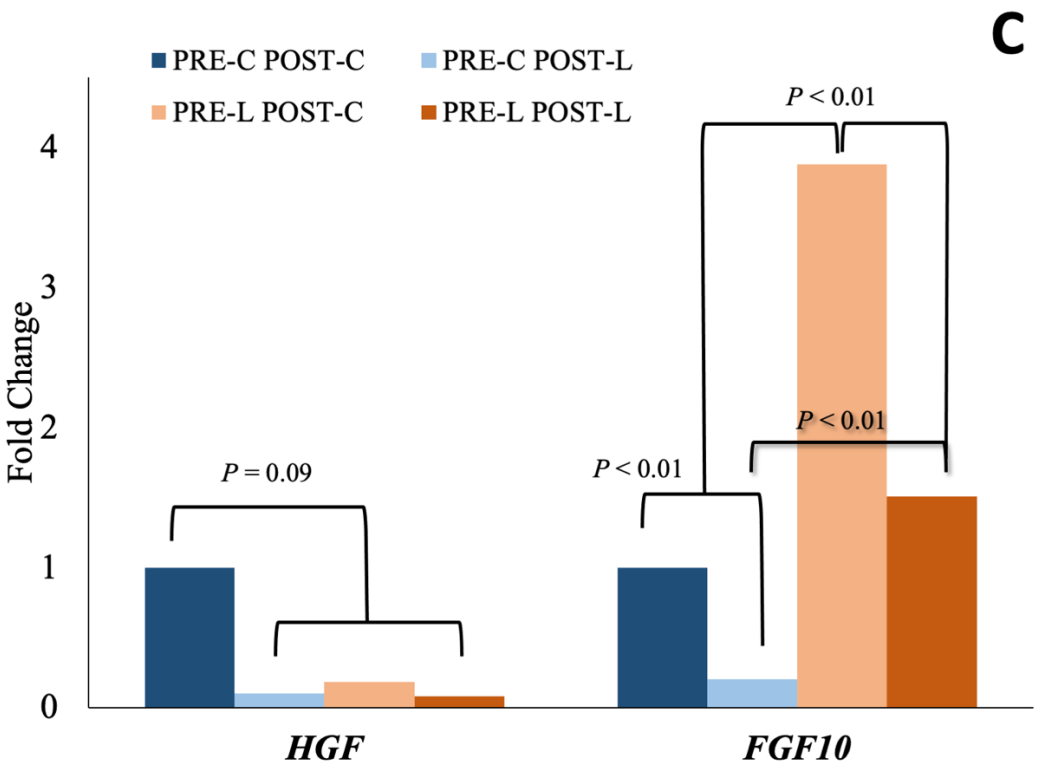

Table 3.1. Least squares means and associated standard error of the mean (SEM) for the ovulation dynamics of the dominant follicle of the first follicular wave.

	Treatment[1]				SEM[2]	*P*-value[3]		
Variable	PRE-L POST-L	PRE-L POST-C	PRE-C POST-L	PRE-C POST-C		PRE	POST	PRE × POST
Diameter of dominant follicle at first measurement, mm[4]	8.25	7.31	7.97	7.40	-	0.83	0.13	0.73
Diameter of dominant follicle at first ovulation, mm[5]	15.2	17.7	15.5	18.2	1.7	0.79	0.14	0.95
Growth rate of dominant follicle before first ovulation, mm/d[6]	0.7	1.3	1.0	1.1	0.2	0.89	0.10	0.23
Diameter of corpus luteum after ovulation, mm[7]	20.6	18.8	20.7	22.7	1.6	0.17	0.98	0.21

[1]Dietary treatments included at top dress with RPL prepartum and postpartum (PRE-L POST-L), with RPL prepartum and without RPL postpartum (PRE-L POST-C), without RPL prepartum and with RPL postpartum (PRE-C POST-L), and without RPL prepartum and postpartum (PRE-C POST-C) in a carrier of 300 g of dried molasses.

[2]Greatest value of standard error of the mean among treatments.

[3]For the main effects of RPL prepartum (PRE), RPL postpartum (POST), and their interaction.

[4]PRE-L POST-L (n = 18); PRE-L POST-C (n = 20); PRE-C POST-L (n = 19); PRE-C POST-C (n = 18). Log transformed least square means ± SEM: PRE-L POST-L = 2.06 ± 0.07; PRE-L POST-C = 1.94 ± 0.06; PRE-C POST-L = 2.06 ± 0.07; PRE-C POST-C = 1.97 ± 0.07.

[5]PRE-L POST-L (n = 13); PRE-L POST-C (n = 9); PRE-C POST-L (n = 16); PRE-C POST-C (n = 12).

[6]Growth rate was calculated as the difference in diameter from the last measurement by the first measurement, divided by the difference in days from those two measurements. PRE-L POST-L (n = 13); PRE-L POST-C (n = 9); PRE-C POST-L (n = 16); PRE-C POST-C (n = 12).

[7]PRE-L POST-L (n = 13); PRE-L POST-C (n = 9); PRE-C POST-L (n = 16); PRE-C POST-C (n = 12).

Table 3.2. Least squares means and associated standard error of the mean (SEM) for the proportion of polymorphonuclear cells (PMN) in the uterus and for glandular morphology of uterine endometrial tissue samples from Holstein cows.

Variable	n	Treatment[1]				SEM[2]	*P*-value[3]		
		PRE-L POST-L	PRE-L POST-C	PRE-C POST-L	PRE-C POST-C		PRE	POST	PRE × POS
PMN %	68								
15 days in milk		9.15[a]	23.2[b]	27.8[b]	23.3[b]	4.10	<0.01	0.33	<0.01
28 days in milk		10.4[a]	15.8[b]	12.9[ab]	17.2[b]	3.68	<0.01	<0.01	<0.01
Glandular morphology									
Glandular area, μm^2 [4]	53	4,048	3,595	3,887	3,768	-	0.92	0.77	0.38
Glandular perimeter, μm	53	61.2	57.4	59.4	59.7	2.70	0.92	0.80	0.43
Glandular epithelial cell height, μm	53	7.84	8.67	7.95	8.21	0.27	0.47	0.06	0.25
Number of cells per gland[5]	53	69.2	53.5	67.6	56.8	-	0.90	0.09	0.72
Number of glands	53	50.7	46.6	66.0	78.9	20.6	0.20	0.83	0.64

[1]Dietary treatments included at top dress with RPL prepartum and postpartum (PRE-L POST-L), with RPL prepartum and without RPL postpartum (PRE-L POST-C), without RPL prepartum and with RPL postpartum (PRE-C POST-L), and without RPL prepartum and postpartum (PRE-C POST-C) in a carrier of 300 g of dried molasses.

[2]Greatest value of standard error of the mean within treatment.

[3]Consists of the main effect of RPL prepartum (PRE), the main effect of RPL postpartum (POST), and their interactions.

[4] Log transformed least square means ± SEM: PRE-L POST-L = 8.27 ± 0.13; PRE-L POST-C = 8.11 ± 0.13; PRE-C POST-L = 8.17 ± 0.13; PRE-C POST-C = 8.24 ± 0.13.

[5] Log transformed least square means ± SEM: PRE-L POST-L = 4.19 ± 0.11; PRE-L POST-C = 3.93 ± 0.11; PRE-C POST-L = 4.13 ± 0.10; PRE-C POST-C = 4.03 ± 0.12.

[a-b]Means within a row with different superscripts differ ($P \leq 0.05$).

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CHAPTER 4: PREPARTUM SUPPLEMENTATION WITH RUMEN-PROTECTED LYSINE ALTERS PLACENTAL METABOLISM AT A TRANSCRIPTIONAL LEVEL

ABSTRACT

Lys and Met are the most limiting amino acids in dairy cows' diets. Increasing Met supply during late gestation improves offspring development through modulation of uteroplacental transport of amino acids and glucose and upregulation of genes involved in the mTOR (mechanistic target of rapamycin) pathway. Feeding RPL to prepartum dairy cows results in a greater intake and improved health of their calves during the first six weeks of life. However, whether increased supply of Lys in late gestation can influence placental tissue and, if so, which pathways are impacted remain to be investigated. Therefore, we hypothesize that feeding RPL during late gestation can influence the offspring due to changes in placental metabolism, which allow for the improved passage of nutrients to the fetus and immunity. Therefore, we aimed to determine the effects of feeding rumen-protected Lys (RPL, AjiPro-L Generation 3, Ajinomoto Health & Nutrition North America, Itasca, IL) prepartum (0.54% DM of TMR) on mRNA gene expression profiles of placental samples of Holstein cows. Seventy (n = 70) multiparous Holstein cows were randomly assigned to 1 of 2 dietary treatments, consisting of TMR top-dressed with RPL (**PRE-L**) or without (control, **CON**), fed from 27 ± 5 d prepartum until calving. After natural delivery (6.87 ± 3.32 h), placentas were rinsed with physiological saline (0.9% sodium chloride solution) to clean any dirtiness from the environment and weighted. Placental weights did not differ from cows in PRE-L (15.5 ± 4.03 kg) and cows in CON (14.5 ± 4.03 kg). Then, three placentomes were collected, one from each placental region (cranial, central, and caudal), combined and flash-frozen in liquid nitrogen to evaluate the

expression of transcripts and proteins related to protein metabolism and inflammation. Feeding RPL prepartum downregulated the expression of *NOS3* (nitric oxide synthase 3), involved in vasodilation processes, and *SOD1*, which encodes the enzyme superoxide dismutase, involved in oxidative stress processes. Additionally, feeding RPL prepartum upregulated the expression of transcripts involved in energy metabolism (*GLUT3*, glucose transporter 3; and *PCK1*, phosphoenolpyruvate carboxykinase 1), placental metabolism (*FGF2*, fibroblast growth factor 2; *FGF2R*, fibroblast growth factor 2 receptor; and *PGF*, placental growth factor), Met metabolism (*MAT2A*, methionine adenosyltransferase 2-α), and tended to upregulate *IGF2R* (insulin-like growth factor 2 receptor). Placental FGF2 and LRP1 (low-density lipoprotein receptor-related protein 1) protein abundance were greater for cows that received RPL prepartum than cows in CON. In conclusion, feeding RPL to prepartum dairy cows altered uteroplacental transport of glucose and metabolism, modulated by changes in gene transcription and protein expression. Such changes are illustrated by increased *GLUT3* and *PCK1* expression and increased protein abundance of FGF2 and LRP1 for cows consuming RPL.

Keywords: amino acids, dairy cow, glucose, placenta.

INTRODUCTION

The dry period is critical in the production cycle of a dairy cow because it directly impacts the future lactation performance (Drackley, 1999; Grummer and Rastani, 2004), but also because it coincides with the greatest fetal growth rate, which is primarily determined by nutrient availability (Jones et al., 2007). Fetal nutrient availability is directly related to maternal nutrition and placental nutrient and oxygen transport (Jones et al., 2007). The importance of uteroplacental tissue in supplying metabolic substrates required for fetal growth has long been recognized in all Eutheria mammals (Ramsey, 1982; Morriss and Boyd, 1988; Reynolds and Redmer, 1995). In bovine placenta, the placentomes are the areas of maternal-fetal interface (Schlafer et al., 2000). The placentomes consist of maternal caruncles interdigitating with fetal cotyledons (Bridger et al., 2007). Although the number of cotyledons is determined early in the gestation period (Laven and Gross, 2001), cotyledon growth continues throughout gestation (Reynolds et al., 1990). In the fetal cotyledon, the chorionic villi that interdigitates with the maternal caruncular crypts is covered by an epithelium formed of trophoblast cells and binuclear trophoblast giant cells (Wooding, 1992). The binuclear trophoblast giant cells migrate toward the caruncle epithelium, traversing the placental barrier and fusing using epithelial cells from the maternal side, in a process that releases molecules of fetal origin into the maternal compartment (Wooding and Wathes, 1980; Polei et al., 2020). This process of trophoblast cells proliferation and migration require resources, mostly in the form of glucose, for cellular energy production and biosynthesis (Nishitani et al., 2019).

Other functions of the placenta include to act as the first barrier between maternal environment and the fetus (Sandovici et al., 2012), and hormonal production, impacting the development and metabolism of the utero-placental tissue itself, besides the fetus (Anthony,

1995; Reynolds and Redmer, 1995). Besides being an energy-dependent process, protein synthesis requires AA, and therefore, require amino acid transporters to be working properly (Suryawan and Davis, 2011). Amino acid transporter activity can affect intracellular AA concentration, indirectly affecting mTOR pathway. Additionally, mTOR can also affect the activity of placental AA transporters in response to nutrient supply (Roos et al., 2009). For instance, increased Met supply to dairy cows during late gestation results in increased expression of genes involved in neutral AA transport, glucose transport, and the mTOR pathway of the placenta (Batistel et al., 2017). The bovine placenta produces a range of proteins throughout the whole gestation period, therefore requiring an adequate supply of energy source and AA (Reynolds and Redmer, 1995). For example, the bovine placenta can produce enough progesterone to support pregnancy during the last 70 days of gestation (Chew et al., 1979). Steroidogenic acute regulatory protein (STAR) is the main agent involved in the transfer of cholesterol into the mitochondria for the steroid hormone synthesis (Miller, 2007). Amino acids are also key nutrients for the fetus, and fetal acquisition of amino acids happens through an asymmetric bidirectional transfer, with net movement occurring in favor of the fetal circulation (Shennan and Boyd, 1987). Therefore, ensuring adequate maternal nutritional during the dry period is of utmost importance to guarantee proper function of placental metabolism and fetal development.

The importance of maternal nutrition to the placental nutrient transfer to the fetus can be exemplified by the association between feeding restriction during late gestation and consequent decrease in postnatal calf birth weight (LeMaster et al., 2017). Additionally, beef heifers born to grazing beef cows that were supplemented with 0.45 kg/d of a 42 % CP supplement had greater BW at weaning and at prebreeding season and had greater pregnancy rates compared with

heifers born to cows with no supplementation (Martin et al., 2007). In dairy cows, the effects of late gestation maternal nutrition on the offspring development are also reported. Particularly related to the most limiting amino acids in dairy cows' diets (NRC, 2001), Met and Lys, increasing the maternal supply of Met during the last month prior to calving enhances fetal growth in utero, as well as in the pre- and post-weaning periods (Alharthi et al., 2018). These effects are attributed to modifications in the uteroplacental transport of glucose and AA, and modulation of genes involved in the mTOR (mechanistic target of rapamycin) pathway (Batistel et al., 2017). Mechanistic target of rapamycin is an intermediate in a critical translational control pathway that regulates the cell cycle, proliferation, and growth (Raught et al., 2001). JAK2/STAT5 also plays an important role in growth, differentiation, and protein synthesis (Schmidt et al., 2014). Lin et al. (2018) reported an increase in the mRNA and phosphorylation levels of signal transducer and activator of transcription 5 (STAT5) and mTOR in response to Lys. These nutrient-sensing pathways were already reported in placental tissue (Batistel et al., 2017; Jansson & Powell, 2013; Roos et al., 2009).

Catabolism of Lys can play a role in biosynthesis processes, such as synthesis of non-essential amino acids, as described to happen in the mammary gland (Lapierre et al., 2009), or protein synthesis. During the peripartum period, the immune response of the dairy cow is active, as the animal is under a state of systemic inflammation (Bogado Pascottini et al., 2020). Protein synthesis is a crucial process whenever the immune response is activated, and Lys is mainly required to produce acute-phase proteins in response to increase circulating cytokines (Iseri & Klasing, 2014). Greater metabolic protein and Lys intake during the pre-calving period are attributable to increased DMI postpartum (Fehlberg, 2020; Girma et al., 2019) by alleviating the deficiency of those components in the transition diets. Feeding rumen-protected Lys (RPL) to

prepartum dairy cows tends to increase DMI and crude protein intake for their calves in their first six wk of life and tends to increase average daily gain during pre-weaning phase (Thomas et al., 2022). Additionally, calves born to cows fed with RPL during prepartum tend to have a greater percentage of phagocytic neutrophils than calves born to cows that are not fed with RPL (Thomas et al., 2022). However, the mechanisms by which increased prepartum supply of metabolizable Lys would affect offspring development and immunity remain unclear. Thus, we hypothesize that these effects are possible due to changes in placental metabolism, which allow for the improved passage of nutrients to the fetus, and improved immunity. This enhanced exchange of nutrients between the dam and the fetus, particularly amino acids, would allow for better use by the fetus but probably by the placenta itself. Therefore, we aim to determine the effects of maternal supplementation with RPL during late pregnancy on protein and gene expression of transcripts involved in the placental amino acid transport system, protein metabolism, energy metabolism, and immune metabolism.

MATERIAL AND METHODS

Animal Care and Housing, and Experimental Design

All experimental procedures were approved by the University of Illinois (Urbana-Champaign) Institutional Animal Care and Use Committee (#18157). Details of the animal handling, experimental design, and diets were previously described by Fehlberg et al. (2020). Briefly, 89 multiparous Holstein cows were categorized into low, intermediate, or high mature-equivalent milk production (ME305) based on the tertiles for ME305, and a similar concept was used for BCS as well. Then, they were blocked by lactation number (3.3 ± 1.1), previous 305-d mature-equivalent milk production (11,363 ± 1,860 kg), expected calving date, and BCS during

the far-off period (3.76 ± 0.84). Each block had 4 cows in it, except for one block that had 6 cows. Cows were then assigned to 1 of 2 dietary treatments [TMR with or without RPL (AjiPro-L Generation 3; 42% L-Lys-HCl, Ajinomoto Health & Nutrition North America, Itasca, IL)]. The number of cows per treatment was calculated to detect a minimum of 7% difference in postpartum DMI between groups, assuming a power of 0.9 and a two-tailed α of 0.05. Additional calculations were made to ensure that the experimental design had the power to detect a minimum of 10% increase in transcript expression (mRNA) from placental samples of two different independent groups, assuming a power of 0.8 and a two-tailed α of 0.05. This additional power analysis determined that, to detect such a difference among groups, it would be required a minimum of 32 cows per treatment group, which was met by the experimental design. Prepartum (-28 d to calving), cows were fed a diet with RPL [PRE-L (n = 35); 0.54 % RPL of dietary DMI] or without RPL [CON (n = 35)] top dressed in a carrier of 300 g of dried sugarcane molasses. The amount of RPL top-dressed was adjusted daily and for each cow based on their individual DMI of the previous day. Cows that were not receiving RPL were top-dressed with 300g of sugarcane molasses. According to the manufacturer, there is 80% rumen bypass and 80% intestinal digestibility of this encapsulated RPL product, which would provide 1.4 g of intestinally available Lys prepartum per kg of DMI. Prepartum diet was formulated using AMTS.Cattle.Pro version 4.7 (2017, AMTS, LLC, Groton, NY) to meet or exceed recommendations for dry cows at 694 kg of BW and a predicted DMI of 13 kg/d. According to AMTS.Cattle.Pro prediction, cows in PRE-C received 1.17 kg/d of MP, resulting in 6.86% MP as Lys and 2.98% MP as Met with a Lys:Met ratio of 2.30, while cows in PRE-L received 1.19 kg/d of MP, resulting in 8.24% MP as Lys and 2.94% MP as Met with a Lys:Met ratio of 2.80. The exclusion criteria included calving with twins or not having consumed the treatment for at

least 16 d during the prepartum period. Four cows were excluded due to twins (PRE-C n = 2; PRE-L n = 2) and 1 cow was excluded for calving too early (PRE-L n = 1). Nine cows were excluded postpartum due to health problems (PRE-C n = 2; PRE-L n = 7). A total of 75 cows concluded the study. Five cows had retained placenta (PRE-C n = 3; PRE-L n = 2), therefore their placentas were not collected.

Placental collection

Placentas were only collected after natural expel and there was no human intervention in the process. After natural at-term delivery (6.87 ± 3.32 h), placentas were rinsed with physiological saline (0.9% sodium chloride solution) to clean any dirtiness from the environment and weighted (PRE-L = 15.54 kg and CON = 14.54 kg, P = 0.43). Then, three placentomes from each placenta were dissected and rinsed with physiological saline. Placentomes were collected from the cranial, central, and caudal regions of the placenta (Figure 4.1). A subset of each of the placentomes was combined and flash-frozen in liquid nitrogen. Samples were stored at -80°C for further RNA and protein extraction.

Placental RNA extraction and transcript expression

For RNA extraction, the Direct-zol® RNA Miniprep system (Zymo Research, CA, USA) was used following the manufacturer's protocols. Briefly, 600µl of TRI-Reagent® was added to pellet and homogenized completely. 600µl of ethanol 100% was then directly added to the solution and homogenized. After, each sample was added to Zymo-Spin IIC® column with the collection tube and centrifuged, the column moved to a new collection tube and the collection tube containing the filtrate, discarded. The RNA samples were treated by DNase. After, 400µl Direct-zol® RNA, a prewash, was added to the column and centrifuged. Subsequently, the filtrate was discarded, and this step was repeated. 700µl RNA wash buffer was added to the column and

centrifuged. The column was carefully transferred from the collection tube into the RNase-free tube and 20µl DEPC-treated nuclease-free water was added to it (Life Technologies, Madrid, Spain), followed by centrifugation. One and a half microliters of solution were then used to measure concentration of RNA in NanoDrop ND-1000 (NanoDrop Technologies, Rockland, DE). Complementary DNA was synthesized using 100 ng RNA. Firstly, random primers (10 mM) (Invitrogen Corp. CA) and DNase/RNase free water was mixed and incubated at 65 °C for 5 min and kept on ice for 3 min. Then a second mix containing DNase/RNase free water, first strand buffer (5%), oligo dT18 (Operon Biotechnologies, AL), dNTP mix (10 mM) (Invitrogen Corp.), RevertAid Reverse Transcriptase (200 U/mL) (Fermentas Inc., MD) and RNase Inhibitor (20 U/mL) (Promega, WI) was added to the sample. The reaction was performed in an Eppendorf Mastercycler (Eppendorf North America, Hauppauge, NY) using the following temperature program: 25 °C for 5 min, 42 °C for 60 min, and 70 °C for 5 min. RNA integrity was measured using and Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA). Messenger RNA expression was analyzed on BioMark 96.96 Dynamic Array platform (Fluidigm, San Francisco, CA) using primers designed using Primer Express 2.0 with minimum amplicon size of 70 bp (when possible, amplicons of 100e120 bp were chosen) and limited 30 G þ C (Applied Biosystems). Primers were aligned against publicly available databases using BLASTN at NCBI (Nucleotide BLAST, 2008) and UCSC's Cow (Bos taurus) Genome Browser Gateway. All evaluated genes and primer information are reported in the supplemental material (Supplemental Table B.1-B.2). To control analytical and tissue sampling variation, the final data were normalized to the expression of the geometric mean of GAPHD (Batistel et al., 2017). The delta CT (ΔCT, cycle threshold) for each gene was calculated following guidelines reported by Schmittgen and Livak (2008), by subtracting the geometric mean of the CT of GAPDH, ACTB,

H2AFZ, RPS9, and UXT from the CT of the gene of interest. Fold change was calculated using the $2^{-\Delta\Delta CT}$ method, as described by Schmittgen and Livak (2008).

Placental protein extraction and expression

Proteins were extracted from placental samples using a tissue protein extraction reagent (catalog no. 78510; Thermo Fisher Scientific, Waltham, MA) containing Halt protease and phosphatase inhibitor cocktail (100×, catalog no. 78442; Thermo Fisher Scientific). Concentration of total protein was determined using a Nano-Drop ND-1000 spectrophotometer (Nano-Drop Technologies, Wilmington, DE). Protein samples were denaturated by heating at 95ºC for 5 min before loading 75 µg of protein into each lane of a 4 to 20% SDS-PAGE gel (catalog no. 4561096; Bio-Rad, Hercules, CA). Reactions were run for 10 min at 180 V, followed by 60 min at 110 V. After protein samples were transferred to the polyvinylidene fluoride membrane (catalog no. 1620261; Bio-Rad) in a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (catalog no. 170-3940; Bio-Rad). Membranes were then blocked in 1 × Tris-buffered saline (TBST) containing 5% nonfat milk for 2 h at room temperature. Membranes were then incubated in 1 × TBST containing primary antibodies to GAPDH, SLC7A7, AKT, FGF2, IGFBP3, LRP1, TMLHE, JAK2, phosphorylated-JAK2, mTOR, and phosphorylated-mTOR overnight at 4ºC. Catalog numbers and dilution ratios are included in Supplemental Table B.3. The membranes were then washed 6 times with 1 × TBST, incubated with antirabbit horseradish peroxidase-conjugated secondary antibodies (catalog no. 7074S; dilution 1:2000; Cell Signaling Technology, Danvers, MA) for 1 h at room temperature, and then washed again 6 times with 1 × TBST. Subsequently, membranes were incubated with enhanced chemiluminescence reagent (catalog no. 170-5060; Bio-Rad) for 3 min in the dark before image acquisition using ChemiDOC MP Imaging System (Bio-Rad). The intensities of the bands were

measured with Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD). Specific target protein band density values were normalized to the internal control (GAPDH) values. Equal amounts of protein (25 μg) were separated on SDS-PAGE gels, transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA), and then incubated with the primary antibodies (rabbit anti-) against β-actin, total mechanistic target of rapamycin (mTor), phosphorylated mTor, Y+L amino acid transporter 1 (SLC7A7), total Janus kinase 2 (JAK2), phosphorylated JAK2, fibroblast growth factor 2 (FGF2), Trimethyl-lysine Hydroxylase-Epsilon (TMLHE), Sodium- and chloride-dependent neutral and basic amino acid transporter B(0+) ($ATB0^{+}$), Insulin-like growth factor binding protein-3 (IGFBP-3), and phosphorylated IGFBP-3. Secondary antibodies will be Goat polyclonal Secondary Antibody to Rabbit IgG – H&L (Abcam, cat. #ab6721). Beta-actin will be used as an internal reference protein to normalize protein expression. Images will then be acquired using the ChemiDOC MP Imaging System (Bio-Rad). The intensities of the bands will be measured with Image-Pro Plus 6.0 software (Media Cybernetics Inc., Rockville, MD).

Statistical Analysis

Statistical analysis was performed using SAS 9.4 (SAS Institute Inc. Cary, NC, USA). The MIXED procedure of SAS was used to model the fixed effects of treatment and block using the following model:

$$Y_{jkl} = \mu + A_j + B_k + C_l + \varepsilon_{jkl}$$

where; Y_{jkl} = the observations for dependent variables; μ = the overall mean; A_j = the fixed effect of treatment (PRE); B_k= the fixed effect of block; C_l= the random effect of cow; and ε_{jkl}= the random residual error. Cow was used as the experimental unit. Residual distribution was evaluated for normality and homoscedasticity variance in all analysis. The Kenward-Roger

degrees of freedom approximation was used to determine the denominator degrees of freedom for tests of fixed effects. Statistical analysis of transcript expression was performed after the $2^{-\Delta CT}$ transformation was calculated to obtain the mean ± SD, following guidelines reported by Schmittgen and Livak (2008). Statistical significance was declared at $P \leq 0.05$ and tendency at $0.05 > P \leq 0.10$.

RESULTS

Cow performance data is reported elsewhere (Fehlberg et al. 2020; Fehlberg et al., 2020(Abstr); and Fehlberg et al., 2021(Abstr.)). Briefly, there was no difference in dry matter intake (DMI, PRE-L 12.1 ± 0.21 kg; CON 11.8 ± 0.21 kg; P = 0.80) or BW (PRE-L 808 ± 2 kg; CON 803 ± 2 kg; P = 0.12) prepartum due to dietary treatment. Plasma concentrations of Lys prepartum (69.8 ± 1.8 μM) increased for cows consuming RPL compared to those that did not (62.46 ± 1.3 μM). Plasma concentrations of total AA, total dispensable AA, total branched-chain AA, total sulfur AA, and total urea cycle AA decreased, and total indispensable AA tended to decrease prepartum for cows in PRE-L (Fehlberg et al., 2020). An increase in hepatic protein abundance of SLC7A7 (solute carrier family 7 member 7) indicated increased uptake of Lys by the liver of cows in PRE-L (Fehlberg et al., 2020(Abstr.)). Moreover, cows that consumed RPL had an improved liver functionality due to decreased inflammation, indicated by greater negative acute-phase proteins (albumin and globulin) and cholesterol, and decreased haptoglobin and aspartate aminotransferase (AST) concentrations prepartum (Fehlberg et al., 2021(Abstr.)). Additionally, downregulation of hepatic expression of interleukin 1-β (IL-1β) for cows in PRE-L further supports the hypothesis of a decreased inflammatory response upon RPL consumption

(Fehlberg et al., 2020(Abstr.)). Occurrence of retained placenta did not differ between dietary treatments (Fehlberg et al., 2020).

Placental Gene and Protein Expression

The mRNA fold change of transcripts is in Figure 4.2. Feeding RPL prepartum downregulated the expression of NOS3 (nitric oxide synthase 3, $P = 0.05$), involved in vasodilation processes, and SOD1 ($P = 0.02$), which encodes the enzyme superoxide dismutase 1, involved in oxidative stress processes. Additionally, feeding RPL prepartum upregulated the expression of transcripts involved in energy metabolism (GLUT3, glucose transporter 3; and PCK1, phosphoenolpyruvate carboxykinase 1; $P \leq 0.05$), in placental metabolism (FGF2, fibroblast growth factor 2; FGF2R, fibroblast growth factor 2 receptor; and PGF, placental growth factor; $P \leq 0.05$), Met metabolism (MAT2A, methionine adenosyltransferase 2 α; $P < 0.01$), and tended to upregulate IGF2R (insulin-like growth factor 2 receptor; $P = 0.08$).

Placental FGF2 and LRP1 (low-density lipoprotein receptor related protein 1) protein abundance was greater ($P = 0.03$) for cows that received RPL prepartum in comparison with cows in CON (Table 4.1). Phosphorylated Janus kinase 2 (phosphorylated-JAK2) protein expression was not detected in the placental tissue samples. No difference in SLC7A7 (solute carrier family 7 member 7), TMLHE (trimethyl-lysine hydroxylase, epsilon), IGFBP3 (insulin-like growth factor-binding protein 3), LRP1 (low-density lipoprotein receptor-related protein 1), AKT (protein kinase B), mTOR (mechanistic target of rapamycin) or JAK2 (Janus kinase 2) protein abundance were observed between treatments.

DISCUSSION

Our objectives were to determine the effects of feeding RPL to prepartum dairy cows on protein and gene expression of transcripts involved in placental amino acid transport system, protein metabolism, energy metabolism, and immune metabolism.

The greater transcriptional and protein abundance of FGF2 and the greater expression of *FGFR2* could indicate a more significant cell differentiation and, consequently, greater metabolic activity in the placenta of cows consuming RPL during late gestation. Fibroblast growth factor-2 is mainly involved in stimulating trophoblasts' migratory activity and binucleation (Taniguchi et al., 1998) and serves as an angiogenic factor for vascular endothelial cells (Zhang et al., 1997). Additionally, FGF2 also affects the production of hormones by the binucleate trophoblast giant cells, as demonstrated by in vitro studies with stabilizing the endocrine phenotype of PC12 cells (catecholamine cell line) (Grothe et al., 1998). As previously mentioned, binucleate trophoblast giant cells proliferation and migration process requires energy sources and synthesis of protein (Nishitani et al., 2019). Glucose is used as an energy resource by the trophoblast cells and is crucial for cell viability and proliferation processes in the placenta (Han et al., 2015) and glucose transporters are rate-limiting for overall glucose disposal in vivo (Fink et al., 1992). The greater expression of *GLUT3* in placentomes of cows in PRE-L could indicate increased glucose uptake capacity by the placenta, to be used either for cell proliferation processes or to be transferred to the fetus. Placental growth factor is involved in the proliferation and migration of cell, due to its strong angiogenic and mitogenic properties (Maglione et al., 1993; Cao et al., 1997). Thus, the upregulation of *PGF* expression in placenta of cows that were fed RPL is also likely involved in the proliferation and migration of trophoblasts.

The fetal-glucose requirements are met by an increase in the transplacental glucose gradient and a concomitant increase in placental glucose transfer capacity (Desoye & Nolan, 2016; Jr, 2006; Ward et al., 2004). Ticiani et al. (2020) associated a greater expression of glucose transporters bovine placentomes with greater glucose uptake by the conceptus when investigating placental metabolism of bovine pregnancies established after superovulation, in vitro fertilization, and cloning by nuclear transfer. Since it is possible that the increased glucose uptake, denoted by the increased expression of *GLUT3* in the placenta from cows consuming RPL, is being transferred to the fetus, then the increase in *PCK1* expression could be resulting in either increased gluconeogenesis (Chakravarty et al., 2005) or energy generation through recycling carbon skeletons of amino acids back into the TCA cycle (Yang et al., 2009). However, the decarboxylation of amino acids for oxidative metabolism is more likely to happen in situations of hypoglycemia (Limesand et al., 2009), which was not the case for neither cows in PRE-L nor cows in CON (Fehlberg et al., 20209(Abstr.) and 2021(Abstr.)). Thus, *PCK1* expression increased concomitantly with the increase in *GLUT3* expression, indicating that the placenta relied on gluconeogenic processes while the fetus took up glucose from the maternal circulation.

Increased glucose availability increased cell proliferation on bovine mammary epithelial cells while decreasing Lys:Met ratio had no effect (Wang et al., 2019). The increased hepatic protein expression of *SLC7A7* of cows consuming RPL in prepartum (Fehlberg et al., 2020(Abstr), combined with no differences in transcripts related to Lys-metabolism in uterine tissue (Guadagnin et al., 2020 (Abstr)) nor in placental tissue (present study), indicates a "preference" for hepatic uptake of Lys, most likely for protein synthesis. In corroboration with this hypothesis, Fehlberg et al. (2021(Abstr)) reported enhanced liver function for cows that were

fed RPL. Thus, it is likely that the increase in liver functionality during prepartum resulting from increased Lys availability allowed for greater fetal uptake of glucose, demonstrated by the increased transcription of *GLUT3* in placentomes. Moreover, the increased protein abundance of LRP1 is likely related to the increased *GLUT3* expression. LRP1 is necessary for *GLUT3* translocation to the plasma membrane in neurons and hepatocytes upon insulin signaling (Dato & Chiabrando, 2018). However, *GLUT3* is an insulin-independent transporter of glucose (Mora & Pessin, 2013). Additionally, LRP1 could be concomitantly serving as a sensor of the placental nutritional status, especially lipid composition (Au et al., 2017), possibly linked to the greater maternal circulation of cholesterol in cows that consumed PRE-L. As additional support of no peripheral oxidation of Lys, Lobley et al. (2003) reported no catabolism of Lys across the gastrointestinal tract of sheep. Wang et al. (2019) also demonstrated that extra-hepatic cells, particularly bovine mammary epithelial cells, do not respond to different Lys:Met ratios but increase cell proliferation upon glucose supplementation. Thus, the effects on extra-hepatic tissues are probably an indirect effect of improved liver functionality arising from RPL supplementation.

Additionally, RPL supplementation resulted in greater utilization of all other amino acids, as denoted by the upregulation of *MAT2A* in placental tissue of cows in PRE-L. Guadagnin et al. (2020(Abstr)) also reported upregulation of *MAT2A* along upregulation of *AHCY* in uterine tissue of cows supplemented with RPL. Methionine-adenosyltransferase 2A upregulation increases supply of S-adenosylmethionine, linking Met metabolism to mTOR through SAMTOR protein (Coleman et al., 2020). As previously mentioned, mTOR is an intermediate in a translational control pathway that regulates the cell proliferation (Raught et al., 2001), which is a continuous process linked to trophoblast giant cells migration and metabolism. Although the exact

mechanism explaining how increased supply of Lys can affect Met metabolism is not clear, this could be a result of increasing the supply of a limiting AA resulting in increased utilization of other AA. Within the theory of limiting AA, protein synthesis depends on the availability and efficiency of use of the most limiting AA in a diet; thus, when a limiting AA is not provided at sufficient amounts, protein synthesis is limited to the rate at which this limiting AA is available (Wolfe, 2017). Furthermore, Lys methyltransferases catalyze the transfer of methyl groups from S-adenosylmethionine to Lys residues, resulting in different forms of methylated Lys (Smith and Denu, 2009). These methylated Lys act as regulators of different process, including the transcription factor tumor suppressor protein p53, thus being involved in the regulation of cell-cycle processes and apoptosis, for instance (Scoumanne and Chen, 2008). Upon fusing of the binuclear trophoblast giant cells with the caruncular epithelial cells, the formed short-lived fetomaternal hybrid cells undergo apoptosis after degranulation (Wooding, 1992). As so, the increased migration and proliferation process could result in greater apoptosis.

CONCLUSIONS

We confirmed our hypothesis of increased uteroplacental exchange of nutrients at least in part through changes in the transcription of genes (*GLUT3* and *PCK1*) and expression of proteins (LRP1) involved in placental glucose uptake and metabolism. Increased transport of amino acids was not confirmed, as there were no differences in the amino acid transport systems evaluated. However, there was an increased expression of *MAT2A* in the placental tissue of cows consuming RPL, indicating increased Met catabolism. Placental immune metabolism was modestly affected by the maternal supplementation of RPL, with the main effects being the downregulation of transcription of *NOS3* and *SOD1*. We suggest minimal biological

significance to the latter findings due to the slight level of downregulation. The increased expression of *FGF2*, *FGF2R*, *PGF*, and *IGF2R* transcripts, along increased protein abundance of FGF2, indicate enhanced placental metabolic activity, possibly linked to trophoblasts proliferation and migration processes.

FIGURES AND TABLE

Figure 4.1. Schematic figure depicting placenta collection after natural delivery. Black arrows indicate the placentomes, which were harvested and dissected for collection.

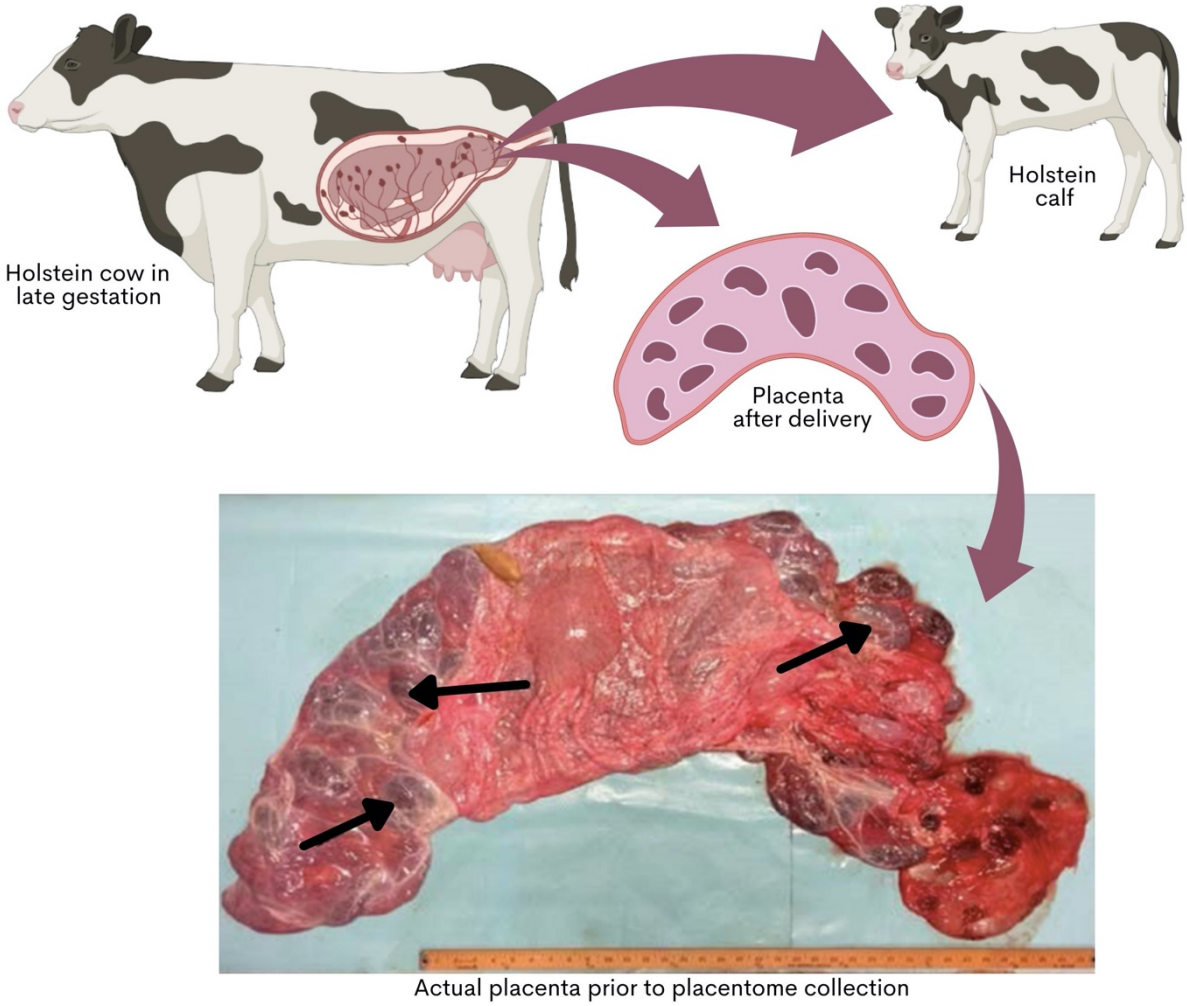

Figure 4.2. Effects of feeding rumen-protected lysine (RPL) in prepartum diets of dairy cows on placental mRNA expression, expressed as fold change. *MAT2A* = methionine adenosyltransferase 2 A; *FGF2* = Fibroblast growth factor 2; *FGFR2* = fibroblast growth factor 2 receptor; *GLUT3* = glucose transporter 3; *NOS3* = nitric oxide synthase 3; *SOD1* = Superoxide dismutase 1; *PGF* = Placental growth factor; *PCK1* = phosphoenolpyruvate carboxykinase 1; *IGF2R* = Insulin –like growth factor 2 receptor.

Figure 4.2 (cont.).

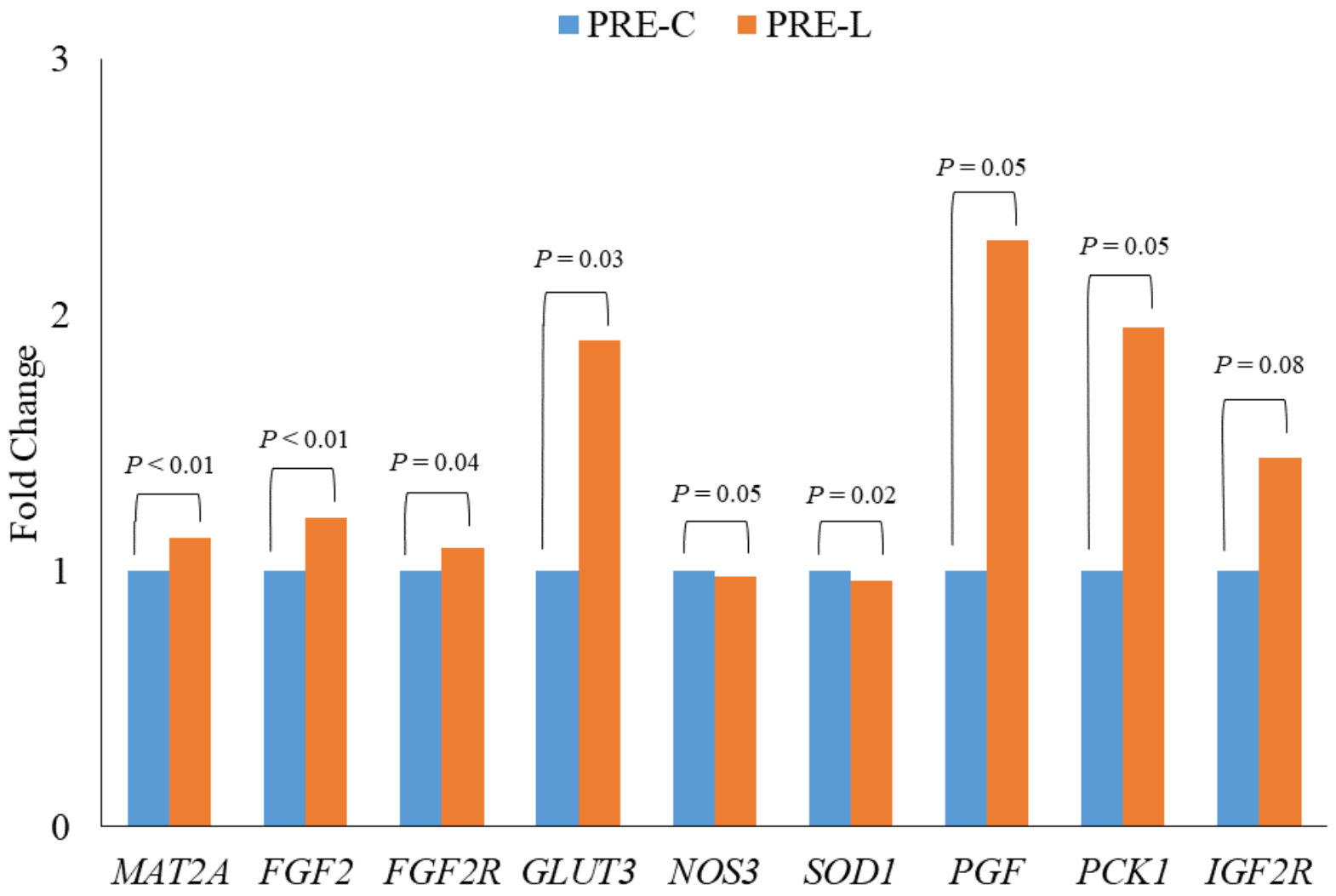

Table 4.1. Least squares means and SEM for protein abundance of placenta samples from Holstein cows fed with rumen-protected Lys (PRE-L) during peripartal period or not (CON).

	Treatment[1]			
Protein[3]	PRE-L	CON	SEM[2]	P-value
SLC7A7	0.87	0.74	0.15	0.55
TMLHE	0.97	0.92	0.22	0.70
FGF2	7.47	5.27	0.73	0.03
IGFBP3	9.17	10.4	0.93	0.35
LRP1	0.43	0.25	0.06	0.03
AKT	0.35	0.32	0.05	0.68
mTOR	0.25	0.21	0.05	0.64
Phosphorylated mTOR	0.23	0.22	0.03	0.91
mTOR:phosphorylated mTOR ratio	0.95	0.92	0.17	0.92
JAK2	0.57	0.60	0.09	0.85

[1]Dietary treatments consisted of a crossover design in which cows consumed a top dress either with (PRE-L) or without (PRE-C) RPL for 4 wk prior to calving in a carrier of 300 g of dried molasses.
[2]Greatest value for standard error of the mean within treatment.
[3]SLC7A7 = Light chain of the second Na^+ dependent Lys transporter; TMLHE = Trimethyllysine hydroxylase, epsilon; FGF2 = Fibroblast growth factor 2; IGFBP3 = Insulin-like growth factor-binding protein 3; LRP1 = Low-density lipoprotein receptor-related protein 1; AKT = Protein kinase B; mTOR = Mammalian target of rapamycin; JAK2 = Janus kinase 2.

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CHAPTER 5: ASSOCIATION OF DRY MATTER INTAKE, MILK YIELD, AND DAYS TO FIRST OVULATION WITH CYTOLOGICAL ENDOMETRITIS IN THE EARLY POSTPARTUM OF HOLSTEIN COWS

ABSTRACT

Dry matter intake (**DMI**) is closely related to the magnitude of negative energy and protein balance in the transition period and to the metabolic adaptations to support lactation in dairy cows. Thus, DMI might be an important factor for the development of cytological endometritis in the early postpartum, since difficulty to adapt to these metabolic changes are related to impaired immune function and increased occurrence of reproductive disorders. However, the association of DMI and cytological endometritis at 15 DIM has not been explored yet. We aimed to determine the association of pre- and postpartum DMI (kg/d and as % of BW), body weight (**BW**), body condition score (**BCS**), early postpartum milk yield and milk composition, and days to first ovulation with cytological endometritis at 15 (**CYT15**) and at 30 DIM (**CYT30**). A second objective was to associate vaginal discharge with CYT15 and CYT30 and production parameters. We conducted a pooled statistical analysis of five studies, including data from 394 multiparous Holstein cows in the pre- and postpartum period. Based on the cutoffs for the percentage of uterine PMN, determined by taking the median value of the data set for 15 and 30 DIM, cows were categorized as follows: **LOW15** (PMN % at 15 DIM ≤ 24%), **HIGH15** (PMN % at 15 DIM > 24%), **LOW30** (PMN % at 30DIM ≤ 7%); and **HIGH30** (PMN % at 30DIM > 7%). Cows in HIGH15 consumed on average 1.97 ± 0.5 kg of DM/d less than cows in LOW15 during prepartum, and 3.01 ± 0.5 kg of DM/d less during postpartum. Dry matter intake (% of BW) was greater for cows in LOW15 during pre- and postpartum than for cows in

HIGH15. Moreover, cows in HIGH15 tended to have lower milk yield than cows in LOW15 from the third until the fifth week postpartum. Although DMI (kg/d) was not associated with CYT30, DMI (% of BW) was lower for cows in LOW30 pre- and postpartum than for cows in HIGH30. There was no association between CYT30 and milk production. Cows in LOW15 had greater days to first ovulation than cows in HIGH15, while cows in LOW30 also had greater days to first ovulation than cows in HIGH30. Simple regression analyses demonstrated linear associations of increased DMI, particularly in the postpartum, with decreased uterine PMN percentage and improved vaginal discharge. Additionally, increased units of vaginal discharge score and increased percentage units of uterine PMN were linearly associated with decreased milk yield. In conclusion, the association of DMI and milk production with CYT15 suggests that uterine health diagnostics at an earlier stage may demand nutritional adjustments to help prevent the negative impact of cytological endometritis on cows' performance. Corroborating with the notion of ovarian function being associated with uterine inflammatory status, cows in HIGH15 and HIGH30 ovulated on average 3 days before cows in LOW15 and LOW30, respectively. Finally, although the evaluation of vaginal discharge using the Metricheck device might not be a direct function of uterine cytology at both 15 and at 30 DIM, its association with milk yield suggests it should be a practice performed at the farm.

Keywords: dry matter intake, PMN, endometritis

INTRODUCTION

The transition period is characterized by the progression from gestation into lactation and it is a unique period in a dairy cow's productive life. In this period, the decreased DMI concomitant to an increased maintenance and milk production costs, results in a state of negative energy balance (NEB) (Drackley, 1999). Thus, to support milk production in such a physiological state, metabolic adaptations take place. Briefly summarizing, due to the decreased DMI and increased mammary gland's requirement for milk production, almost all glucose available is taken up by the mammary gland in the early lactation (Bell, 1995). As a result, circulating non-esterified fatty acids (NEFA) increase due to increased lipolysis in response to reduced systemic insulin sensitivity along decrease circulating insulin (Rhoads et al., 2004). Difficulty to adapt to the nutrient needs for lactation is related to increased occurrence of health problems in this period (Ingvarsten and Moyes, 2013). As so, intake has a great impact in this scenario, as it is directly related to nutrient availability and it is one of the main determinants of the magnitude of the NEB (Drackley, 1999).

A prompt and robust inflammatory response with a substantial influx of PMN (polymorphonuclear cells) in the uterus early postpartum is associated with a reduced incidence of reproductive disorders (Galvao et al., 2011; Gilbert and Santos, 2016; Hammon et al., 2006). However, when excessive, this inflammatory response can lead to cytological endometritis (Pascottini and LeBlanc, 2020), which is characterized by increased proportion of PMN in uterine cytology characterizes cytological endometritis (Kasimanickam et al., 2004; Gilbert e al., 2005; Dubuc et al., 2010). The specific cause for cytological endometritis has not been described yet, but reduction of immune function and impairment of inflammation regulatory mechanisms are fundamental players in the development of uterine diseases (Sheldon et al., 2019), and were

reported to precede the diagnosis of diseases (Hammon et al., 2006). Dairy cows face impairment of PMN function during the transition period, which is attributed to increased exposure to circulating NEFA and BHB (Ster et al., 2012; Ingvartsen and Moyes, 2013), and decreased availability of glucose (Garcia et al., 2015; LeBlanc, 2020) and calcium (Ducusin et al., 2003; Martinez et al., 2012). Therefore, DMI might be an important factor in the development of cytological endometritis, since it is closely related to the metabolic adaptations leading to increased NEFA in circulation and decreased glucose availability.

Uterine health is crucial for reproductive success of the postpartum dairy cows (Fonseca et al., 1983; Sheldon, 2004). Thus, studies often use reproductive performance measurements as a main outcome of interest for the association with uterine cytology, such as pregnancy status after first service (Denis-Robichaud and Dubuc, 2015), hazard of pregnancy or time to first pregnancy after calving (Bicalho et al., 2016; Galvão et al., 2009; Gilbert et al., 2005). Endometrial cytology is most performed at 5 wk postpartum to diagnose cytological endometritis, which is associated with decreased fertility (Galvão et al., 2009). However, as previously mentioned, the uterine immunity has been dynamically active since the calving date. Few studies have evaluated the association of uterine cytology at earlier days postpartum with measurements of fertility. For example, a study with 406 Holstein cows from 5 herds of the United State reported that cows with ≥ 8.5 % of PMN at 21 DIM have similar intervals from calving to pregnancy than cows with < 8.5 % of uterine PMN (Galvão et al., 2009). Only a few works have attempted to determine the association of dry matter intake with cytological endometritis, and as far as the authors know, the association of intake and milk yield with cytological endometritis at 15 DIM has not been explored yet. Additionally, uterine inflammatory status can impact ovarian resumption and cyclicity in the early postpartum,

through inhibition of hypothalamic GnRH release and pituitary LH secretion by inflammatory mediators (Williams et al., 2001). Nevertheless, associations of DMI during the transition period, uterine cytology in the early postpartum, and ovulation of the first dominant follicle postpartum are not yet explored.

Therefore, we aimed to determine the association of DMI, lactation performance, days to first ovulation, and vaginal discharge with cytological endometritis at 15 DIM and at 30 DIM. Thus, our objectives are two-folded: the primary objective of this study is to evaluate the association of prepartum and postpartum DMI (kg/d and as % of BW), BW, and BCS, early postpartum milk yield and milk composition, and days to first ovulation with cytological endometritis at 15 and at 30 DIM. We hypothesize that these productive parameters and days to first ovulation would be associated with CYT15 as well as with CYT30, and whenever a cow would develop both, this would incur in an additive negative effect. A second objective is to associate vaginal discharge with CYT15 and CYT30 and the production parameters. We hypothesize that vaginal discharge is not necessarily associated with cytological endometritis, but it is associated with production parameters.

MATERIAL AND METHODS

Experimental design and sample size

The experimental design was a retrospective longitudinal study using production and health data for 404 multiparous Holstein cows assembled from 5 studies conducted at the University of Illinois at Urbana-Champaign (Table 5.1). Therefore, sample size calculations were not performed a priori. For continuous variables, a minimum of 45 cows would be required in each group to provide sufficient power ($\alpha = 0.05$; $\beta = 0.20$) to detect significant associations between cytological endometritis and DMI (difference between groups of at least 1 kg/d when

SD is 1.8 kg/d of DMI), milk yield (difference between groups of at least 1 kg/d when SD is 2 kg/d of milk yield), and days to first ovulation (difference between groups of 1 ± 3 days at first ovulation). From the 404 cows originally assembled, ten cows were removed due to incomplete data for DMI postpartum. Therefore, the final dataset consisted of 394 cows.

Data collection and dataset construction

In all studies, dry cows were housed in an enclosed ventilated barn with access to sand-bedded free-stalls, where they were fed once a daily using individual feeding system (American Calan Inc. Northwood, NH). After calving, cows were moved into a tie-stall barn where they were fed once a day. In all studies, cows were provided a total mixed ration (**TMR**; Supplemental Table C.1), had free access to water, and were milked on a set schedule (2 or 3 × /d). Daily DMI was determined for each cow by weighing refusals and total amount of feed and determining the difference on a DM basis. To avoid bias due to inaccuracy in records of DMI of d 0 due to a change from a prepartum diet to a postpartum diet, DMI of d 0 was not included. Weekly composited milk samples were analyzed for contents of fat, true protein, MUN, and SCC using mid-infrared procedures (AOAC, 1995b) by a commercial laboratory (Dairy One Cooperative Inc., Ithaca, NY). Body condition score was assigned independently by 3 individuals weekly using a 5-point scale (Ferguson et al., 1994) and the average score was used for each cow. Body weight was measured (Ohaus digital scale, model CW-11, Newark, NJ) for each cow weekly. Retained placenta was defined as a placenta that failed to deliver completely more than 12 h after calf delivery.

Cytology of the endometrium was performed using a cytology brush (Andwin Scientific, CA) at 15 ± 2 DIM and at 30 ± 2 DIM. Cytology slides were prepped immediately following the procedure described in Ryan et al. (2020). A minimum of 100 cells were counted within the

individual areas (ImageJ, National Institutes of Health, MD) to determine the percentage of PMN to epithelial cells. Cytological endometritis at 15 DIM (**CYT15**) and at 30 DIM (**CYT30**) were defined based on the percentage of polymorphonuclear cells (**PMN**) from endometrial cytology samples. Cut-off values for samples being classified as having cytological endometritis were obtained from the median values of the data set for each specific day and used to categorize all cows as follows: **LOW15** (PMN percentage at 15 DIM ≤ 24%), **HIGH15** (PMN percentage at 15 DIM > 24%), **LOW30** (PMN percentage at 30 DIM ≤ 7%), and **HIGH30** (PMN percentage at 30 DIM > 7%).

Evaluations of vaginal discharge were performed at 4 ± 1, 7 ± 1, 10 ± 1, 13 ± 1, 15 ± 1, and 17 ± 1 DIM via the Metricheck® device (MC, Simcro, New Zealand) following guidelines reported by Stella et al. (2018). The vaginal contents were scored according to the smell (0 = no odor or 3 = fetid odor) and appearance: score 0 = clear or translucent mucus; score 1 = mucus containing flecks of white or off-white pus; score 2 = discharge containing ≤ 50% white or off-white mucopurulent material; and score 3 = discharge containing ≥ 50% purulent material, may be white, yellow or sanguineous (Sheldon et al., 2006). Vaginal discharge was defined as Metricheck Smell + Score > 3 (MS), following guidelines reported in LeBlanc and Bicalho, 2017.

The first postpartum follicular cycle was monitored via transrectal ultrasonography (7.5-MHz linear array probe, E.I. 141 Medical Imaging, Loveland, Colorado) every other day from 7 DIM until 28 DIM. Ovulation was classified as the disappearance of the previously identified dominant follicle (DF) and the appearance of a *corpus luteum* in the subsequent examinations. Ultrasonography examinations were performed until the putative DF (≥ 10 mm in diameter) disappeared or until 28 DIM.

Health disorders included retained placenta, milk fever, displaced abomasum, and clinical mastitis. Retained placenta was defined as a placenta that failed to deliver completely more than 12 h after calf delivery; milk fever and displaced abomasum were diagnosed by a veterinarian; and clinical mastitis was diagnosed by altered milk composition confirmed by positive microbiological culture.

Statistical analyses

Cutoff values for the classification of samples as having cytological endometritis were obtained from the median values of the data set for each specific day. Cytological endometritis at 15 DIM was classified if the percentage of PMN was > 24% and at 30 DIM if the percentage of PMN was > 7%.

Mixed multivariable linear regression models were built using the MIXED procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC). The effect of time (day or week), reproductive tract inflammatory disease status (LOW15, HIGH15, LOW30, and HIGH30), and their interaction were forced into each model to test their effects on DMI, BW, BCS, milk yield, and milk composites. Cow nested within experiment was the random error term. Models account for repeated measures of time [DIM was used for the variables DMI (kg/d), DMI (% of BW), milk yield (kg/d), and week was used for the variables BW, BCS, and milk composition). The following model was used:

$$Y_{jklm} = \mu + A_j + B_k + T_l + (AT)_{jl} + (BT)_{kl} + (AB)_{jk} + (ABT)_{jkl} + C_m + \varepsilon_{jklm}$$

where; Y_{jklm} = the observations for dependent variables; μ = the overall mean; A_j = the fixed effect of CYT15; B_k = the fixed effect of CYT30; T_l = the repeated effect of T; $(AT)_{kl}$ =

the interaction of CYT15 and T; $(BT)_{km}$ = the interaction of CYT30 and T; $(AB)_{lm}$ = the interaction of CYT15 and CYT30; $(ABT)_{klm}$ = the interaction of CYT15 and CYT30 and T; C_m= the random effect of cow nested within experiment; and ε_{jklm}= the random residual error. Cow was utilized as the experimental unit. Parity (primiparous or multiparous), BCS at enrollment (≤ 3.5 and > 3.5), and retained placenta were offered as covariates and retained when $P \leq 0.05$. Numerator degrees of freedom was 1. Denominator degrees of freedom was estimated using Kenward-Rogers method (Littell, 2002). Model residuals were assessed using a scatterplot of the studentized residuals for homoscedasticity, linear predictor for linearity, and a Shapiro-Wilk test for normality. The covariance structure having the smallest Akaike information criterion and Schwarz Bayesian criterion was used (Littell et al., 2002). A Q-Q plot and box plot were used to determine outliers (calculated by multiplying the interquartile range by 3 and subtracting or adding from the corresponding quartile). A log transformation was used for the variable milk SCC since residuals were not normally distributed and with homogenous variance. Least-squares means were back transformed for this variable.

Simple regression models were built using the MIXED procedure of SAS. Metricheck (score plus smell, averaged from evaluations performed at 4, 7, 10, 13, 15, and 17 DIM), PMN percentage at 15 DIM, PMN percentage at 30 DIM, and days to first ovulation were considered as outcomes. Weekly DMI (kg/d) and DMI (% of BW) were considered as explanatory variables. Univariable associations for each explanatory variable with each outcome of interested were tested. Additional simple regression models considering milk yield (kg/d) as the outcome of interest and was Metricheck (score plus smell, averaged from evaluations performed at 4, 7, 10, 13, 15, and 17 DIM), PMN percentage at 15 DIM, PMN percentage at 30 DIM as the explanatory variables were built. For all univariable linear regression models, cow was

considered as the experimental unit and cow nested within experiment was considered as a random effect. Statistical parameters including coefficient of determination and root mean square error (RMSE) were calculated to evaluate model fit. Assumptions of normality and homoscedasticity of variance were evaluated with normal probability plots and plots of residuals versus predicted values.

Models for the development of cytological endometritis at 15 DIM, cytological endometritis at 30 DIM, vaginal discharge (by day) were evaluated by logistic regression using a binomial distribution in the GLIMMIX procedure of SAS fitting a binary distribution and a logit link function. The following model was used:

$$Y_{ij} \sim bin\left[P(Y_{ij})\right]$$

$$Logit\left[P(Y_{ij})\right] = \beta_{0ij} + \beta_1 A_{ij} + \mu_j$$

where $P\left(Y_{ij}\right)$ is the probability of the outcome of interest of cow i in herd j and is function of the predictor variable through the logit function; β_{0ij} is the intercept; β_1 is the regression coefficient for the predictor; and μ_j is the random herd effect. Odds ratio was used to compare the likelihood of high or low intake pre- and postpartum, and RP to incur in the event. Dichotomization of DMI values was performed using the median value of DMI for prepartum (11 kg/d) and for postpartum (13 kg/d). The Kenward-Roger method was used to calculate the approximate denominator degrees of freedom for the *F* tests in the statistical models. Goodness of fit was evaluated to test for over-dispersion.

The interval from calving to ovulation of the first dominant follicle was analyzed using PHREG procedure. Cox proportional-hazards regression models included the fixed effects of endometrial cytology (LOW15, HIGH15, LOW30, and HIGH30). Cows that did not ovulate and

cows that developed a follicular cyst by 30 DIM were censored. The Cox's hazard regression analysis used the following model:

$$Ln\left\{\frac{h(t)}{ho(t)}\right\} = \beta_1 \times Endometrial\ Cytology + \beta_2 \times Experiment,$$

where logarithm of the ratio of the hazard of time over the basal hazard was a function of the set of predictors representing the fixed effect of endometrial cytology (β_1) and the random effect of experiment (β_2). Time interval (median ± 95% CI) for days to first ovulation was obtained using the LIFETEST procedure of SAS.

Data are presented as least squares means ± standard error of the mean, unless otherwise stated. Close diamonds (♦) within graphs represent the results of the mean separation procedure PDIFF within MIXED procedure at $P < 0.05$. Open diamonds (◊) within graphs represent the results of the mean separation procedure PDIFF within MIXED procedure at $0.05 < P \leq 0.10$. Significance was declared at $P \leq 0.05$ and tendencies were reported if $0.05 < P \leq 0.10$.

RESULTS

1. ***Primary objective.*** To evaluate the association of prepartum and postpartum DMI (kg/d and as % of BW), BW, and BCS, early postpartum milk yield and milk composition, and days to first ovulation with cytological endometritis at 15 and at 30 DIM.

1.1. Dry matter intake, BW, and BCS

Intake, BW, and BCS data are in Table 4. Cows in LOW15 had greater DMI than cows in HIGH15 during prepartum and early postpartum ($P < 0.01$, Figure 5.1A). There was no effect for the interaction CYT15 × CYT30 ($P = 0.97$). Dry matter intake did not differ between cows in LOW30 and HIGH30 prepartum nor postpartum (Figure 5.2A). Dry matter intake as a percentage of BW prepartum was greater for cows in LOW15 than for cows in HIGH15 prepartum (1.24 ± 0.06 and 1.10 ± 0.06, respectively; $P < 0.01$) and postpartum (2.34 ± 0.07 and

2.14 ± 0.07, respectively; $P < 0.01$). Additionally, DMI as a percentage of BW was lesser for cows in LOW30 than for cows in HIGH30 prepartum (1.05 ± 0.06 and 1.30 ± 0.06, respectively; $P < 0.01$) and postpartum (2.13 ± 0.07 and 2.35 ± 0.07, respectively; $P < 0.01$). There was a tendency for an effect of CYT15 × CYT30 ($P = 0.07$), where cows in HIGH15-LOW30 had lesser average DMI as a percentage of BW (1.26 ± 0.13 %) than cows in LOW15-LOW30 (1.57 ± 0.13 %), LOW15-HIGH30 (1.74 ± 0.13 %), and in HIGH15-HIGH30 (1.62 ± 0.13 %) throughout the pre- and postpartum. Cows in HIGH15-LOW30 had lower BW than cows in LOW15-LOW30, LOW15-HIGH30, and HIGH15-HIGH30 during 2 weeks prior to calving and from weeks 2 through 5 postpartum ($P < 0.01$; Figure 5.44). Additionally, cows in LOW15-HIGH30 had the largest BW than the rest from weeks 3 through 5 postpartum ($P < 0.01$; Figure 4). Cows in LOW15-LOW30 had the lowest BCS one week prior to calving than cows in LOW15-HIGH30, HIGH15-LOW30, and in HIGH15-HIGH30 ($P < 0.01$; Figure 5.5). Additionally, cows in LOW15-LOW30 also had the lowest BCS than cows in LOW15-HIGH30, HIGH15-LOW30, and in HIGH15-HIGH30 on weeks 2 and 3 postpartum ($P < 0.01$; Figure 5.5).

1.2. Lactation performance

Lactation performance data is in Table 5.4. There was a tendency for an interaction of CYT15 × TIME ($P = 0.08$), where cows in HIGH15 tended to have lower milk yield from 15 to 24 DIM and from 26 to 30 DIM than cows in LOW15 ($P = 0.09$; Figure 5.1B). Milk yield (kg/d) did not differ ($P = 0.87$) between cows in LOW30 and HIGH30 (Figure 5.2B). Energy-corrected milk (ECM) was greater for cows in LOW15 (41.7 ± 0.00 kg/d) than for cows in HIGH15 (39.5 ± 0.00 kg/d; $P < 0.01$) and was lesser for cows in LOW30 (40.4 ± 0.00 kg/d) than for cows in HIGH30 (40.8 ± 0.00 kg/d; $P < 0.01$). Milk composition data are in Table 5.4 and illustrated in Figure 5.6. Milk fat was greater for cows in HIGH30 (4.99 ± 0.10 %) than for cows in LOW30

(4.64 ± 0.09 %) on the first week postpartum ($P = 0.03$), but it did not differ on the subsequent weeks (Figure 5.6A). Milk fat yield tended to be greater ($P = 0.06$; Figure 5.6B) for cows in LOW15 than for cows in HIGH15 on week 4 (1.70 ± 0.06 and 1.58 ± 0.06 kg/d, respectively) and on week 5 (1.75 ± 0.10 and 1.65 ± 0.10 kg/d, respectively). Milk protein yield was greater ($P = 0.01$; Figure 5.7C) for cows in LOW15 than for cows in HIGH15 during weeks 3 (1.35 ± 0.04 and 1.26 ± 0.05 kg/d, respectively) and 4 (1.37 ± 0.04 and 1.27 ± 0.05 kg/d, respectively) postpartum. Somatic cell count was greater ($P = 0.03$; Figure 5.7D) for cows in LOW30 than for cows in HIGH30 on week 4 postpartum (200 ± 105 and 449 ± 111 × 1,000/mL, respectively), but inverted on week 5 postpartum (LOW30 = 833 ± 328 × 1,000/mL; HIGH30 82.8 ± 270 × 1,000/mL).

1.3. Vaginal discharge and days to first ovulation

Metricheck score plus smell, averaged from the evaluations performed at 4 ± 1, 7 ± 1, 10 ± 1, 13 ± 1, 15 ± 1, and 17 ± 1 DIM, was greater ($P < 0.01$) for cows in HIGH15-HIGH30 (2.68 ± 0.06) than for cows in LOW15-LOW30 (2.51 ± 0.06), LOW15-HIGH30 (2.45 ± 0.06), and HIGH15-LOW30 (2.45 ± 0.06; Table 5.4). Cows in LOW15 had greater days to first ovulation (19 ± 0.07 d) than cows in HIGH15 (16 ± 0.07 d; $P < 0.01$; Figure 5.8A), while cows in LOW30 also had greater days to first ovulation (19 ± 0.08 d) than cows in HIGH30 (16 ± 0.07 d; $P <0.01$; Figure 5.8B). Prevalence of retained placenta was 13.5% (21/155) for cows in LOW15, 17.9% (15/84) for cows in HIGH15, 13.2% (19/144) for cows in LOW30, and 18.3% (17/93) for cows in HIGH30 ($P = 0.61$).

1.4. Regression analyses

Linear regression results for the association of DMI (kg/d) by week relative to calving with Metricheck, PMN percentage at 15 DIM, PMN percentage at 30 DIM, and days to first ovulation are in Table 6 and scatter plots are in Figure 5.7. For every 1 kg increase in weekly DMI on the third week prior to calving, PMN percentage at 15 DIM decreased by 13% (P = 0.01; R^2 = 0.06). For every 1 kg increase in weekly DMI on the first week postpartum, Metricheck score plus smell decrease 0.06 units ($P < 0.01$; R^2 = 0.11), and PMN percentage at 30 DIM decreased by 0.72 % (P = 0.05; R^2 = 0.02). For every 1 kg increase in DMI on wk 2 postpartum, the Metricheck score plus smell decreased by 0.03 units ($P < 0.01$; R^2= 0.09). For every 1 kg increase in DMI on wk 3 postpartum, the PMN percentage at 15 DIM decreased by 0.90 % (P = 0.04; R^2 = 0.15). Finally, for every 1 kg increase in DMI on wk 4 postpartum, the Metricheck score plus smell was lower by 0.04 units (P = 0.02; R^2 = 0.09).

Linear regression results for the association of DMI (% of BW) by week relative to calving with Metricheck, PMN percentage at 15 DIM, PMN percentage at 30 DIM, and days to first ovulation are in Table 5.7 and Figure 5.8. For every unit of increase in DMI (% of BW) on the fourth week prior to calving, the Metricheck score plus smell decreased by 0.45 units (P = 0.01; R^2 = 0.12). For every 1 unit increase in DMI (% of BW) on the first week prior to calving, PMN percentage at 30 DIM increased by 6.49 % (P = 0.04; R^2 = 0.04). For every 1 unit increase in DMI (% of BW) on the first week postpartum, Metricheck score plus smell decreased by 0.43 units ($P < 0.01$; R^2 = 0.14). For every 1 unit increase in DMI (% of BW) on wk 3 postpartum, the PMN percentage at 15 DIM decreased by 4.45 % (P = 0.03; R^2 = 0.07). For every 1 unit increase in the DMI (% of BW) on wk 4 postpartum, the Metricheck score plus smell was lower by 0.27 units (P = 0.02; R^2 = 0.07).

Linear regression results for the association of Metricheck, PMN % at 15 DIM, and PMN % at 30 DIM with milk yield (kg/d) by week postpartum are in Table 5.8 and Figure 5.9. Cows with vaginal discharge ≥ 3 were associated with a decrease of 1.77 kg/d of milk yield on wk 1 postpartum ($P < 0.01$; $R^2 = 0.49$) and a decrease of 1.19 kg/d of milk yield on wk 3 postpartum ($P = 0.03$; $R^2 = 0.45$); and an increase of 1.47 kg/d of milk yield on wk 4 postpartum ($P < 0.01$; $R^2 = 0.21$). For every 1 unit of increase in the Metricheck score plus smell, the milk yield decreases by 1.84 kg/d on the first week postpartum ($P < 0.01$; $R^2 = 0.54$), 2.26 kg/d on the second week postpartum ($P < 0.01$; $R^2 = 0.50$), 2.18 kg/d on the third week postpartum ($P < 0.01$; $R^2 = 0.49$), and 1.73 kg/d on the fourth week postpartum ($P < 0.01$; $R^2 = 0.47$). For every 1 % increase in the PMN percentage at 30 DIM, the milk yield decreased by 0.06 kg/d on the first week postpartum ($P < 0.01$; $R^2 = 0.95$), 0.04 on the second week postpartum ($P < 0.01$; $R^2 = 0.95$), 0.11 kg/d on the third week postpartum ($P < 0.01$; $R^2 = 0.95$), and 0.10 kg/d on the fourth week postpartum ($P < 0.01$; $R^2 = 0.43$).

Logistic regression data are in Tables 5.9 and 5.10. Not having FVD at 4 ($P = 0.09$; OR = 0.35; 95% CI = 0.10-1.20) and 7 DIM ($P = 0.09$; OR = 0.43; 95% CI = 0.17-1.12) tended to be associated with lesser odds of developing cytological endometritis at 15 DIM. Additionally, not having FVD at 15 DIM was associated with lesser odds of having cytological endometritis at 15 DIM ($P = 0.02$; OR = 0.33; 95 % CI = 0.13-0.80). Dry matter intake < 11 kg/d during prepartum was associated with lesser odds of developing cytological endometritis at 30 DIM ($P = 0.05$; OR = 0.77; 95 % CI = 0.44-1.00). Dry matter intake < 13 kg/d during early postpartum was also associated with lesser odds of developing cytological endometritis at 30 DIM ($P = 0.04$; OR = 0.65; 95 % CI = 0.43-0.98).

2. Second objective. To determine the association of vaginal discharge with endometrial cytology (CYT15 and CYT30) and production parameters (prepartum DMI, DMI as a percentage of BW, BW, and BCS, and postpartum DMI, DMI as a percentage of BW, BW, BCS, and milk production).

2.2. Dry matter intake, BW, BCS, and Milk yield

Production parameters associated with vaginal discharge are in Table 5.5. Prepartum DMI and DMI as a percentage of BW was greater ($P \leq 0.05$) for cows with a MC ≤ 3 at 15 DIM (12.3 ± 1.53 kg/d and 1.53 ± 0.24 %, respectively) than for cows with a MC > 3 at 15 DIM (9.91 ± 1.53 kg/d and 1.19 ± 0.24 %, respectively). Prepartum BW was lesser ($P < 0.01$) for cows with a MC ≤ 3 at 15 DIM (790 ± 11.0 kg) than for cows with MC > 3 at 15 DIM (812 ± 11.0 kg). Prepartum BCS was lesser ($P = 0.02$) for cows with a MC ≤ 3 at 10 DIM and at 13 DIM (3.77 ± 0.18 and 3.76 ± 0.16, respectively) than for cows with a MC > 3 at 10 DIM and at 13 DIM (4.06 ± 0.18 and 4.01 ± 0.16, respectively). Postpartum DMI as a percentage of BW was lesser ($P = 0.04$) for cows with a MC ≤ 3 at 10 DIM (2.14 ± 0.04 %) than for cows with a MC > 3 at 10 DIM (2.24 ± 0.04 %); and tended to be greater ($P = 0.10$) for cows with a MC ≤ 3 at 15 DIM (2.21 ± 0.03 %) than for cows with a MC > 3 at 15 DIM (2.16 ± 0.03 %). Postpartum BW was greater ($P < 0.01$) for cows with a MC ≤ 3 at 10 DIM and at 15 DIM (680 ± 1.88 kg and 683 ± 1.68 kg, respectively) than for cows with a MC > 3 at 10 DIM and at 15 DIM (671 ± 1.88 kg and 672 ± 1.68 kg, respectively). Milk yield was greater ($P < 0.01$) for cows with a MC ≤ 3 at 7 DIM (38.5 ± 0.42 kg/d), at 13 DIM (38.3 ± 0.85 kg/d), at 15 DIM (38.2 ± 0.59), and at 17 DIM (38.5 ± 0.83) than for cows with a MC > 3 at 7 DIM (36.6 ± 0.42 kg/d), at 13 DIM (37.1 ± 0.85 kg/d), at 15 DIM (37.5 ± 0.59), and at 17 DIM (37.5 ± 0.83), respectively.

2.3. Endometrial cytology

Percentage of endometrial PMN cells at 15 DIM was greater ($P < 0.01$) for cows with a MC ≤ 3 at 10 DIM (37.1 ± 0.93 %) than for cows with a MC > 3 at 10 DIM (32.3 ± 0.93 %). Percentage of endometrial PMN cells at 30 DIM was lesser ($P = 0.05$) for cows with a MC ≤ 3 at 17 DIM (21.9 ± 3.75 %) than for cows with a MC > 3 at 17 DIM (30.1 ± 3.75 %).

DISCUSSION

The primary objective of this study was to evaluate the association of prepartum and postpartum DMI (kg/d and as % of BW), BW, and BCS, early postpartum milk yield and milk composition, and days to first ovulation with cytological endometritis at 15 and at 30 DIM. We hypothesize that these productive parameters and days to first ovulation would be associated with CYT15 as well as with CYT30, and whenever a cow would develop both, this would incur in an additive negative effect. A second objective was to associate vaginal discharge with CYT15 and CYT30 and the production parameters. We hypothesize that vaginal discharge is not necessarily associated with uterine cytological endometritis, but it is associated with production parameters.

Dry matter intake, DMI as a percentage of BW, BW, and BCS

Herein, we report an association of prepartum and postpartum DMI (kg/d and as % of BW) with CYT15 from 4 wk prepartum until 4 wk postpartum, where cows in HIGH15 had lower DMI than cows in LOW15. Additionally, for every 1 kg increase in DMI 3 wk prior to calving, or for every unit increase in DMI (% of BW) 3 wk postpartum, the percentage of PMN cells at 15 DIM decreased 13% and 4.45%, respectively. Until now, there is no defined cause for cytological endometritis in dairy cows. Some studies report an association of metritis and DMI (Huzzey et al., 2007; Pérez-Báez et al., 2019). Alternatively, prepartum energy intake is associated with changes in postpartum blood neutrophil function (Grauhnard et al., 2014; Moyes

et al. 2014). Moyes et al. (2014) reported lower postpartum blood PMN phagocytosis capacity in cows fed a prepartum diet formulated for 1.62 Mcal/kg of DM in comparison with cows fed a prepartum diet formulated for 1.34 Mcal/kg of DM. Impaired PMN function characterizes immunosuppression, which is observed at some degree in dairy cows in the peripartum period (Weber et al., 2001; Goff, 2006; Hammon et al., 2009). Although a physiological event, this immunosuppression might be aggravated by nutritional aspects, such as excess of energy intake in the prepartum period (Graugnard et al., 2012; Moyes et al., 2014) or increased negative energy balance metabolites (Ingvartsen and Moyes, 2013). In the present study, cows were consuming a diet averaging 1.45 ± 0.05 Mcal of NE_L/kg of DMI during prepartum, which could be considered a moderate-energy diet (Cardoso et al., 2020). Data indicate that over-consuming energy during prepartum period may predispose dairy cows to health problems when DMI is limited (Cardoso et al., 2013). Thus, it is possible that the lower prepartum intake of cows in HIGH15 lead to greater mobilization of adipose tissue fatty acids, elevating blood concentration of NEFA, potentially influencing immune cells in the circulation and inflammation indices (LeBlanc, 2020). Migration of PMN cells is activated upon stimulation, for example by IL-8 or tumor necrosis factor α (TNFα) released from macrophages, or by pathogen associated molecular patterns detected by toll-like receptors (Sheldon et al., 2019). Negative energy balance metabolites, such as NEFA, can activate toll-like receptor 4, leading to secretion of TNFα and IL-8 (Ingvartsen and Moyes, 2013). At the same time, negative energy balance metabolites are associated with decreased function of PMN cells. Thus, we hypothesize that the association of lower intake pre- and postpartum and HIGH15 is possibly explained by an increased migration of PMN cells in the uterus to compensate a potential lower PMN function.

The lack of association of CYT30 and milk yield can be explained by the lack of association of CYT30 and DMI, since milk yield is mostly driven by intake (Bell and Roberts, 2007). Conversely, Akbar et al. (2014) reported that cows with uterine PMN > 18% between 22 and 25 DIM had lesser milk yield through the first 3 wk postpartum than cows with uterine PMN < 18%. Akbar et al. (2014) suggested the lower milk yield to be, at least in part, a consequence of increased utilization of glucose by the immune system, thus limiting the glucose available for milk production. It is possible that differences in the PMN percentage cutoff values and sampling date contributed to differences in results. For instance, Gobikrushanth et al. (2016) reported no association of DMI and milk yield with different categories of endometritis (cytological, clinical, or both) assessed at 25 ± 1 DIM. Additionally, it is plausible that DMI is a main explanatory factor for milk yield differences, although DMI data is not provided by Akbar et al. (2014).

Wittrock et al. (2011) used vaginal discharge associated with fever to classify cows as having metritis or not in the first 3 wk postpartum and reported that multiparous cows with metritis ate less during 3 wk after calving (12.2 ± 1.2 vs.14.0 ± 0.8 kg/d). Safeguarding the limitations of directly comparing our results with the reported by Wittrock et al. (2011), since the evaluation of the vaginal discharge was performed using a different scoring, we report no differences in postpartum DMI (kg/d). However, the vaginal discharge evaluated at 10 DIM and at 15 DIM were associated with differences in the DMI as a percentage of BW. At 10 DIM, cows with a MS ≤ 3 ate less than the cows with MS > 3, but when vaginal discharge was evaluated at 15 DIM, cows with MS ≤ 3 ate more than cows with a MS > 3. These could be indicating that at 10 DIM the vaginal discharge could be more associated with the natural reproductive tract cleansing process, which physiologically happens after calving. Conversely, when evaluated at 15 DIM, the vaginal discharge could be more associated with a uterine inflammatory process,

which would concomitantly have an increased influx of PMN cells in the uterus. Additionally, we highlight that the timing of evaluating the vaginal discharge matters, since they are associated with different responses for the outcome.

Lactation performance

Cytological endometritis at an earlier stage of the postpartum period (15 DIM) is associated with decreased milk yield. We attribute the decreased milk yield for cows in HIGH15 to be a consequence of a decrease in DMI. A decline in milk yield was previously reported to be driven by decreased DMI (Huzzey et al., 2007; Bell and Roberts, 2007). In contrast, CYT30 was not associated with milk production, agreeing with Dubuc et al. (2010). Dubuc et al. (2010) suggested this lack of association to be due to cytological endometritis being a condition localized at the reproductive tract and therefore, not having systemic effects. However, the present study only used milk yield records until 30 DIM, thus, prospective effects of cytological endometritis on milk yield are not discarded. Additionally, for every unit increase in MS, milk yield decreases up to 2.26 kg/d in the early postpartum. Since the evaluation of vaginal discharge using MS is a tool for the on-farm diagnosis of metritis (Sheldon et al., 2006), this result agreed with previous studies reporting reduction in milk yield due to metritis (Wittrock et al., 2011; Giuliodori et al., 2013; Stangaferro et al., 2016). Although out of the scope of the present study to determine the origin of vaginal discharge, it is important to highlight that it could be originating from the uterus or cervix (LeBlanc et al., 2011; LeBlanc, 2014). Moreover, for every unit increase in uterine PMN percentage at 30 DIM, milk yield decreases up to 0.11 kg/d, which indicates a greater impact of purulent vaginal discharge than uterine cytology on milk yield.

Differences in milk composition were associated with CYT15 and CYT30. The greater milk protein yield for cows in LOW15 at wk 3 and 4 postpartum are likely a result of the greater

DMI (kg/d and as % of BW) observed for these cows since DMI and milk protein yield are interrelated (NRC, 2001; Roseler et al., 1997a, 1997b). A meta-analysis reported by Hristov et al. (2004) identified DMI as the major variable influencing milk yield and milk protein yield. A greater DMI leads to greater outflow of microbial protein from the rumen, leading to greater absorption of indispensable AA (IAA) and a greater supply of glucose or propionate (Rode et al., 1985; Raggio et al., 2006). Thus, milk protein yield is stimulated by both IAA and insulin activation of mTORC1 (mechanistic target of rapamycin complex 1) in mammary epithelial cells (Apuhamy et al., 2011).

Milk fat content was greater during wk 1 postpartum for cows in HIGH30 than for cows in LOW30, which could indicate a greater mobilization of adipose tissue. During a negative energy balance, the greater mobilization of adipose tissue for energy would result in a greater concentration of preformed FA than de novo and mixed FA (Palmquist et al., 1993; Barbano et al., 2019). However, milk fatty acids were not evaluated. Milk fat yield tended to be greater for cows in LOW15 than cows in HIGH15 on wk 4 and 5 postpartum, which is likely an effect of more available energy, since LOW15 cows had greater DMI in the postpartum period and, at 4 wk postpartum the NEB is likely resolved (Drackley, 1999). In the same way, had FA composition of milk fat being available and this hypothesis correct, de novo and mixed FA would likely be in greater concentration due to increased acetate production in the rumen and decreased plasma NEFA (Palmquist et al., 1993; Barbano et al., 2019). Somatic cell count was greater for cows in HIGH30 at wk 4, but lower at wk 5 than cows in LOW30. Because SCC is frequently more related to events occurring in the mammary gland, we suggest minimal biological relationship of these results to uterine cytology. Besides diurnal variation, mammary

gland insult leading to modulation of inflammatory mediators is the main factor significantly impacting SCC (Harmon, 1993, Sharma et al., 2011).

Vaginal discharge, and days to first ovulation

Cows in HIGH15-HIGH30 had higher averaged MS than cows in LOW15-LOW30, LOW15-HIGH30, and HIGH15-LOW30. However, when evaluated by day, only MS at 10 DIM was associated with uterine PMN percentage at 15 DIM, where cows with MS > 3 had lesser PMN percentage and greater DMI (% of BW) than cows with a MS ≤ 3. Alternatively, MS ≤ 3 at 15 DIM was associated with greater DMI (% of BW) and MS > 3 at 17 DIM was associated with greater uterine PMN percentage at 30 DIM. The cutoff value of 3 for the MS is based on the character and odor of the vaginal mucus being associated with the uterine bacterial load, since the presence of mucopurulent, purulent, or fetid vaginal discharge is associated with greater growth of *A. pyogenes*, *F. necrophorum*, *E. coli*, and *M. haemolytica* (Williams et al., 2005). A MS > 3 would mean that a fetid odor is necessarily present and, thus, the characteristic of the vaginal discharge would be more related to a bacterial contaminated uterus. However, a MS ≤ 3 at 10 DIM was associated with greater PMN influx in the uterus at 15 DIM, which could be reflecting physiological mechanisms of uterine involution. However, cows with a MS ≤ 3 also had lower DMI (% of BW) than cows with a MS > 3, which indicates that the lower intake could also be impacting the uterine PMN influx as for cows in HIGH15. When evaluated at 17 DIM, vaginal discharge characterizing a MS > 3 was associated with greater uterine PMN percentage at 30 DIM. Though speculative, one could hypothesize that a fetid vaginal discharge (MS > 3) at 17 DIM being associated with increased PMN percentage at 30 DIM could be reflecting an ascending contamination, since fetid vaginal discharge is highly associated with bacterial contamination (Williams et al., 2005). However, this requires further and controlled studies to

either confirm or refute this concept. Still, it is notable that the evaluation of vaginal discharge at several DIM is differently associated with uterine PMN influx.

Cows in HIGH15 ovulated on average 3 days earlier than cows in LOW15, which was also observed regarding cows in HIGH30 and LOW30. It has been previously reported that cows with uterine infections in the first week postpartum are susceptible to have perturbed ovarian follicular growth, specifically the growth of dominant follicle into a preovulatory size follicle, which leads to delayed ovulation (Sheldon et al., 2002; Williams et al., 2007). The mechanisms behind uterine infections causing ovarian dysfunction appear to be related to pathogenic organisms disrupting the uterine mucosal layer and releasing LPS, which can enter the ovarian follicular fluid through the circulation and disturb ovarian cyclic events (Peter et al., 1989; Huszenicza et al., 1999; Dahiya et al., 2018). Moreover, inflammatory mediators can also disturb cyclic events through impacts on the hypothalamic-pituitary-gonadal-axis (Sheldon, 2009; Williams et al., 2001). However, these events are usually related to delayed ovulation and development of follicular cysts, while cows in HIGH15 and HIGH30 actually had earlier ovulation. In a study that compared the follicular fluid in cystic ovarian follicles from cows in early lactation (≤ 35 DIM) and from cows in midlactation (≥ 118 DIM), Lima et al. (2019) reported that cows in early lactation had disturbances in steroidogenic and metabolic markers in the follicular fluid, while cows in midlactation had disturbances in immunological markers as well, besides steroidogenic and metabolic. Thus, it is possible that the earlier days to first ovulation in HIGH15 and HIGH30 cows is not related to inflammatory status as much as the metabolic status. Thus, the association of uterine inflammation and early postpartum ovarian cyclic events requires further investigation, with the inclusion of other explanatory variables

related to the cows' nutritional and metabolic status. Moreover, whether these events would positive or negatively impact subsequent fertility are yet to be responded.

CONCLUSIONS

We partially confirmed our hypothesis of an association of cytological endometritis and DMI and lactation performance. Cytological endometritis at 15 DIM was associated with lower DMI and a tendency for lower milk yield, while cytological endometritis at 30 DIM was associated with DMI as a percentage of BW, but it was not with milk yield. Additionally, cytological endometritis at both 15 DIM and at 30 DIM is associated with days to first ovulation, and further studies should now investigate this association with subsequent reproductive outcomes, taking into consideration the cows' intake. Furthermore, we confirmed our second hypothesis, since the vaginal discharge association with cytological endometritis is variable and dependent on the day of the vaginal discharge evaluation. However, vaginal discharge is strongly associated with milk yield in the first four weeks postpartum.

ACKNOWLEDGEMENTS

This project was partially supported by the USDA National Institute of Food and Agriculture (Washington, DC; NC-4112). We thank Adisseo (Alpharetta, GA) for the donation of Smartamine M; and Dairy Nutrition Plus (Ralston, IA) for the donation of Soyplus during the course of the experiments. We express sincere appreciation to the Dairy Focus Team at the University of Illinois, along with the University of Illinois Dairy Research Unit staff for assisting with data collection.

FIGURES AND TABLES

Figure 5.1. Association of prepartum and postpartum dry matter intake (DMI, kg/d; A) and milk yield (kg/d; B) with cytological endometritis at 15 days in milk (DIM). Values are least squares means ± SEM. Cytological endometritis at 15 DIM (CYTO) was defined based on the percentage of polymorphonuclear cells (PMN) from endometrial cytology samples. Cut-off values for samples being classified as having cytological endometritis were obtained from the median values of the data set (> 24%). Cows were classified as LOW15 (PMN % 15 DIM ≤ 24%) and HIGH15 (PMN % 15 DIM > 24%). Cows in LOW15 had greater DMI throughout the whole period than cows in HIGH15 ($P < 0.01$; Graph A). In Graph B, (◊) indicates a tendency for a difference at that time point at $P < 0.10$.

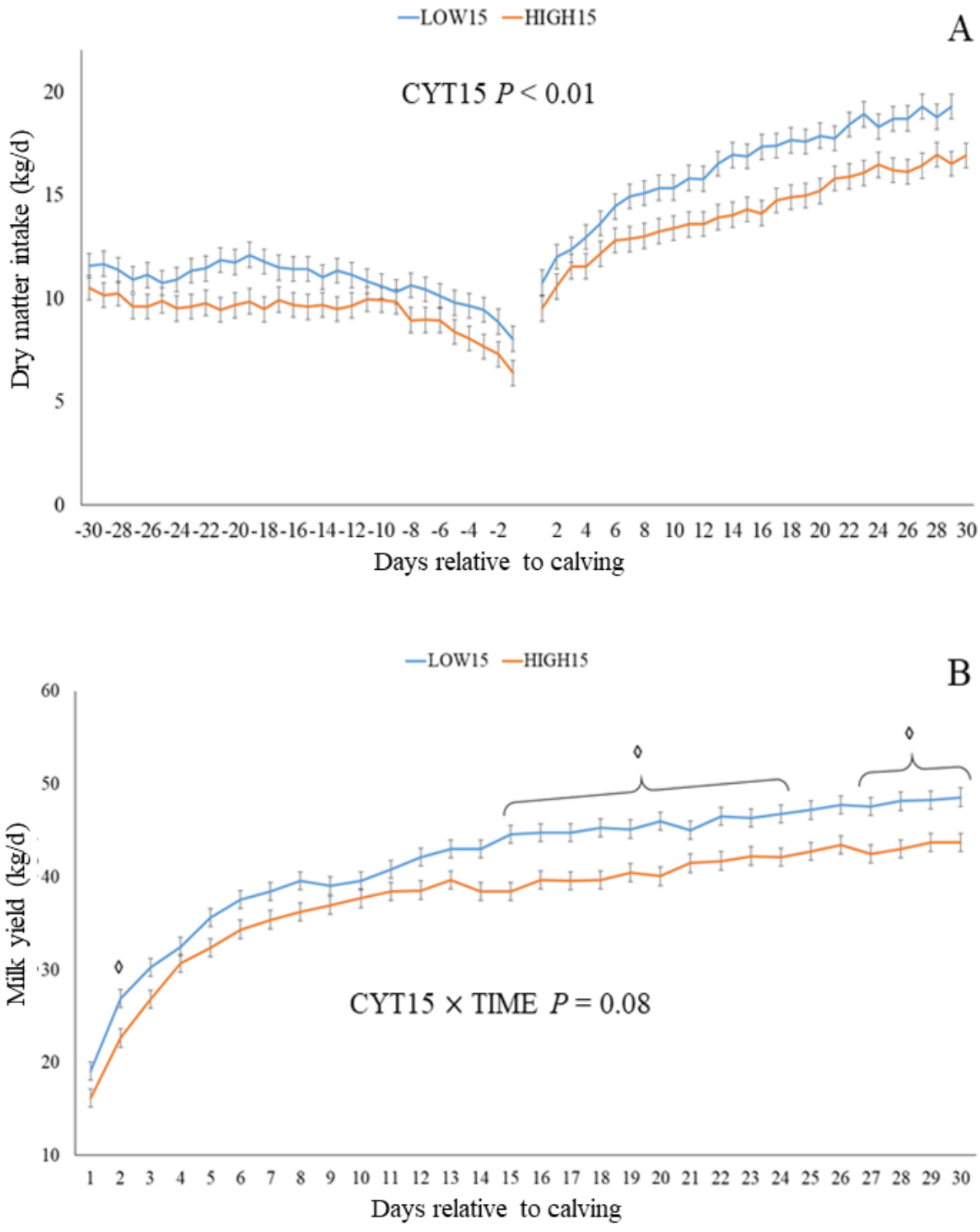


Figure 5.1 (cont.).

Figure 5.2. Association of prepartum and postpartum dry matter intake (DMI, kg/d; A) and milk yield (kg/d; B) with cytological endometritis at 30 days in milk (DIM). Values are least squares means ± SEM. Cytological endometritis at 15 DIM (CYTO) was defined based on the percentage of polymorphonuclear cells (PMN) from endometrial cytology samples. Cut-off values for samples being classified as having cytological endometritis were obtained from the median values of the data set (> 7%). Cows were classified as LOW30 (PMN % 30 DIM ≤ 7%) and HIGH30 (PMN % 30 DIM > 7%). Cows in LOW30 did not differ in DMI throughout the whole period than cows in HIGH30 ($P = 0.99$). No difference between milk yield from cows in LOW30 and HIGH30 ($P = 0.18$).

Figure 5.2 (cont.).

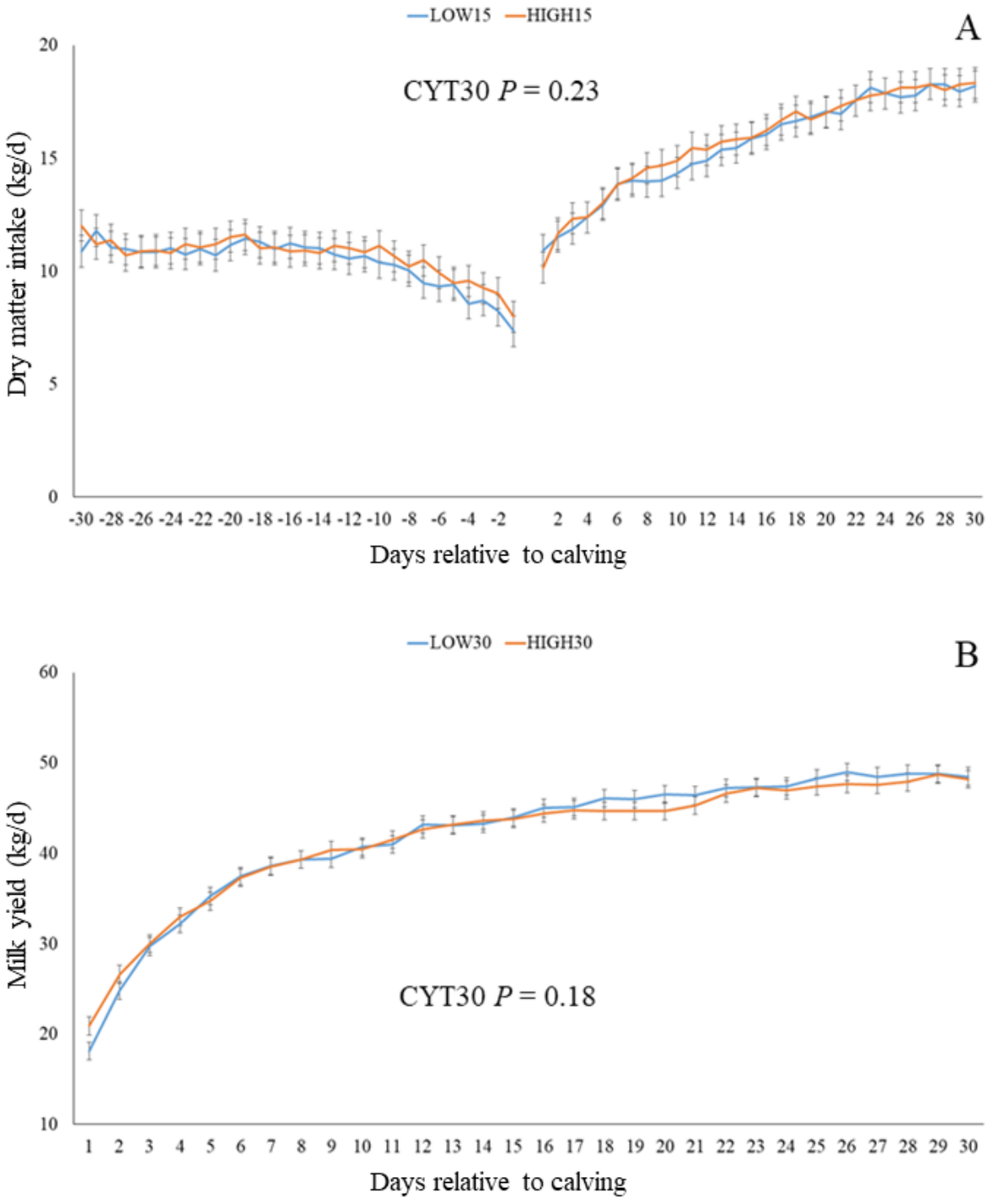


Figure 5.3. Least squares means (± SEM) for weekly body condition score (BCS) from 5 weeks prepartum until 5 weeks postpartum of Holstein cows classified according to their endometrial cytology at days 15 and 30 postpartum. Cytological endometritis at 15 DIM (CYT15) and at 30 DIM (CYT30) were defined based on the percentage of polymorphonuclear cells (PMN) from endometrial cytology samples. Cut-off values for samples being classified as having cytological endometritis were obtained from the median values of the data set for each specific day and were: 15 DIM: > 24%; 30 DIM: > 7%. Cows were classified as LOW15-LOW30 (PMN % 15 DIM ≤ 24% and PMN % 30 DIM ≤ 7%), LOW15-HIGH30 (PMN % 15 DIM ≤ 24% and PMN % 30 DIM > 7%), HIGH15-LOW30 (PMN % 15 DIM > 24% and PMN % 30 DIM ≤ 7%), and HIGH15-HIGH30 (PMN % 15 DIM > 24% and PMN % 30 DIM > 7%). (♦) Indicates a difference at that time point at $P < 0.05$.

Figure 5.3 (cont.).

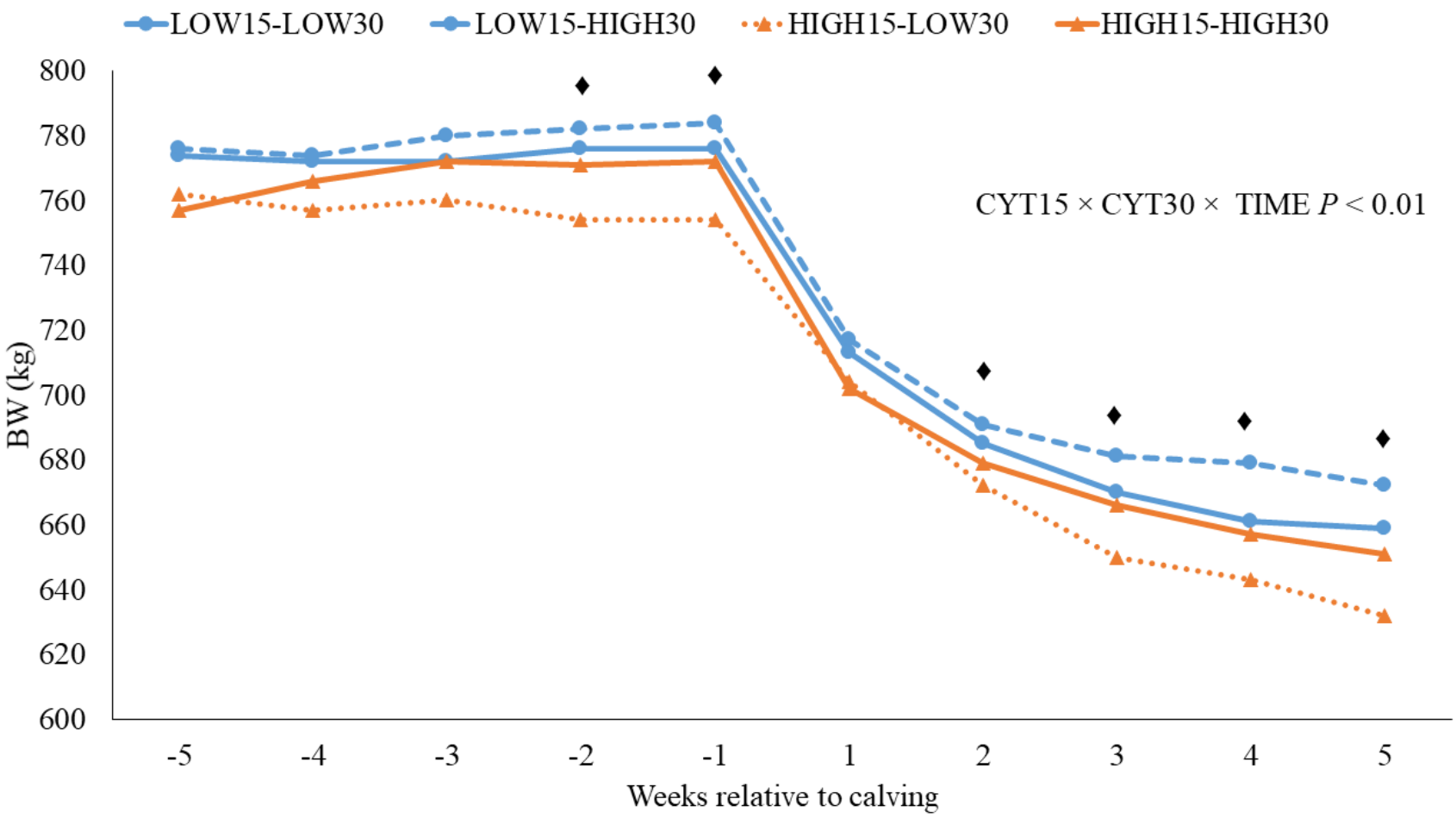


Figure 5.4. Least squares means (± SEM) for weekly body condition score (BCS) from 5 weeks prepartum until 5 weeks postpartum of Holstein cows classified according to their endometrial cytology at days 15 and 30 postpartum. Cytological endometritis at 15 DIM (CYT15) and at 30 DIM (CYT30) were defined based on the percentage of polymorphonuclear cells (PMN) from endometrial cytology samples. Cut-off values for samples being classified as having cytological endometritis were obtained from the median values of the data set for each specific day and were: 15 DIM: > 24%; 30 DIM: > 7%. Cows were classified as LOW15-LOW30 (PMN % 15 DIM ≤ 24% and PMN % 30 DIM ≤ 7%), LOW15-HIGH30 (PMN % 15 DIM ≤ 24% and PMN % 30 DIM > 7%), HIGH15-LOW30 (PMN % 15 DIM > 24% and PMN % 30 DIM ≤ 7%), and HIGH15-HIGH30 (PMN % 15 DIM > 24% and PMN % 30 DIM > 7%). (♦) Indicates a difference at that time point at $P < 0.01$, where cows in LOW15-LOW30 had lower BCS than cows in HIGH15-HIGH30. Additionally, cows in LOW15-HIGH30 tended to have lower BCS than cows in HIGH15-HIGH30 on week 2 ($P = 0.06$).

Figure 5.4 (cont.)

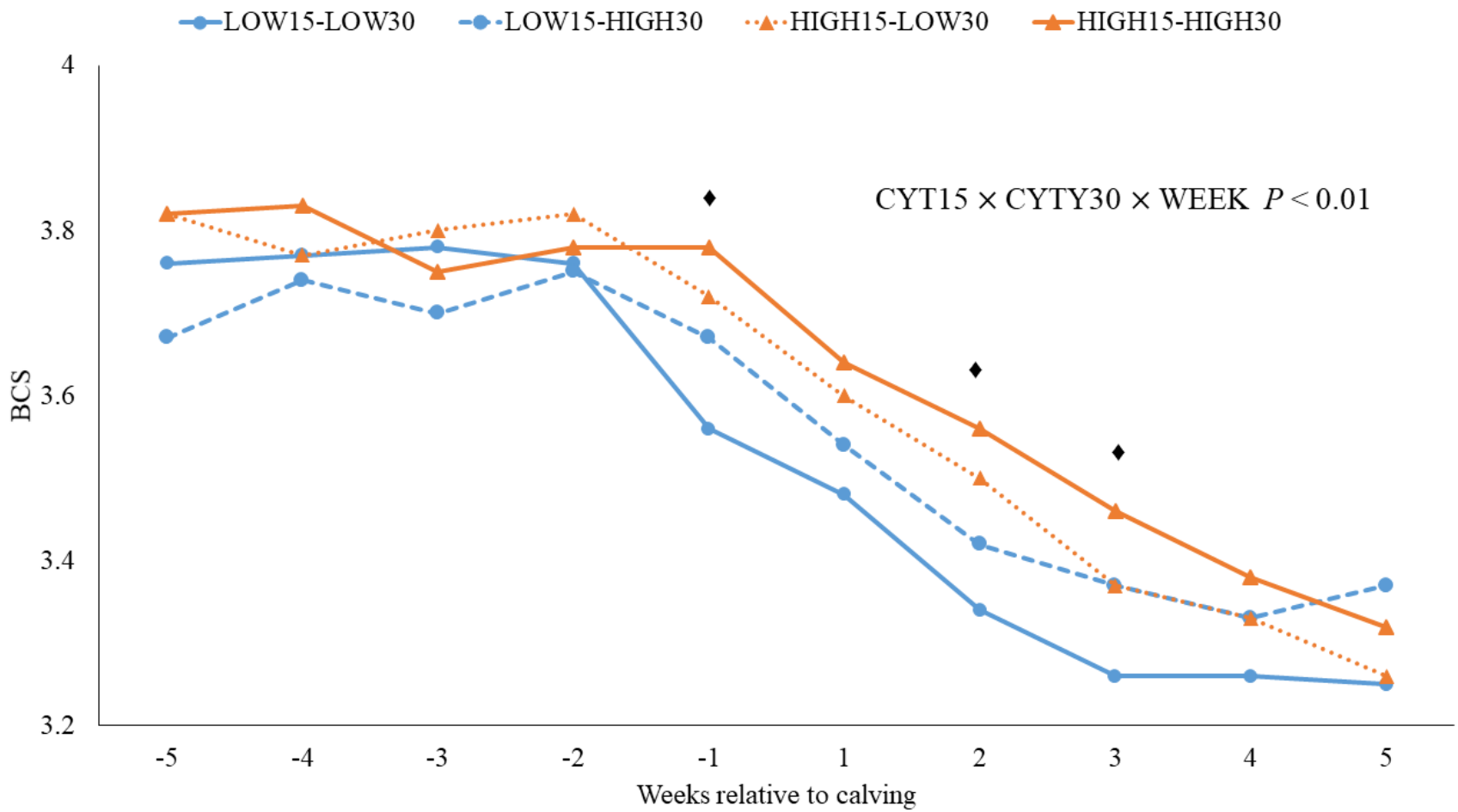


Figure 5.5. Least squares means (± SEM) for weekly milk fat, milk fat yield, milk protein yield, and somatic cell count from 5 weeks prepartum until 5 weeks postpartum of Holstein cows classified according to their endometrial cytology at days 15 and 30 postpartum. Cytological endometritis at 15 DIM (CYT15) and at 30 DIM (CYT30) were defined based on the percentage of polymorphonuclear cells (PMN) from endometrial cytology samples. Cut-off values for samples being classified as having cytological endometritis were obtained from the median values of the data set for each specific day and were: 15 DIM: > 24%; 30 DIM: > 7%. Cows were classified as LOW15-LOW30 (PMN % 15 DIM ≤ 24% and PMN % 30 DIM ≤ 7%), LOW15-HIGH30 (PMN % 15 DIM ≤ 24% and PMN % 30 DIM > 7%), HIGH15-LOW30 (PMN % 15 DIM > 24% and PMN % 30 DIM ≤ 7%), and HIGH15-HIGH30 (PMN % 15 DIM > 24% and PMN % 30 DIM > 7%). (♦) Indicates a difference at that time point at $P < 0.05$. (◊) Indicates tendency for a difference at that time point at $0.05 < P \leq 0.10$.

Figure 5.5 (cont.).

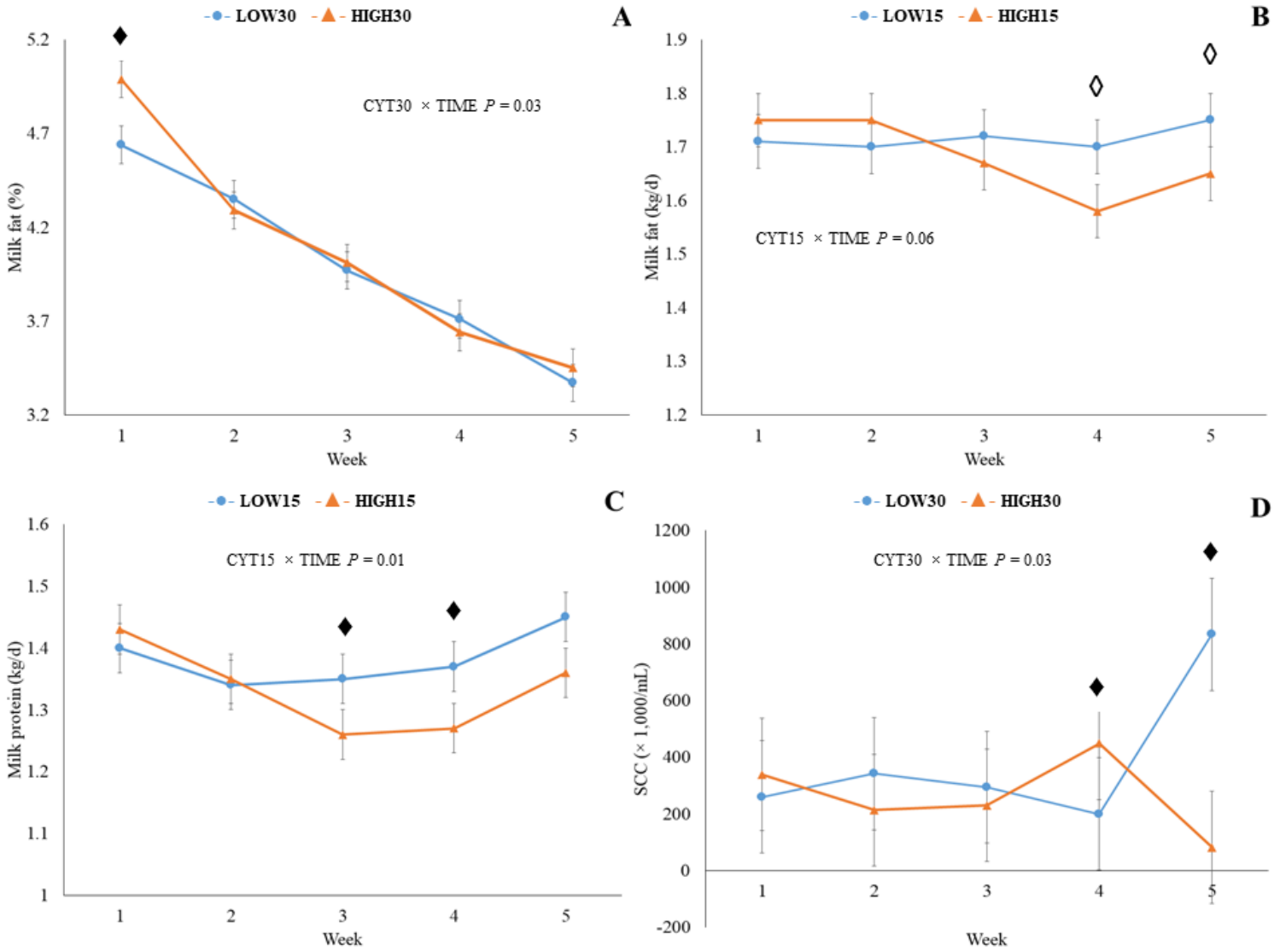
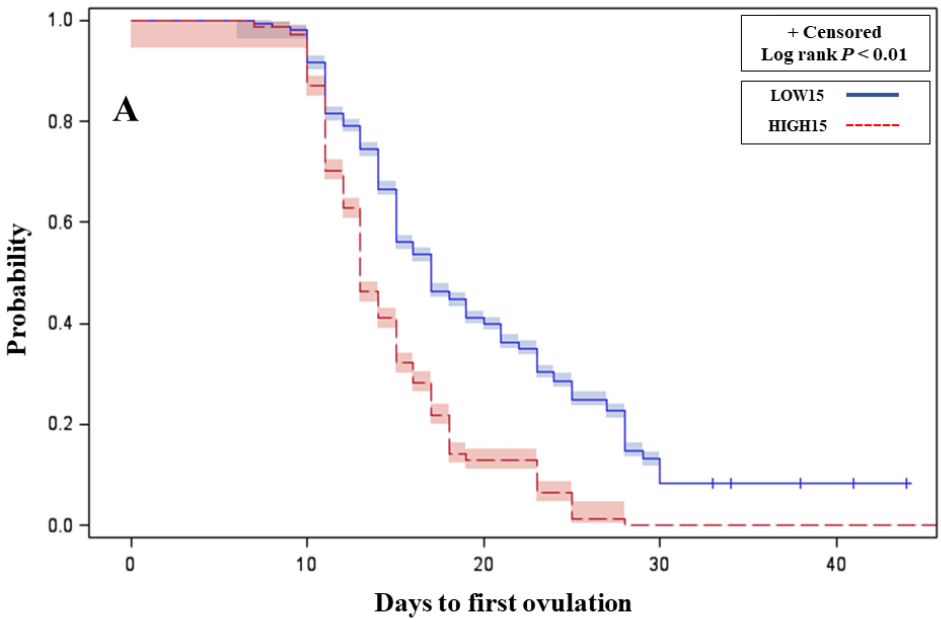


Figure 5.6. Survival curves for cytological endometritis at 15 DIM (A) and cytological endometritis at 30 DIM (B) across days to first ovulation. The y-axis represents the overall probabilities. Cytological endometritis at 15 DIM (CYT15) and at 30 DIM (CYT30) were defined based on the percentage of polymorphonuclear cells (PMN) from endometrial cytology samples. Cut-off values for samples being classified as having cytological endometritis were obtained from the median values of the data set for each specific day and were: 15 DIM: > 24%; 30 DIM: > 7%. The x-axis represents the number of days post-calving. Cows in LOW15 ovulated on average 3 days later than cows in HIGH15; and cows in LOW30 ovulated on average 3 days later than cows in HIGH30 (Log rank $P < 0.01$).

Figure 5.6 (cont.).



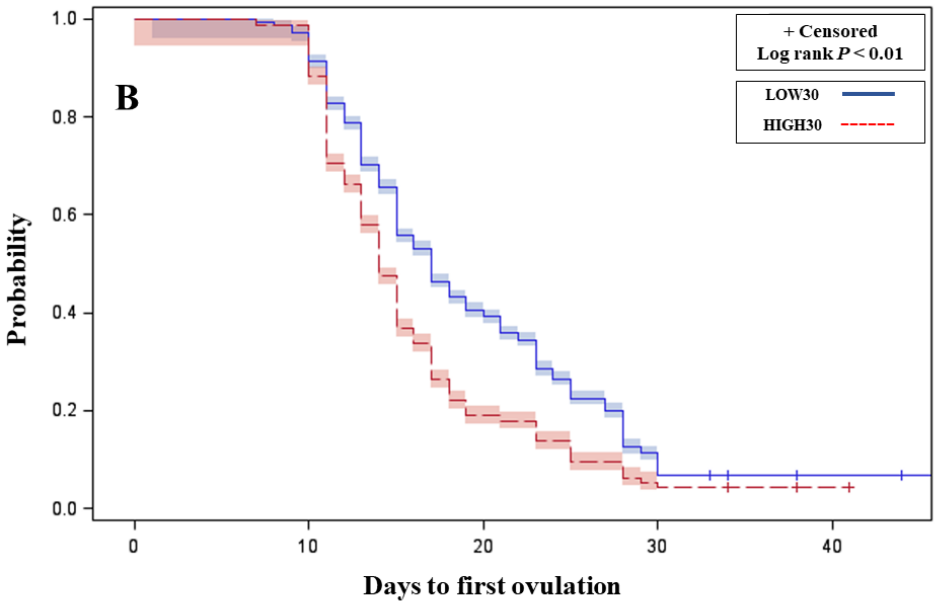
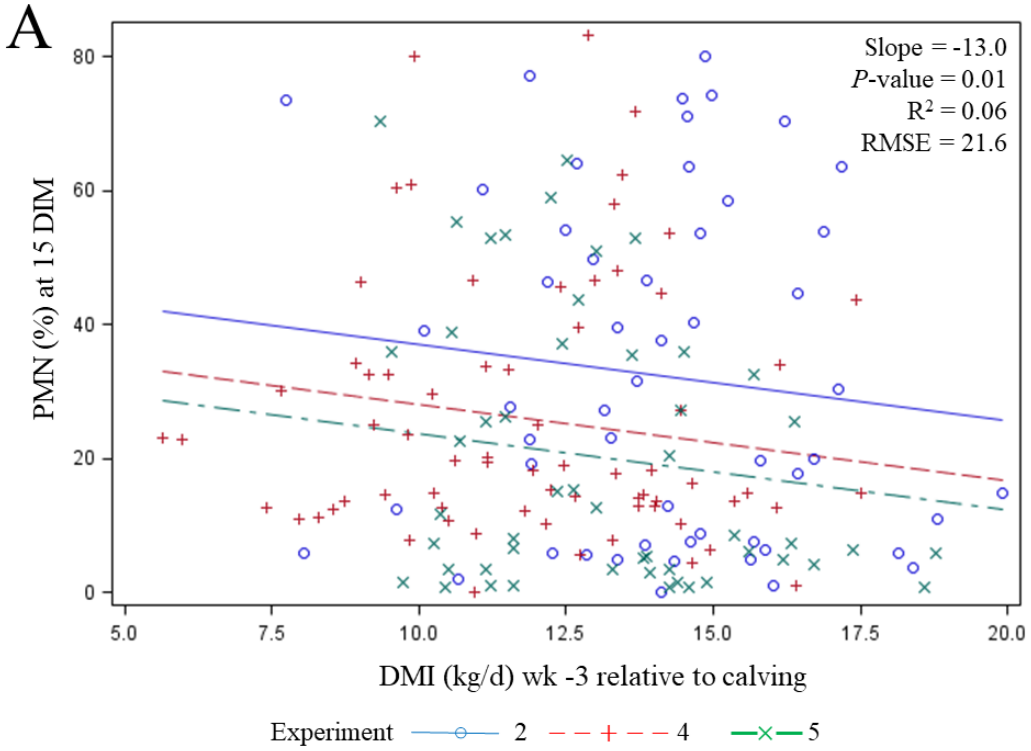


Figure 5.7. Associations of dry matter intake (DMI; kg/d) during the transition period and postpartum vaginal discharge or endometrial cytology of Holstein cows. Evaluation of vaginal discharge was performed using Metricheck device at 4, 7, 10, 13, 15, and 17 days in milk (DIM) following guidelines reported by LeBlanc and Bicalho (2017). Endometrial cytology samples were collected at 15 and 30 DIM and evaluated for the percentage of polymorphonuclear cells (PMN). Scatter diagrams display the specific association of: A) DMI on the third week prior to calving and uterine PMN percentage at 15 DIM; B) DMI on the first week postpartum and Metricheck score plus smell; C) DMI on the first week postpartum and uterine PMN percentage at 30 DIM; D) DMI on the second week postpartum and Metricheck score plus smell; E) DMI on the third week postpartum and uterine PMN percentage at 15 DIM; F) DMI on the fourth week postpartum and Metricheck score plus smell. Data from 5 different studies were combined. Model accounted for the random effects of cow nested within each experiment.

A


Slope = -13.0
P-value = 0.01
R² = 0.06
RMSE = 21.6
PMN (%) at 15 DIM
0
20
40
60
80
5.0
7.5
10.0
12.5
15.0
17.5
20.0
DMI (kg/d) wk -3 relative to calving
Experiment
2
4
5

B

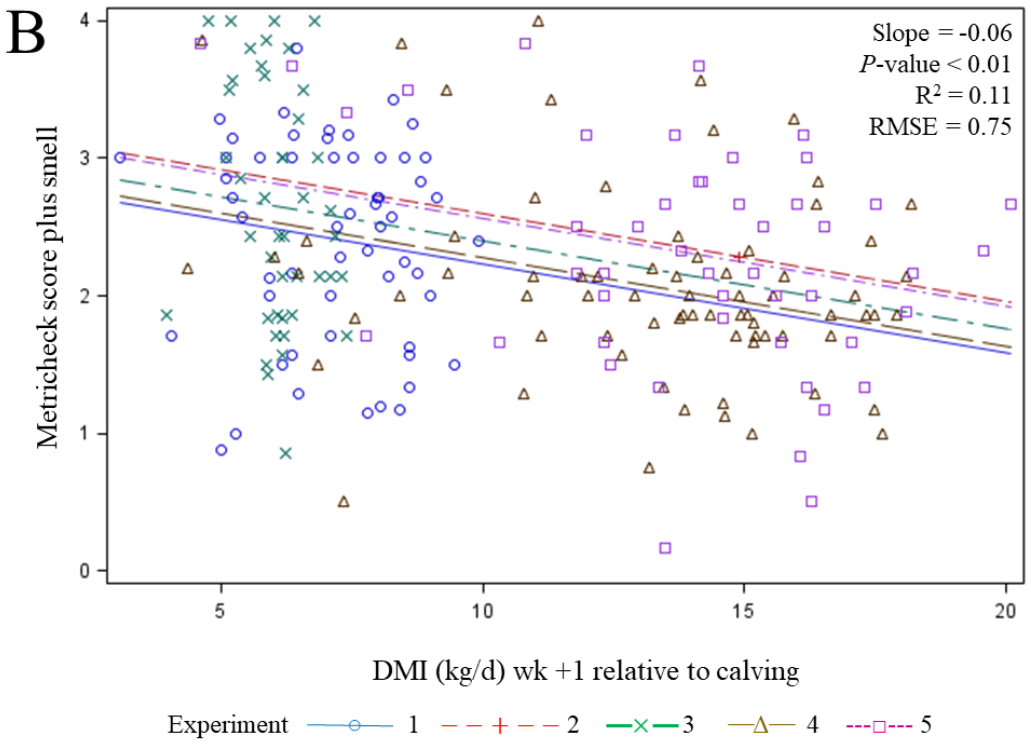
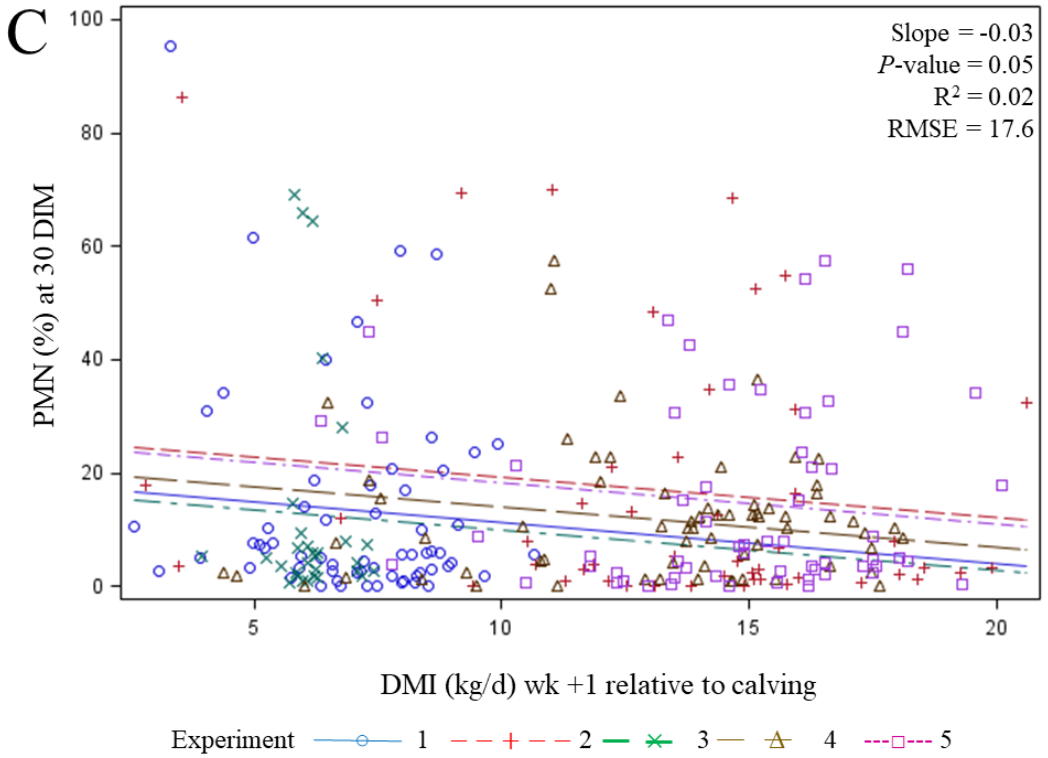

Slope = -0.06
P-value < 0.01
R² = 0.11
RMSE = 0.75
Metricheck score plus smell
0
1
2
3
4
5
10
15
20
DMI (kg/d) wk +1 relative to calving
Experiment
1
2
3
4
5

Figure 5.7 (cont.).



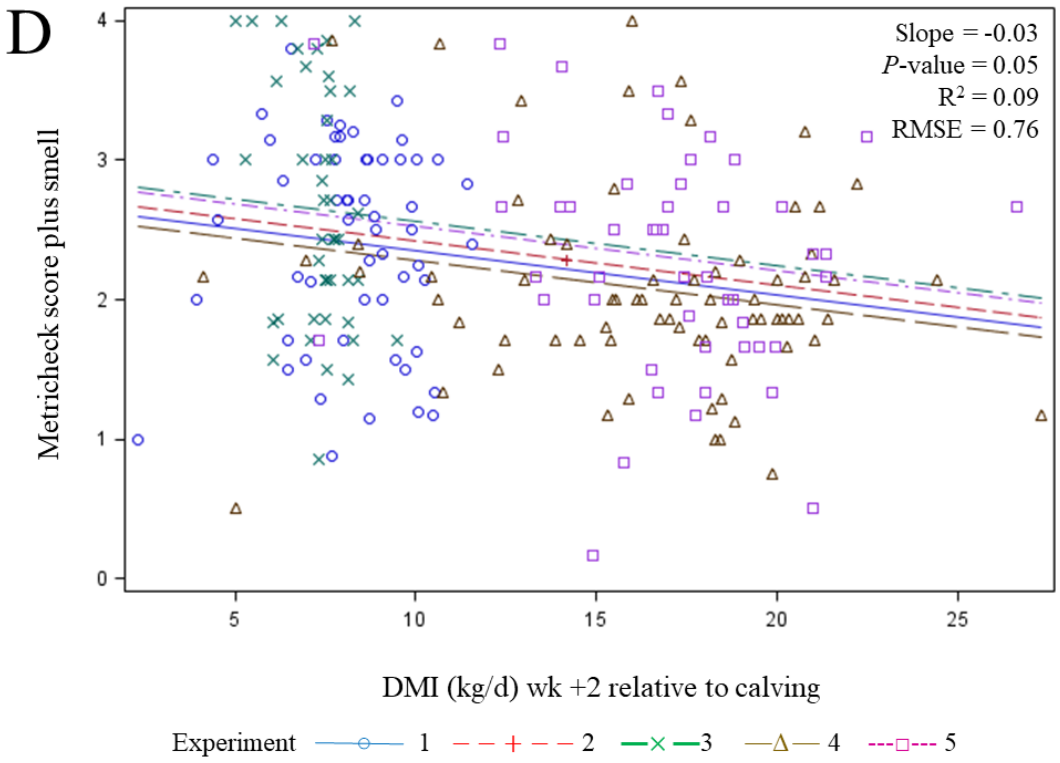
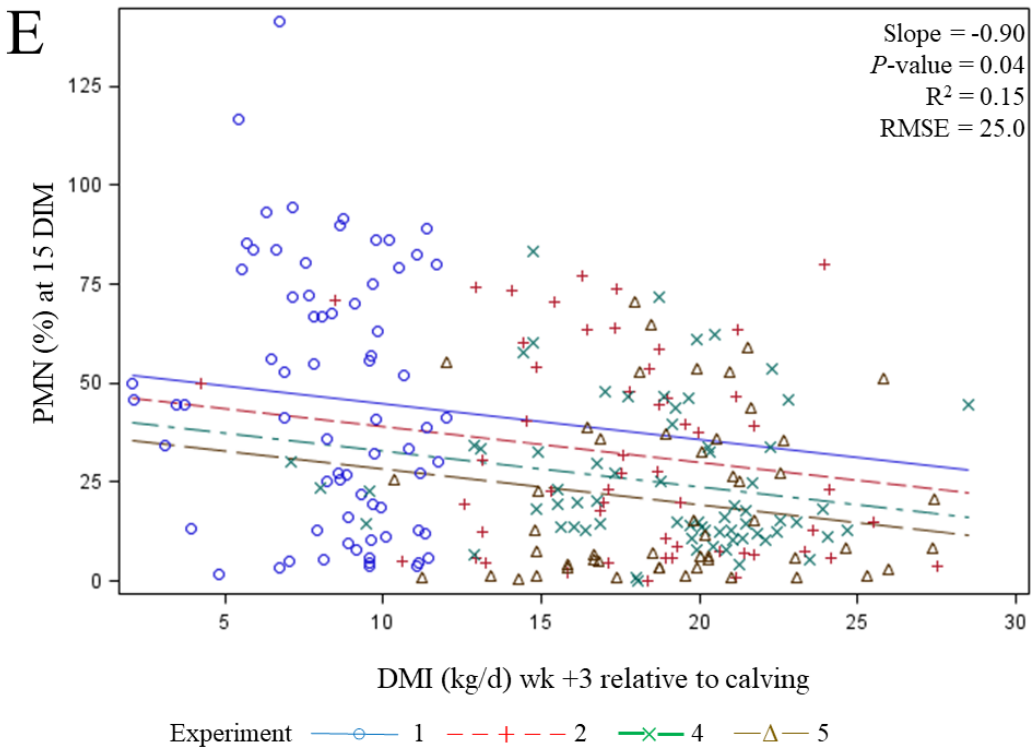


Figure 5.7 (cont.).



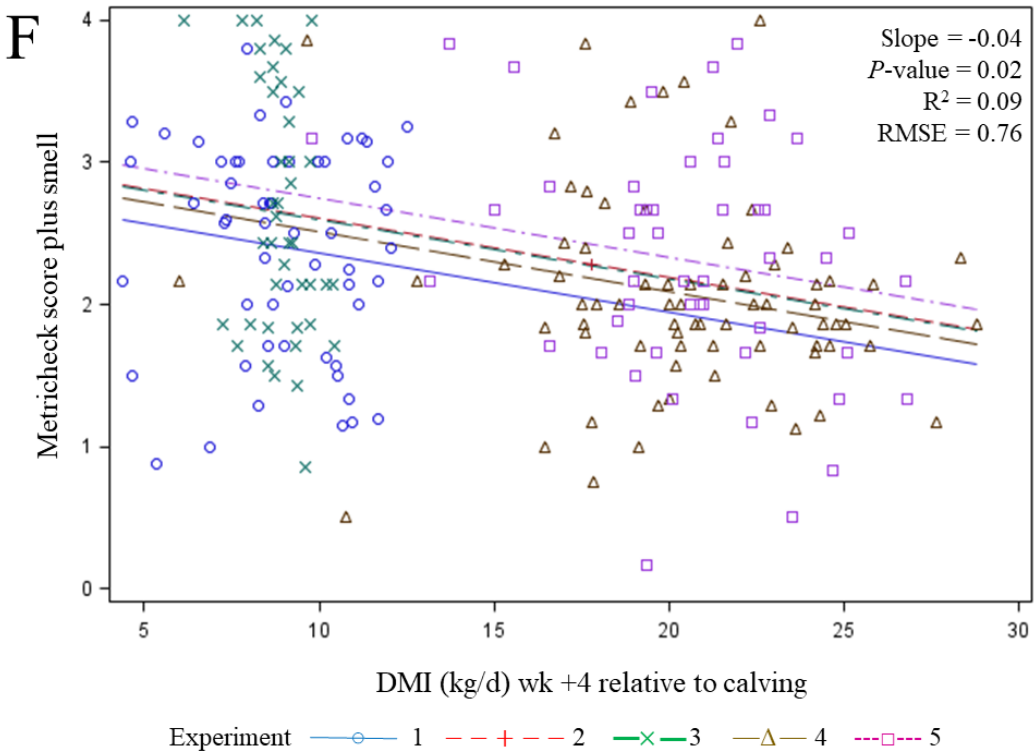


Figure 5.8. Associations of dry matter intake (DMI; % of BW) during the transition period and postpartum vaginal discharge or endometrial cytology of Holstein cows. Evaluation of vaginal discharge was performed using Metricheck device at 4, 7, 10, 13, 15, and 17 days in milk (DIM) following guidelines reported by LeBlanc and Bicalho (2017). Endometrial cytology samples were collected at 15 and 30 DIM and evaluated for the percentage of polymorphonuclear cells (PMN). Scatter diagrams display the specific association of: A) DMI on the fourth week prior to calving and uterine Metricheck score plus smell; B) DMI on the first week prior to calving and uterine PMN percentage at 30 DIM; C) DMI on the first week postpartum and uterine Metricheck score plus smell; D) DMI on the second week postpartum and uterine PMN percentage at 15 DIM; E) DMI on the third week postpartum and Metricheck score plus smell. Data from 5 different studies were combined. Model accounted for the random effects of cow nested within each experiment.

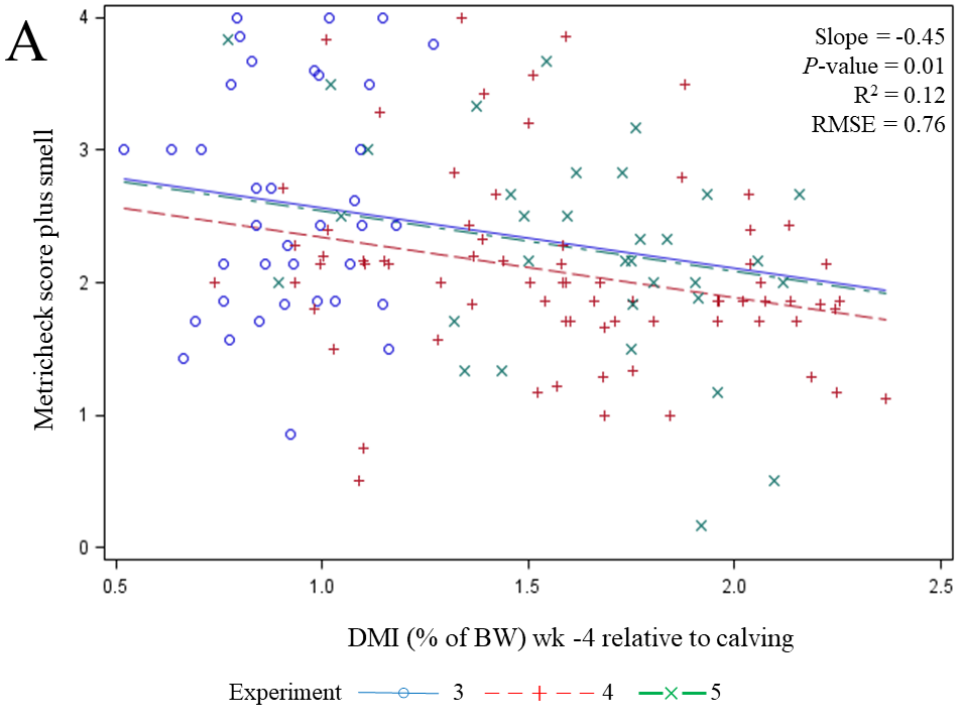
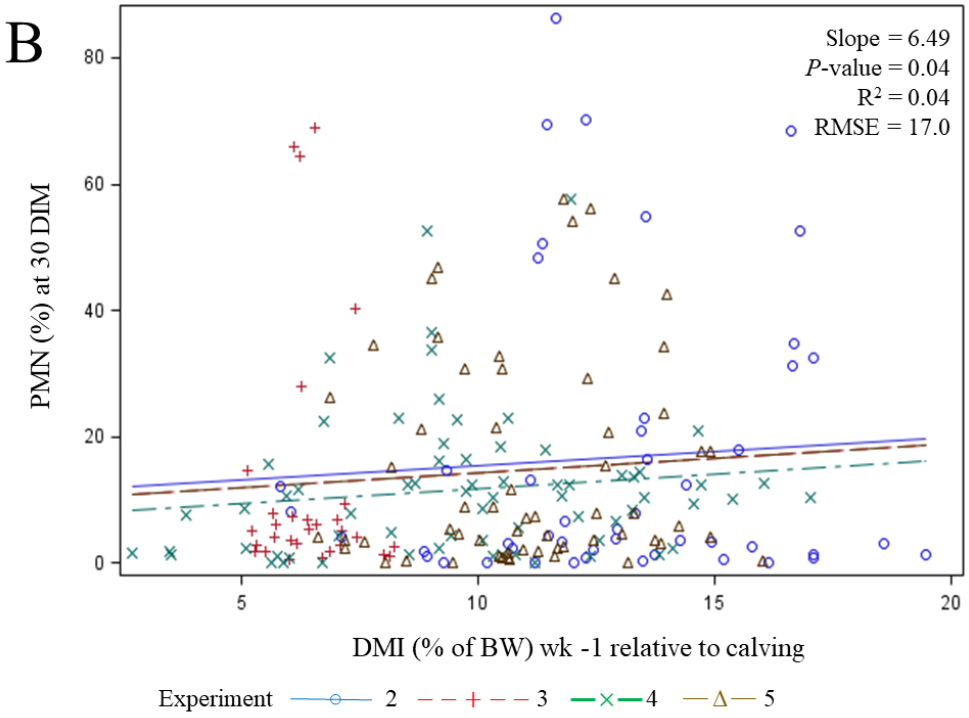
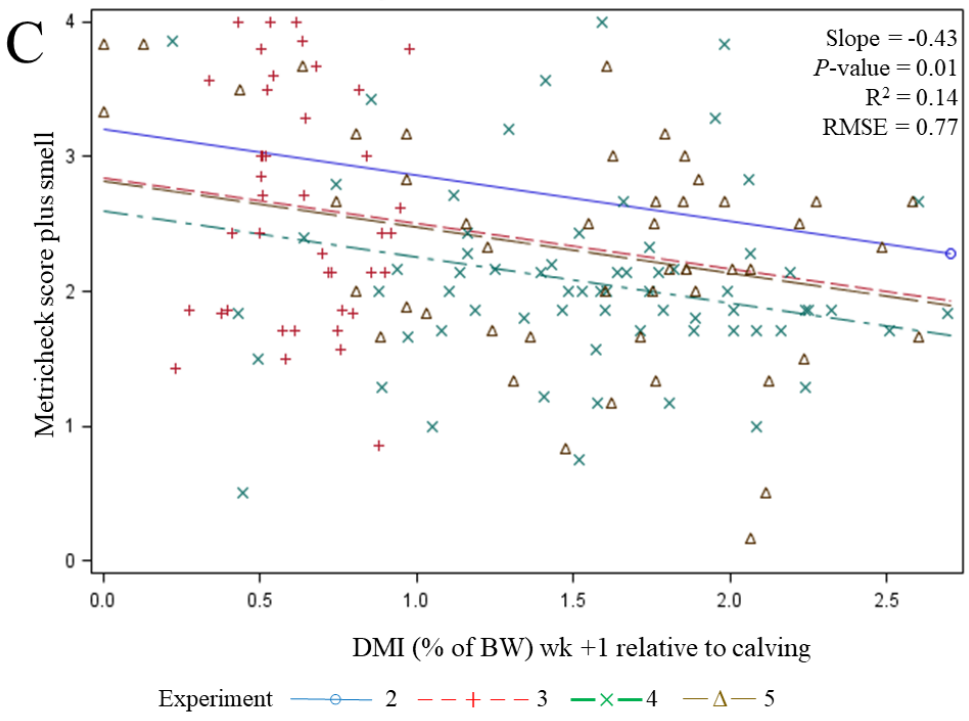


Figure 5.8 (cont.).





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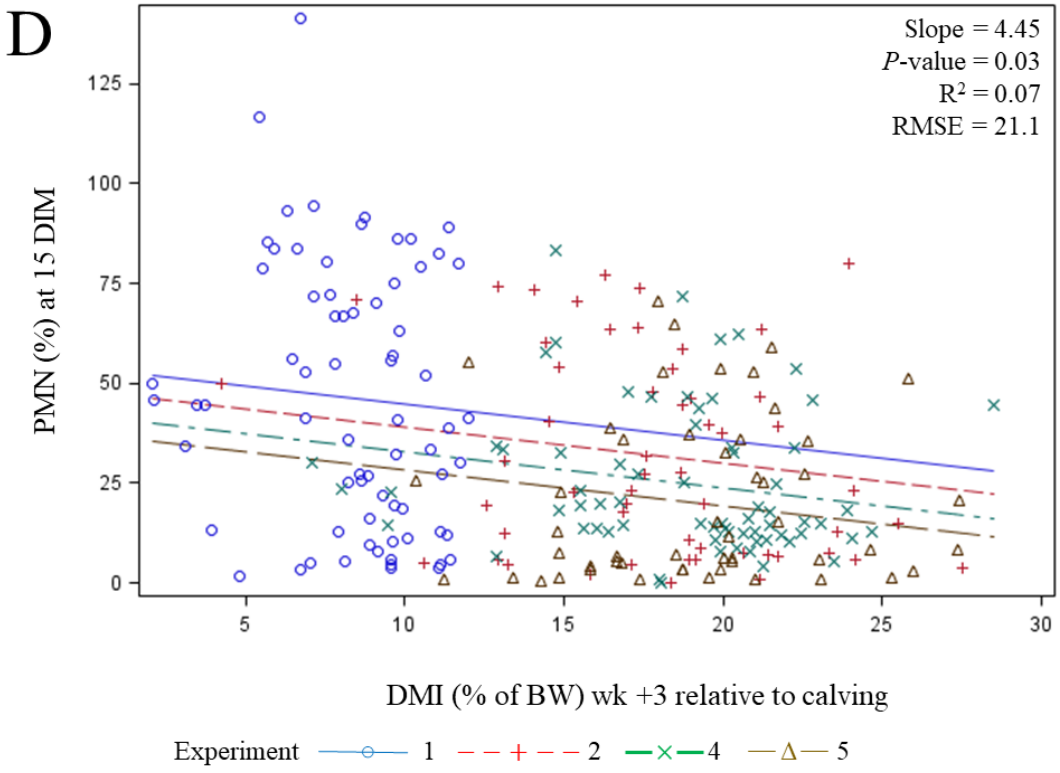


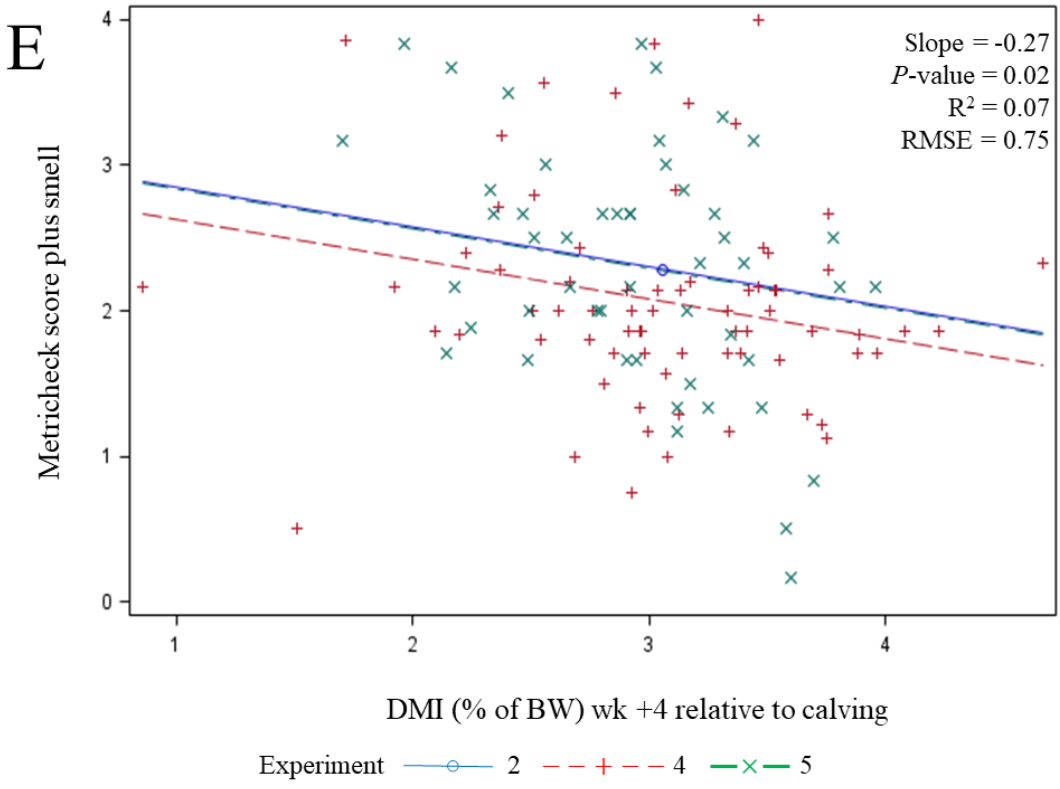


Figure 5.9. Associations of postpartum vaginal discharge or endometrial cytology with milk yield (kg/d) in the first four weeks postpartum of Holstein cows. Evaluation of vaginal discharge was performed using Metricheck device at 4, 7, 10, 13, 15, and 17 days in milk (DIM) following guidelines reported by LeBlanc and Bicalho (2017). Endometrial cytology samples were collected at 30 DIM and evaluated for the percentage of polymorphonuclear cells (PMN). Scatter diagrams display the specific association of: postpartum Metricheck score and smell and milk yield on the first week postpartum (A), milk yield on the second week postpartum (B), milk yield on the third week postpartum (C), and milk yield on the fourth week postpartum (D); and uterine PMN percentage at 30 DIM and milk yield on the first week postpartum (E), milk yield on the second week postpartum (F), milk yield on the third week postpartum (G), and milk yield on the fourth week postpartum (H). Data from 5 different studies were combined. Model accounted for the random effects of cow nested within each experiment.

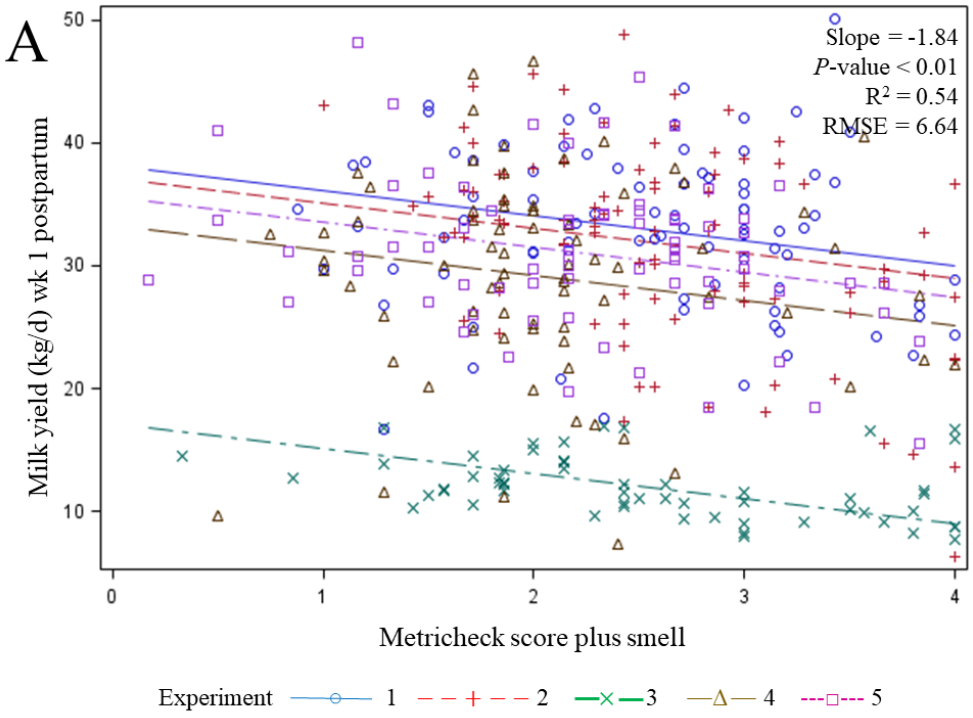
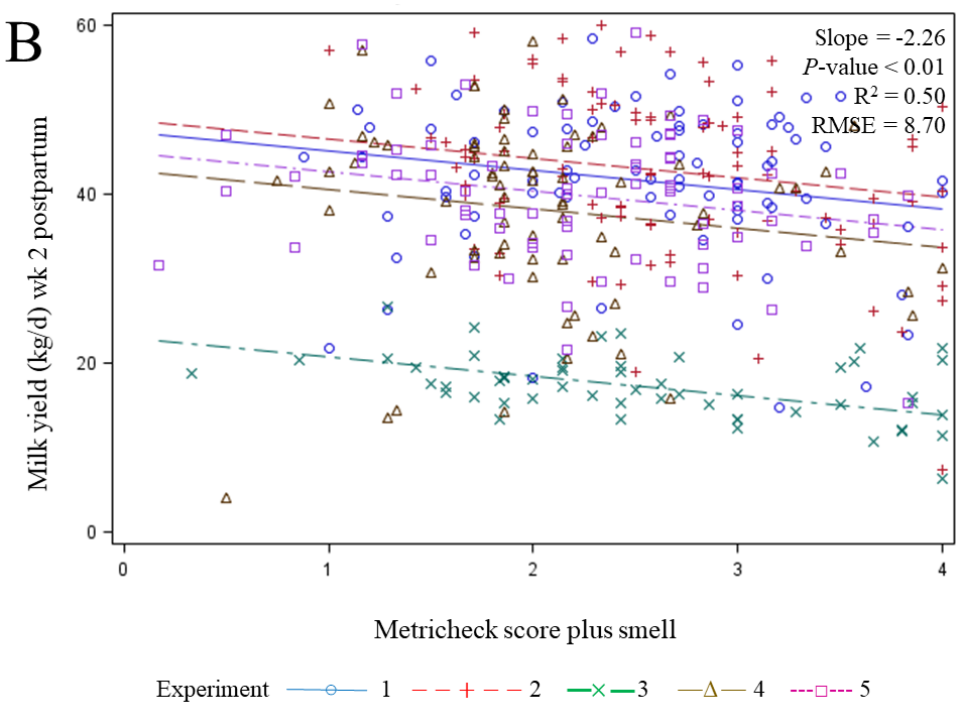
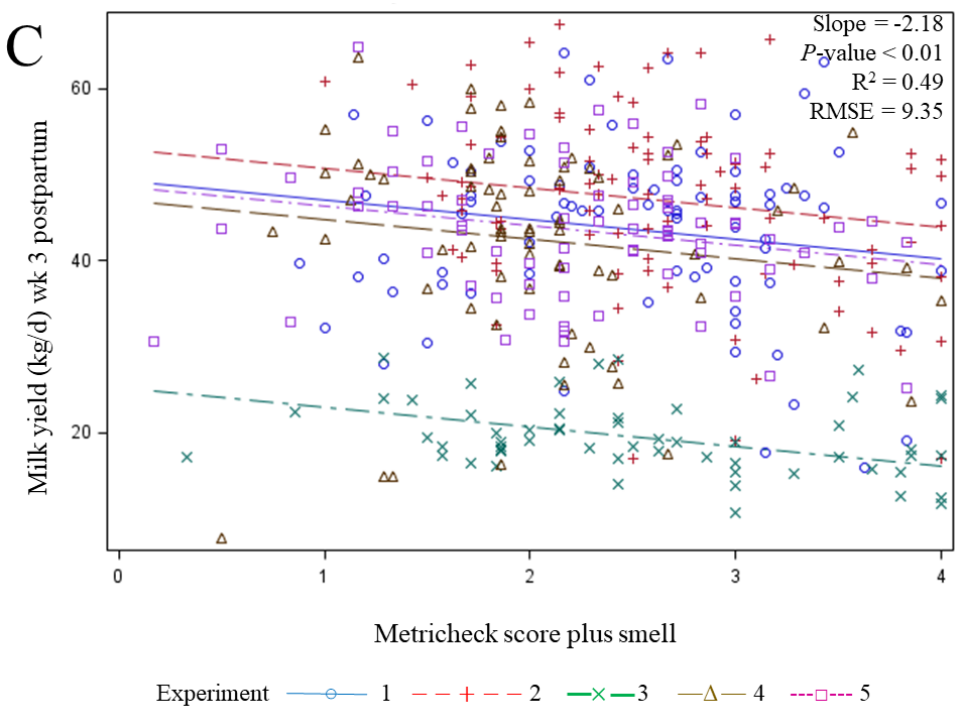


Figure 5.9 (cont.).





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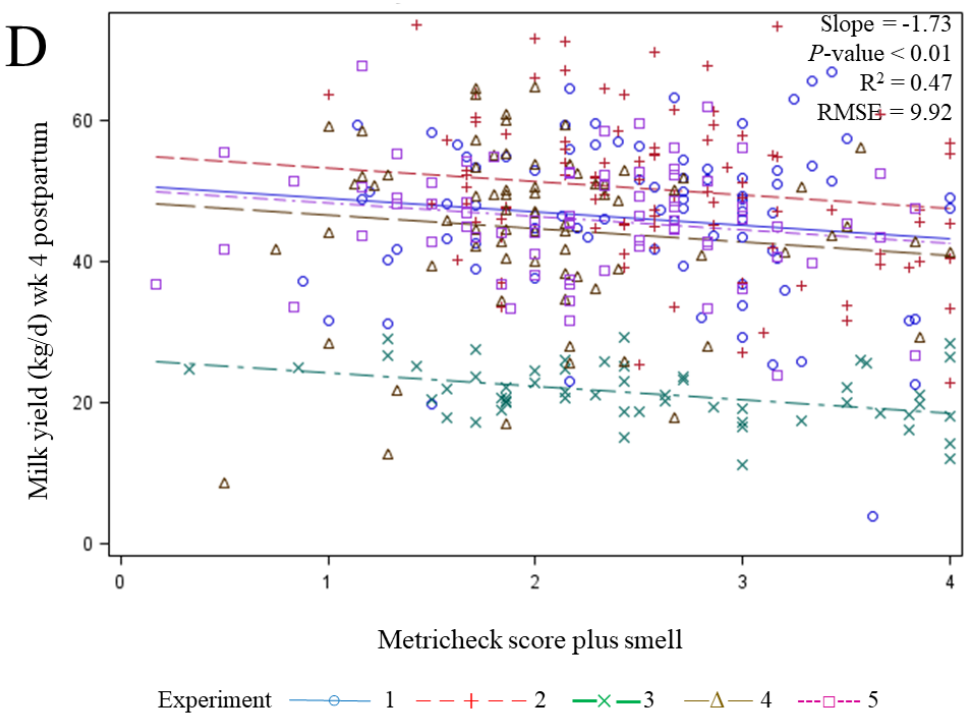


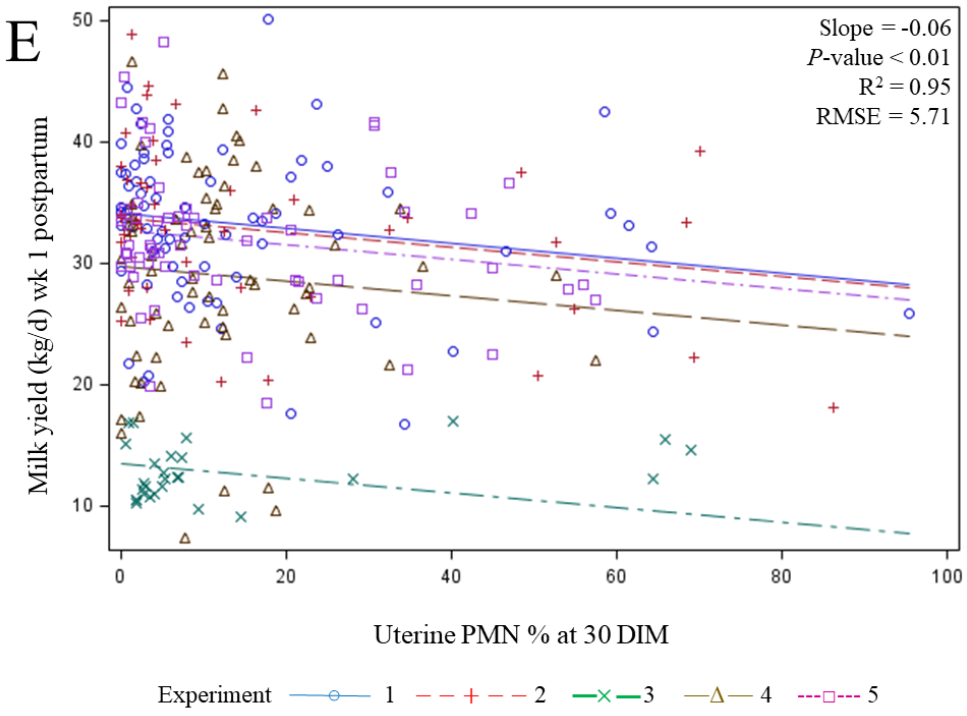
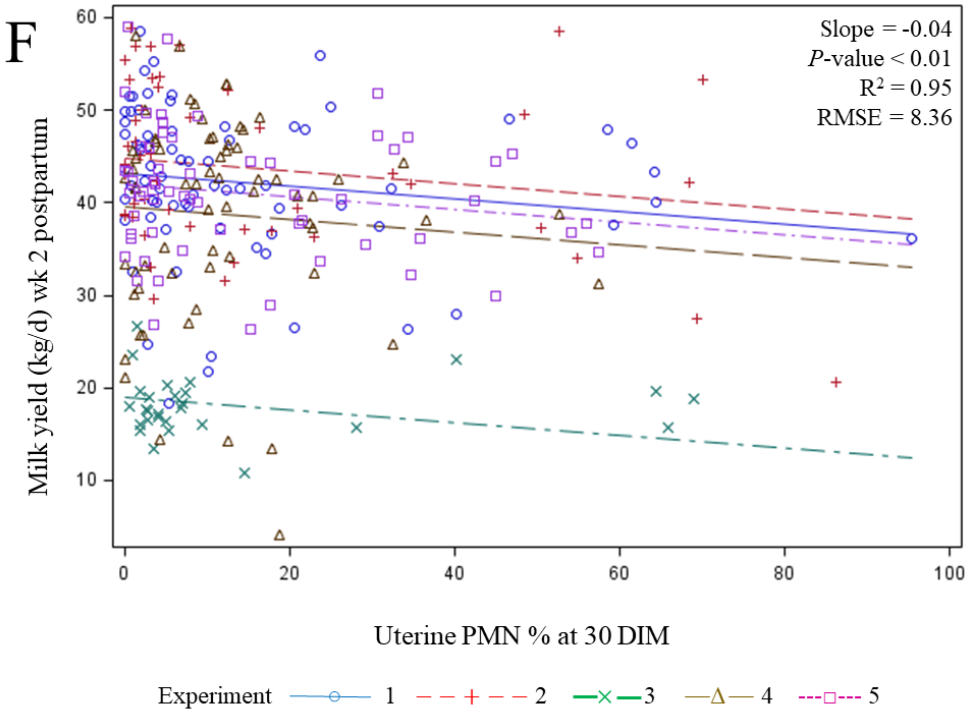
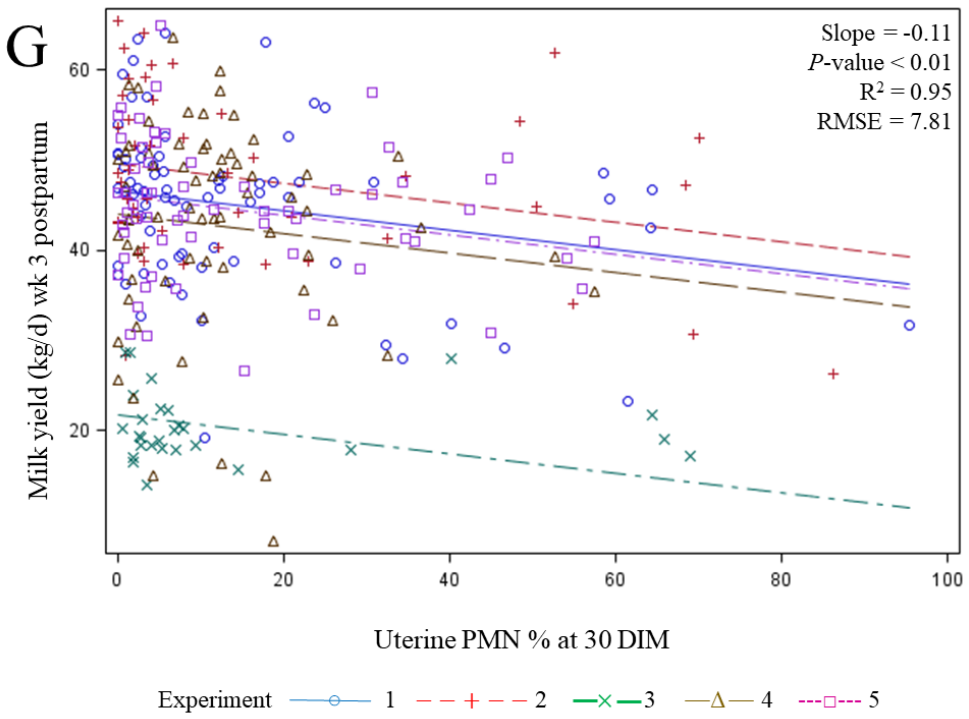


Figure 5.9 (cont.).





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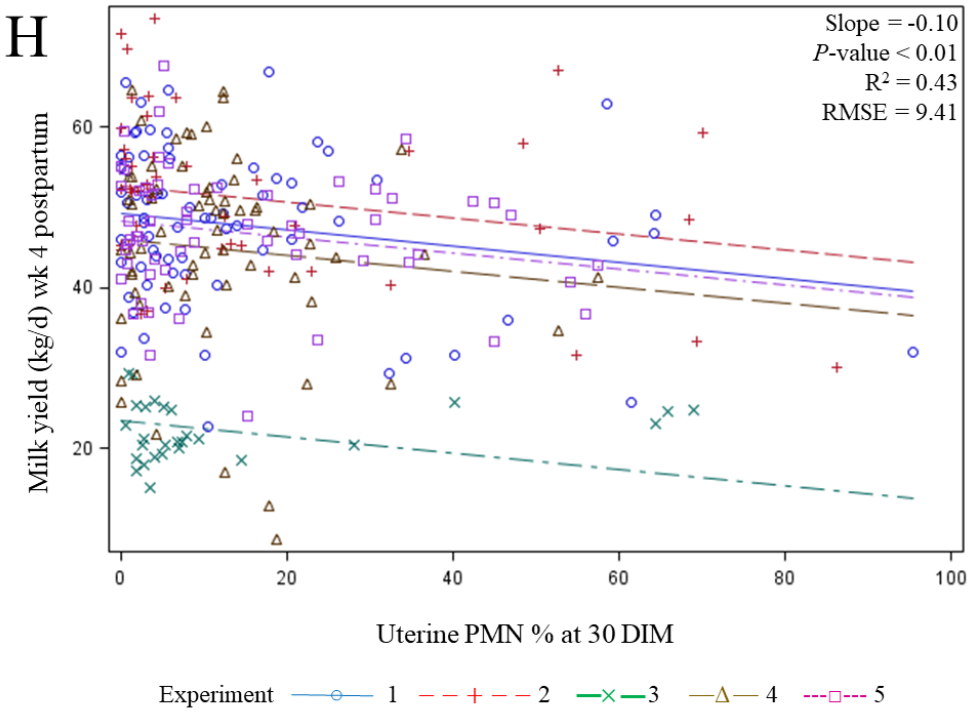

Table 5.1. Description of treatments, diets, and cows utilized in experiments utilized to evaluate the association of uterine health and follicular dynamics with milk composition, dietary energy intake, dietary metabolizable protein intake, and supplementation of rumen-protected amino acids during pre- and postpartum period.

Experiment	n[1]	Treatment characteristics	Diet and cow characteristics[2]	Reference
1	89	Top-dress of rumen-protected methionine (**RPM**) at 0.08% dry matter (**DM**), 60 g/d of choline, both RPM and choline, or not receiving neither RPM nor choline from -28 to 60 days relative to calving (**DRC**).	Multiparous Holstein cows during transition period and early lactation consuming 16% wheat straw and 36% corn silage in the prepartum and 35% corn silage and 25% ground corn during postpartum	Acosta et al. (2017); Stella et al. (2018)
2	99	Top-dress of *Saccharomyces cerevisiae* fermentation products (**SCFP**) at 14 g/d, 19 g/d, 50 g/d or not receiving any SCFP from -26 until 73 DRC.	Primiparous and multiparous Holstein cows during transition period consuming 21% wheat straw and 35% corn silage during the dry period and 40% corn silage and 12% ground corn during the fresh period	Glosson (2018)
3	60	Top-dress of ethyl-cellulose RPM from -28 to 60 DRC at a rate of 0.09% and 0.10% DM.	Multiparous Holstein cows during transition period and early lactation consuming 27% wheat straw and 27% corn silage in the prepartum period and 31% corn silage and 22% corn grain in the postpartum	Batistel et al. (2017)
4	81	Positive DCAD diet [+6 mEq/100 g of DM, target urine pH > 7.5, low dietary Ca (0.40% DM], negative DCAD diet [−24 mEq/100 g of DM, target urine pH 5.5 to 6.0, low dietary Ca (0.40% DM)]; or negative DCAD diet [−24 mEq/100 g of DM, target urine pH 5.5 to 6.0, high dietary Ca (2.0% DM)] from -28 DRC until calving.	Multiparous Holstein cows during the transition period consuming 36% wheat straw and 32% corn silage during the dry period and 28% corn silage and 25% ground corn during the fresh period	Glosson et al. (2020) Ryan et al. (2020)

Table 5.1 (cont.).

5	75	Top-dress of rumen-protected lysine [**RPL**; 0.54% of dietary dry matter intake (**DMI**)] or without RPL prepartum (-28 DRC to calving); after calving, half of the cows from each prepartum treatment group were assigned to a diet with RPL (0.40% RPL of dietary DMI) or without RPL until d 28 postpartum.	Multiparous Holstein cows during the transition period consuming 40% wheat straw and 31% corn silage during the dry period and 40% corn silage, 21% alfalfa hay, and 15% ground corn during the fresh period	Fehlberg et al. (2020)

[1]Number of cows.
[2]Diet characteristics are main ingredients to provide a generalized view of each experimental diet. Full details on all diets can be found in published papers, thesis, or dissertation of each experiment.

Table 5.2. Descriptive statistics of 395 Holstein cows in 5 experiments from -30 days relative to calving until 30 days in milk.

Trait	n[1]	Mean	SD	Min	Max
Parity	336	2.00	1.00	1.00	7.00
Prepartum					
Body weight (kg)	336	790	87.3	535	1022
Body condition score	336	3.80	0.26	2.75	4.50
Dry matter intake (kg/d)	336	11.4	4.08	0.46	41.9
Diet					
Dry matter (%)	339	45.5	2.21	15.2	48.8
Crude protein (%)	394	15.1	1.16	13.8	31.0
Starch (%)	339	16.5	3.54	12.7	22.4
Neutral detergent fiber (%)	394	43.4	3.10	16.1	49.3
NE_L (Mcal/d)	394	1.45	0.05	1.14	1.72
Metabolizable protein (kg/d)	394	1.22	0.16	0.96	2.37
Lys (% of metabolizable protein)	394	7.08	0.55	2.20	8.24
Met (% of metabolizable protein)	394	2.29	0.38	1.73	2.98
Lys (g/d)	394	84.8	8.36	31.0	98.1
Met (g/d)	394	28.1	9.46	20.6	148
Postpartum					
Body weight (kg)	305	693	75.9	422	929
Body condition score	306	3.50	0.31	2.25	4.75
Dry matter intake (kg/d)	394	13.8	6.39	0.00	39.5
Diet					
Dry matter (%)	335	45.0	2.45	38.9	48.3
Crude protein (%)	394	17.4	0.54	14.2	17.9
Starch (%)	335	27.2	3.37	14.0	30.8
Neutral detergent fiber (%)	394	31.7	2.06	29.2	44.8
NE_L[2] (Mcal/d)	394	1.68	0.06	1.44	1.76
Metabolizable protein (kg/d)	394	2.99	1.02	1.17	4.96
Lys (% of MP)	394	6.74	0.47	6.24	8.24
Met (% of MP)	394	2.27	0.39	1.70	2.98
Lys (g/d)	394	164	116	73.0	366
Met (g/d)	394	99.2	41.0	19.8	156
Milk production (kg/d)	382	37.6	14.5	1.30	100
Milk composition					
Milk fat (%)	272	4.13	1.04	1.34	9.55
Milk protein (%)	272	3.20	0.55	2.03	7.41
SCC[3] (x 1000)	216	271	945	4.00	21462
Metricheck score[4]	333	2.20	1.07	0	3.00
Metricheck smell[5]	333	0.59	1.19	0	3.00
PMN at 15 DIM (%)[6]	250	32.1	27.1	0	155
PMN at 30 DIM (%)[7]	280	14.0	17.8	0	95.4
Days at first ovulation[8]	244	18.0	8.00	7.00	44.0

[1]Number of cows with data for the respective trait.
[2]Net energy for lactation.
[3]Somatic cell count,

Table 5.2 (cont.).

[4]Vaginal discharge examined using a Metricheck device and scored on a scale of 0 to 3: score 0 = clear or translucent mucus; score 1 = mucus containing flecks of white or off-white pus; score 2 = discharge containing ≤ 50% white or off-white mucopurulent material; and score 3 = discharge containing ≥ 50% purulent material, which may be white, yellow or sanguineous (Sheldon et al., 2006).

[5]Vaginal discharge examined using a Metricheck device and smell scored (smell 0 = no odor or smell 3 = fetid odor).

[6]Endometrial cytology performed at 15 DIM for the evaluation of polymorphonuclear cells (PMN) percentage.

[7]Endometrial cytology performed at 30 DIM for the evaluation of polymorphonuclear cells (PMN) percentage.

[8]Ovulation of the first dominant follicle postpartum was monitored via transrectal ultrasonography and ovulation was classified as the disappearance of the previously identified dominant follicle, followed by the appearance of a *corpus luteum* in the subsequent examinations.

Table 5.3. Least squares means and associated SEM for dry matter intake (DMI), body weight (BW), body condition score, and production parameters of Holstein cows classified according to the endometrial polymorphonuclear cells (PMN) percentage at 15 and at 30 days in milk.

Variable	Classification[1]				SEM[2]	*P*-value[3]					
	LOW15	HIGH15	LOW30	HIGH30		CYT15	CYT15 × TIME	CYT30	CYT30 × TIME	CYT15 × CYT30	CYT15 × CYT30 × TIME
Prepartum											
DMI, kg/d	9.12	7.89	7.93	9.07	0.33	<0.01	<0.01	0.23	0.73	0.97	0.36
DMI, % BW	1.24	1.10	1.05	1.30	0.06	<0.01	0.96	<0.01	0.26	0.07	0.96
BW, kg	788	771	775	784	7.59	<0.01	<0.01	<0.01	<0.01	0.33	<0.01
BCS	3.75	3.83	3.83	3.74	0.02	<0.01	0.43	<0.01	<0.01	0.05	<0.01
Postpartum											
DMI, kg/d	15.0	13.6	14.0	14.8	1.29	<0.01	<0.01	0.23	0.67	0.97	0.36
DMI, % BW	2.34	2.14	2.13	2.35	0.07	<0.01	0.96	<0.01	0.26	0.07	0.96
BW, kg	684	670	672	681	5.41	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
BCS	3.43	3.36	3.38	3.41	0.00	<0.01	0.99	0.99	1.00	0.99	<0.01
Milk yield											
Milk, kg/d	43.9	39.1	42.2	40.8	0.76	<0.01	0.08	0.87	0.18	0.56	1.00
ECM, kg/d	41.7	39.5	40.4	40.8	0.00	<0.01	0.99	<0.01	1.00	0.99	0.99
Milk composition											
Fat, %	4.04	4.05	4.01	4.08	0.09	0.99	0.84	0.49	0.03	0.78	0.83
Fat, kg/d	1.72	1.69	1.69	1.71	0.06	0.66	0.06	0.70	0.47	0.52	0.90
Protein, %	3.20	3.12	3.15	3.17	0.05	0.09	0.36	0.56	0.38	0.70	0.93
Protein, kg/d	1.38	1.33	1.36	1.36	0.05	0.26	0.01	0.98	0.51	0.48	0.30
MUN, mg/dL	12.5	12.5	12.2	12.8	0.39	0.96	0.72	0.11	0.98	0.26	0.87
SCC, × 1,000/mL	286	330	386	230	96.5	0.72	0.21	0.19	0.03	0.60	0.34

Table 5.3 (cont.).

Metricheck[4]											
Averaged	2.48	2.56	2.48	2.57	0.05	0.08	-	0.02	-	< 0.01	-

[1]Cytological endometritis at 15 DIM (CYT15) and at 30 DIM (CYT30) were defined based on the percentage of polymorphonuclear cells (PMN) from endometrial cytology samples. Cut-off values for samples being classified as having cytological endometritis were obtained from the median values of the data set for each specific day and were: 15 DIM: > 24%; 30 DIM: > 7%.

[2] Greatest value of standard error of the mean within classification.

[3]Consists of the main effect of CYT15, the main effect of CYT30, the main effect of time (day or time), and their interaction. There was an effect of time for all variables and therefore was not included in the table.

[4]Metricheck score and smell evaluations were performed on 4 ± 1, 7 ± 1, 10 ± 1, 13 ± 1, 15 ± 1, and 17 ± 1 DIM and followed guidelines reported in Sheldon et al. (2006) for interpretation.

Table 5.4. Least squares means and associated SEM for dry matter intake (DMI), body weight (BW), body condition score (BCS), milk yield, and polymorphonuclear cells (PMN) percentage of Holstein cows classified according to their vaginal discharge (MS) at different days in milk (DIM).

	MS[1] at 7 DIM[2]				MS at 10 DIM[3]				MS at 13 DIM[4]				MS at 15 DIM[5]				MS at 17 DIM[6]			
Variable	≤ 3	> 3	SEM	*P*-value	≤ 3	> 3	SEM	*P*-value	≤ 3	> 3	SEM	*P*-value	≤ 3	> 3	SEM	*P*-value	≤ 3	> 3	SEM	*P*-value
Prepartum																				
DMI, kg/d	12.4	12.5	1.77	0.93	12.4	12.8	1.67	0.77	12.4	12.1	1.50	0.80	12.3	9.91	1.53	0.03	12.4	12.2	1.13	0.66
DMI, % BW	1.50	1.49	0.42	0.93	1.53	1.52	0.24	0.95	1.50	0.94	0.21	0.78	1.53	1.19	0.24	0.05	1.51	1.47	0.16	0.71
BW, kg[3]	799	796	49.3	0.96	829	857	42.8	0.52	827	829	49.3	0.96	790	812	11.0	< 0.01	802	792	18.6	0.60
BCS[4]	3.76	3.74	0.19	0.91	3.77	4.06	0.18	0.02	3.76	4.01	0.16	0.02	3.73	3.84	0.21	0.41	3.83	3.84	0.15	0.89
Postpartum																				
DMI, kg/d[5]	13.7	13.5	0.51	0.37	13.8	14.1	1.03	0.16	14.1	14.2	0.14	0.69	14.3	14.2	0.06	1.00	14.2	14.1	0.28	0.26
DMI, % BW	2.17	2.19	0.15	0.68	2.16	2.24	0.16	0.04	2.16	2.17	0.16	0.73	2.21	2.16	0.14	0.10	2.18	2.17	0.15	0.71
BW, kg[6]	677	675	6.68	0.32	680	671	1.88	< 0.01	678	676	76.6	0.27	683	672	1.68	< 0.01	678	676	1.15	0.24
BCS [7]	3.35	3.35	0.03	0.97	3.34	3.38	0.03	< 0.01	3.36	3.34	0.03	0.29	3.3	3.40	0.11	< 0.01	3.36	3.35	0.00	0.35
Milk, kg/d[8]	38.5	36.6	0.42	< 0.01	38.0	37.9	0.34	0.61	38.3	37.1	0.85	< 0.01	38.2	37.5	1.59	< 0.01	38.5	37.5	0.83	< 0.01
PMN, %																				
15 DIM[9]	29.5	31.4	10.2	0.75	37.1	32.3	0.93	< 0.01	31.2	32.9	8.54	0.75	37.7	31.7	12.5	0.29	38.6	35.5	15.2	0.66
30 DIM[10]	22.6	29.5	9.21	0.12	27.5	30.9	11.3	0.99	26.7	30.3	8.61	0.44	31.2	26.8	7.65	0.46	21.9	30.1	3.75	0.05

[1]Vaginal discharge was evaluated using the Metricheck (MC) device and following guidelines reported by LeBlanc and Bicalho (2017). Discharge appearance was scored based on a scale of 0 to 3: score 0 = clear or translucent mucus; score 1 = mucus containing flecks of white or off-white pus; score 2 = discharge containing ≤ 50% white or off-white mucopurulent material; and score 3 = discharge containing ≥ 50% purulent material, which may be white, yellow or sanguineous (Sheldon et al., 2006). Discharge smell was scored based on the absence (smell = 0) or presence (smell = 3) of a fetid odor. Sums of MC score plus smell were used.
[2]MS ≤ 3 n = 150; MS > 3 n = 22;
[3]MS ≤ 3 n = 152; MS > 3 n = 28;
[4]MS ≤ 3 n = 152; MS > 3 n = 28;
[5]MS ≤ 3 n = 146; MS > 3 n = 27;
[6]MS ≤ 3 n = 165; MS > 3 n = 20;
MS at 4 DIM differed only for DMI (% BW), where cows with MS ≤ 3 (2.22 ± 0.03 %) had greater DMI as a percentage of BW than cows with MS > 3 (2.16 ± 0.03 %; $P = 0.03$).

Table 5.5. Simple regression of dry matter intake (DMI) averaged by weeks relative to calving to vaginal discharge (Metricheck), endometrial polymorphonuclear cells (PMN) percentage at 15 days in milk (DIM), PMN percentage at 30 DIM, and days to first ovulation of multiparous Holstein cows.

	Metricheck[1]			PMN at 15 DIM			PMN at 30 DIM			Days to first ovulation		
	Slope	RMSE	*P*-value	Slope	RMSE[4]	*P*-value	Slope	RMSE	*P*-value	Slope	RMSE	*P*-value
DMI wk -4	-0.04	0.77	0.13	3.83	21.5	0.17	-0.17	17.0	0.71	0.13	4.69	0.39
DMI wk -3	-0.02	0.78	0.47	-13.0	21.6	0.01	-0.57	17.5	0.28	0.24	5.49	0.21
DMI wk -2	-0.02	0.78	0.35	0.18	21.6	0.74	0.01	17.5	0.98	0.10	5.51	0.58
DMI wk -1	-0.03	0.78	0.24	-0.20	21.6	0.71	0.67	17.4	0.13	0.09	5.51	0.59
DMI wk 1	-0.06	0.75	< 0.01	-0.73	24.9	0.08	-0.72	17.6	0.05	-0.10	5.25	0.45
DMI wk 2	-0.03	0.76	0.05	-0.73	25.4	0.08	0.05	18.0	0.89	-0.07	5.21	0.50
DMI wk 3	-0.03	0.77	0.06	-0.90	25.0	0.04	-0.42	14.3	0.21	-0.10	5.20	0.35
DMI wk 4	-0.04	0.76	0.02	-0.46	25.4	0.35	-0.49	10.2	0.17	-0.04	5.22	0.76

[1]Evaluation of vaginal discharge was performed using Metricheck device at 4, 7, 10, 13, 15, and 17 days in milk (DIM) following guidelines reported by LeBlanc and Bicalho (2017).
[2]Endometrial samples collected at 15 DIM for the evaluation of polymorphonuclear percentage through endometrial cytology analysis.
[3]Endometrial samples collected at 30 DIM for the evaluation of polymorphonuclear percentage through endometrial cytology analysis.
[4]Ovulation of the first dominant follicle (DF) postpartum was monitored via transrectal ultrasonography and ovulation was classified as the disappearance of the previously identified DF and the appearance of a *corpus luteum* in the subsequent examinations.
[5]Root mean square error.
Models accounted for the random effects of experiment and cow.

Table 5.6. Simple regression of dry matter intake as a percentage of body weight (DMI, % BW) averaged by weeks relative to calving to vaginal discharge (Metricheck), endometrial polymorphonuclear cells (PMN) percentage at 15 days in milk (DIM), PMN percentage at 30 DIM, and days to first ovulation of multiparous Holstein cows.

	Metricheck[1]			PMN at 15 DIM[2]			PMN at 30 DIM[3]			Days to first ovulation[4]		
	Slope	RMSE[5]	*P*-value	Slope	RMSE[5]	*P*-value	Slope	RMSE[5]	*P*-value	Slope	RMSE[5]	*P*-value
DMI (% BW) wk -4	-0.45	0.76	0.01	-0.04	19.7	0.99	-2.00	15.2	0.50	0.50	5.18	0.62
DMI (% BW) wk -3	-0.24	0.76	0.21	-6.17	21.5	0.13	0.36	16.7	0.91	1.05	5.46	0.35
DMI (% BW) wk -2	-0.31	0.78	0.11	5.51	21.3	0.14	-2.49	17.5	0.47	0.65	5.50	0.57
DMI (% BW) wk -1	-0.31	0.79	0.10	-1.53	21.7	0.69	6.49	17.0	0.04	0.38	5.54	0.74
DMI (% BW) wk 1	-0.43	0.77	< 0.01	-3.73	20.9	0.19	-3.34	17.6	0.16	-0.36	5.53	0.67
DMI (% BW) wk 2	-0.19	0.75	0.09	-4.07	21.1	0.08	0.31	17.3	0.88	0.20	4.34	0.71
DMI (% BW) wk 3	-0.16	0.76	0.17	-4.45	21.1	0.03	0.01	17.2	0.99	-0.20	4.44	0.70
DMI (% BW) wk 4	-0.27	0.75	0.02	-1.10	21.4	0.62	-0.45	17.2	0.81	0.39	4.33	0.52

[1]Evaluation of vaginal discharge was performed using Metricheck device at 4, 7, 10, 13, 15, and 17 days in milk (DIM) following guidelines reported by LeBlanc and Bicalho (2017).
[2]Endometrial samples collected at 15 DIM for the evaluation of polymorphonuclear percentage through endometrial cytology analysis.
[3]Endometrial samples collected at 30 DIM for the evaluation of polymorphonuclear percentage through endometrial cytology analysis.
[4]Ovulation of the first dominant follicle (DF) postpartum was monitored via transrectal ultrasonography and ovulation was classified as the disappearance of the previously identified DF and the appearance of a *corpus luteum* in the subsequent examinations.
[5]Root mean square error.
Models accounted for the random effects of experiment and cow.

Table 5.7. Simple regression of milk yield (kg/d) averaged by weeks relative to calving to vaginal discharge (Metricheck), endometrial polymorphonuclear cells (PMN) percentage at 15 days in milk (DIM), PMN percentage at 30 DIM, and days to first ovulation of multiparous Holstein cows.

	Milk yield (kg/d) wk 1			Milk yield (kg/d) wk 2			Milk yield (kg/d) wk 3			Milk yield (kg/d) wk 4		
	Slope	RMSE[1]	*P*-value	Slope	RMSE[1]	*P*-value	Slope	RMSE[1]	*P*-value	Slope	RMSE[1]	*P*-value
Purulent and fetid vaginal discharge[2]	-1.77	7.96	< 0.01	0.90	9.63	0.09	-1.19	9.78	0.04	1.47	13.5	< 0.01
Averaged vaginal score	-1.84	6.64	< 0.01	-2.26	8.70	< 0.01	-2.18	9.35	< 0.01	-1.73	9.92	< 0.01
PMN % at 15 DIM[3]	-0.01	4.99	0.65	0.01	0.30	0.93	-0.04	8.50	0.17	-0.02	7.84	0.54
PMN % at 30 DIM[4]	-0.06	5.71	< 0.01	-0.04	8.36	0.01	-0.11	7.81	< 0.01	-0.10	9.41	< 0.01

[1]Root mean square error.
[2]Evaluation of vaginal discharge was performed using Metricheck device at 4, 7, 10, 13, 15, and 17 days in milk (DIM) following guidelines reported by LeBlanc and Bicalho (2017). Vaginal discharge score plus smell was dichotomized based on the threshold of ≥ 3 to classify cows as having purulent and fetid vaginal discharge.
[3]Endometrial samples collected at 15 DIM for the evaluation of polymorphonuclear percentage through endometrial cytology analysis.
[4]Endometrial samples collected at 30 DIM for the evaluation of polymorphonuclear percentage through endometrial cytology analysis.
[5]Ovulation of the first dominant follicle (DF) postpartum was monitored via transrectal ultrasonography and ovulation was classified as the disappearance of the previously identified DF and the appearance of a *corpus luteum* in the subsequent examinations.
Models accounted for the random effects of experiment and cow.

Table 5.8. Logistic regression model, accounting for the random effect of experiment, of variables (by class) associated with subsequent risk of cytological endometritis at 15 days in milk (DIM) in Holstein cows.

Variable	Class	n	OR[1]	95% CI	*P*-value
DMI prepartum	≥ 11 kg/d	99	Referent		
	< 11 kg/d	107	1.06	0.70 – 1.61	0.78
DMI postpartum	≥ 13 kg/d	127	Referent		
	< 13 kg/d	79	1.02	0.67-1.55	0.93
Retained placenta	Yes	17	Referent		
	No	153	0.45	0.15 – 1.33	0.15
Vaginal discharge (MS > 3)					
at 4 DIM	Yes	13	Referent		
	No	143	0.35	0.10-1.20	0.09
at 7 DIM	Yes	22	Referent		
	No	150	0.43	0.17-1.12	0.09
at 10 DIM	Yes	28	Referent		
	No	152	0.66	0.29-1.49	0.31
at 13 DIM	Yes	28	Referent		
	No	152	0.66	0.29-1.49	0.31
at 15 DIM	Yes	27	Referent		
	No	146	0.33	0.13-0.80	0.02
at 17 DIM	Yes	20	Referent		
	No	165	0.98	0.53-1.78	0.92

[1]Odds ratio.

[2]Vaginal discharge characterized by the sum of Metricheck score + Metricheck smell > 3 (LeBlanc and Bicalho, 2017).

Table 5.9. Logistic regression model, accounting for the random effect of experiment, of variables (by class) associated with subsequent risk of cytological endometritis at 30 DIM in Holstein cows.

Variable	Class	n	OR[1]	95% CI	*P*-value
DMI prepartum	≥ 11 kg/d	99	Referent		
	< 11 kg/d	107	0.77	0.44 – 1.00	0.05
DMI postpartum	≥ 13 kg/d	221	Referent		
	< 13 kg/d	201	0.65	0.43-0.98	0.04
Retained placenta	Yes	17	Referent		
	No	153	0.57	0.20 – 1.59	0.28
Vaginal discharge (MS > 4)					
at 4 DIM	Yes	35	Referent		
	No	276	0.54	0.17-1.74	0.30
at 7 DIM	Yes	22	Referent		
	No	150	0.82	0.33-2.03	0.67
at 10 DIM	Yes	28	Referent		
	No	152	0.62	0.27-1.43	0.26
at 13 DIM	Yes	28	Referent		
	No	152	0.62	0.27-1.43	0.26
at 15 DIM	Yes	27	Referent		
	No	146	0.87	0.38-2.00	0.73
at 17 DIM	Yes	20	Referent		
	No	165	0.67	0.26-1.74	0.41

[1]Odds ratio.
[2]Vaginal discharge characterized by the sum of Metricheck score + Metricheck smell > 3 (LeBlanc and Bicalho, 2017).

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CHAPTER 6: SUMMARY AND FINAL CONSIDERATIONS

One of the several challenges that the dairy industry faces is nutrition and all the aspects that it involves, from the need of a rational and effective use of feed ingredients on a dairy cow diet; to the metabolic responses of dietary strategies and their effects on the overall performance of the animal. Additionally, cows are faced with a dysfunctional immune system and an increased inflammatory state during the peripartal period. Furthermore, the increase in the energy demands that the dairy cows face due to the transition from gestation to lactation results in mobilization of body stores, which increase the risk for developing disorders, including reproductive tract inflammatory disorders. This could negatively impact cows' reproductive health and performance, further increasing the economic losses caused by a poor management of transition period. Therefore, the overall objective was to determine the effects of nutrition related strategies, such as feeding rumen-protected sources of Lys (RPL) and Met (RPM), on the uterine metabolism, immunity, and health, and on the ovarian resumption and cyclicity in the early postpartum of Holstein cows.

To address this challenge, we evaluated the effects of feeding limiting amino acids in a rumen-protected form to dairy cows in the transition period. Experiment 1 (Chapter 2) was designed to investigate whether effects of rumen-protected Met (RPM) in improving postpartum vaginal discharge and uterine health were due to modulation of genes involved in uterine metabolism and immunity. Cows fed RPM decreased the expression of transcripts involved in inflammatory processes in cytological samples and increased expression of genes involved in overall tissue metabolism in the endometrium. Thus, feeding RPM during the transition period and early lactation modulates the uterine metabolism and immune defense system, which could decrease the susceptibility to reproductive tract inflammatory diseases. Overall, the amino acids

metabolism can vary substantially depending on the tissue, which may also respond differently according to the AA provided and the diet formulated. Thus, a next step would be to investigate whether the uterine metabolism and immunity would respond differently according to increased supply of Lys plus Met, or Lys plus Met plus His, for example.

In Experiment 2 (Chapter 3), we fed rumen-protected Lys (RPL) through the transition period, so we could detect whether the most beneficial effects on reproductive health and ovarian resumption would come from feeding RPL in the prepartum, postpartum, both, or even neither. Postpartum uterine health was assessed through a range of methods that encompassed sophisticated techniques, such as evaluating the expression of transcripts involved with immunometabolism in the endometrial tissue and mucus samples; but also measurements that can be done at a farm level, such as the evaluation of the vaginal discharge using the Metricheck device. Results indicated that feeding cows with RPL improved uterine health postpartum, impacting different immune responses in the reproductive tract, but it did not change days to first ovulation.

Experiment 3 (Chapter 4) determined the immune and metabolic modulatory potential of maternal supplementation with Lys on uteroplacental tissue. Our preliminary data indicated that feeding prepartum cows with RPL improved intake of their calves during the first six wk of life and their average daily gain during the pre-weaning phase. We demonstrated that feeding rumen-protected Lys (RPL) during the last month of gestation resulted in increased uteroplacental expression of genes involved in placental glucose uptake and metabolism, and increased expression of transcripts involved in placental metabolic activity.

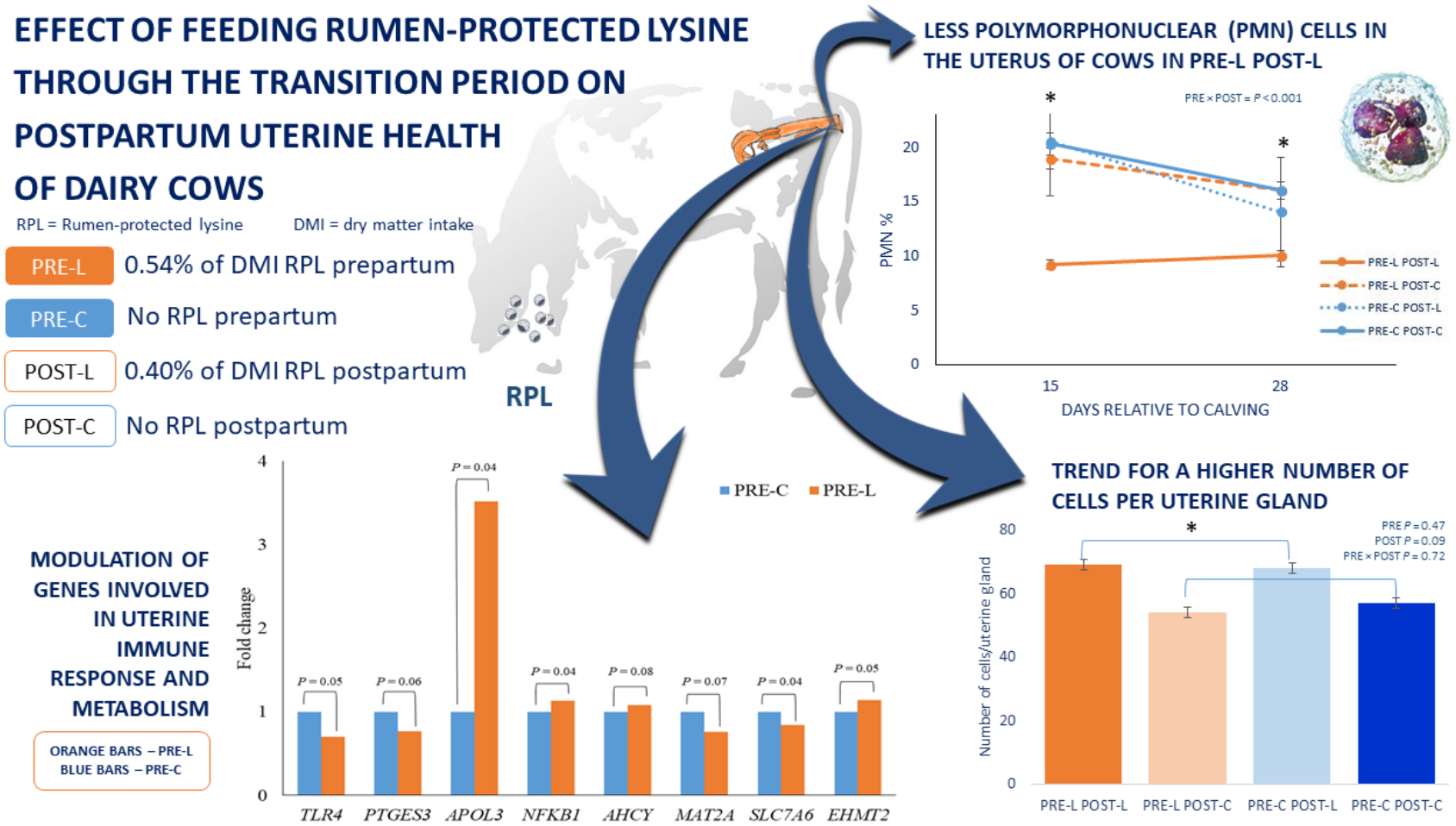
Finally, Experiment 4 (Chapter 5) aimed to determine whether there is an association among of prepartum and postpartum DMI, lactation performance, and days to first ovulation

with measurements of uterine health in the early postpartum. Uterine health, which is crucial for the reproductive success of the postpartum dairy cows, is affected by the impairment of PMN function faced by transitioning cows. The impairment in PMN function is attributed to increased exposure to metabolites of homeorhetic mechanisms to support lactation, such as NEFA, and to decreased availability of glucose. Thus, intake might be associated cytological endometritis because it is closely related to nutrient availability, and it is one of the main determinants of the NEB. In the present work, cytological endometritis at 15 days in milk was associated with decreased DMI from 4 wk prepartum until 4 wk postpartum, and a tendency for decreased milk yield from 3-5 wk postpartum. Cytological endometritis at 30 DIM was associated with DMI as a percentage of BW, but it is no associated with milk yield. Additionally, increase in the vaginal discharge score (evaluated using the Metricheck device) was associated to decrease in up to 2.26 kg/d in milk yield. Moreover, the association of uterine inflammation and early postpartum ovarian cyclic events requires further investigation, with the inclusion of other explanatory variables related to the cows' nutritional and metabolic status. Whether these events would positive or negatively impact subsequent fertility are yet to be responded. Thus, further studies should now investigate this association with subsequent reproductive outcomes, taking into consideration the cows' intake.

With this holistic view of the importance of dairy nutrition and knowing that a dairy cow goes through several challenges during her life, the interest of this research focused on nutrition as a strategy to improve reproductive health and alleviate the detrimental effects of stressors in dairy cows. This was approached by the link between metabolism and immunity as the major factor to be understood and explored, to optimize animal reproductive performance and disease response.

APPENDIX A. SUPPLEMENTAL MATERIAL FOR CHAPTER 3

Graphical Abstract



Supplemental Table A.1. Gene symbol and primer sequences of primers analyzed in uterine tissue of Holstein cows.

Gene[1]	Forward Sequence	Reverse Sequence
GAPDH	TTGTCTCCTGCGACTTCAACA	TCGTACCAGGAAATGAGCTTGAC
ACTB	ACCGCAACCAGTTCGCCAT	GATGGACGGGAAGACGGC
H2AFZ	CGGAATTCGAAATGGCTGGC	GAACTGCAAACCGGCTCTCT
CPT1A	CCTATTTTGGACACGGGAAA	TCAAACCACCTGTCGAAACA
GLUT4	ATGTACGTGGGGGAGATTGC	CTAGCACCTGGGCGATTAGG
FGF10	TGGCTACAATGTCAGGGGC	CAGCCTTTTGGGAGGTAGGA
HGF	AGCTACAAGGGGACGGTATC	GCTCGAAGGCAAAAAGCTGTG
AHCY	TGGCATTCCAGTGTACGCC	CCACCGTCGTCCAGAATCAT
EHMT2	CTCAGGAGGTGGCTTGTCAG	CTACCACCGTTTCCCACTCC
S100A4	CCCTCGATGTGATGGTGTCC	CATCCGTCCTTTTCCCCAAGA
IL1β	ATTCTCTCCAGCCAACCTTCATT	TTCTCGTCACTGTAGTAAGCCATCA
IL-10	ACTTTAAGGGTTACCTGGGTTG	GAAAGCGATGACAGCGCCGC
TLR4	TGCGTACAGGTTGTTCCTAACAT	TAGTTAAAGCTCAGGTCCAGCATC
IL-6	CCAGAGAAAACCGAAGCTCTCAT	CCTTGCTGCTTTCACACTCATC
IL-8	GTGAAGAGAGCTGAGAAGCAAG	CACCAGACCCACACAGAACAT
OCLN	ATCAACCCCGGTGCCGGAAG	GTGGTCTTGCTCTGCCCGCC
EDN2	GAGCGTGCCTCACCACTG	CTCTTGCCTTCGTGCAGGG
SOD1	TCCACGTCCATCAGTTTGGA	GGTCTCCAACATGCCTCTCTT
GPX1	AACGCCAAGAACGAGGAGATC	CATTCACCTCGCACTTTTCGA
NFKB1	AGGACCAACCAGACCG	TGTCACCAGGCGAGTTAT
MUC1	CAGGAGCTGCAGAGAAGCAT	CCACAGATCCTGGCCTGAAC
MUC4	AGATGTCAATGCCTCGGTGG	TGGTGCCGTTGAGGGTTTAG
PTGES3	AAGGGCAAAGCTTAATTGGC	CCACCCATGTTGTTCATCATCT
IGF-I	TGCACTTCAGAAGCAATGGG	TGATGGGCATCTTCACCTTC
NOS2	TGTCACTGGTCTGAAACCTCTC	TTCTTCTCGGCAGGCTTGG
DUOX2	ATCCAGAGAGGAGGGGTCAA	TCCCAGGTCAGTGAGAGTGA
TSPO	GACAGCAGAGCTCACAAGCATCT	GAACCATACCCCATGGCCG
MAT2A	ACTACCACCACCATGAACGG	CAGATCTTATCTGGGTGGCCT

Supplemental Table A.1 (cont.).		
SLC7A1	GATGGCCTTCCTCTTCGACC	CGGGCTGGTATCGTAAGACC
SLC3A2	GGCGTCATCTCGGTTCCAA	TCATGGTGCCTGAGTCGC
SLC7A6	GTGTGGGAAGTAAGGCTGTAG	GGCCTCAGGAATGAGATGGT
SLC7A7	TCAGGGCTGGGGAAGGG	TGCTGGGAGGCCACTTCATA
AASS	ACTTGGGCTGAGGAGAGAGA	AACCTGGTGACATGGGTGAC

[1]*GAPDH* = Glyceraldehyde-3-phosphate dehydrogenase; *ACTB* = Actin beta; *H2AFZ* = H2A histone family, member z; *CPT1A* = Carnitine palmitohyltransferase 1 A; *GLUT4* = Glucose transporter type 4; *FGF10* = fibroblast growth factor 10; *HGF* = Hormone growth factor; *SAHH* = S-Adenosyl-L-Homocysteine hydrolase; *AHCY* = adenosylhomocisteinase; *EHMT2* = Histone-lysine 9 N-trimethyltransferase; *S100A4* = S100 calcium binding protein A4; *IL1β* = Interleukin 1 Beta; *IL-10* = Interleukin 10; *TLR4* = Toll-like receptor 4; *Tκα* = Tumor necrosis factor alpha; *IL-6* = Interleukin 6; IL-8 = Interleukin 8; *OCLN* = Occludin; *EDN2* = Endothelin 2; *SOD1* – Superoxide dismutase 1; *GPX* = Glutatione peroxidase; *NFKB1* = Nuclear factor kappa B 1; *MUC1* = Mucin 1, cell surface associated; *MUC4* = Mucin 4, cell surface associated; *PGES3* = Prostaglandin E synthase 2; *IGF-1* = Insulin-like growth factor I; *NOS2* = Nitric oxide synthase 2; *DUOX2* = Dual oxidase 2; *TSPO* = Translocator protein; *MAT2A* = Methionine adenosyltransferase 2 alpha; *SLC7A1* = Arginine, lysine, and ornithine transporter; *SLC3A2* = Heavy chain of the second Na^+ dependent lysine transporter; *SLC7A6* = Light chain of the second Na^+ dependent lysine transporter (1); *SLC7A7* = Light chain of the second Na^+ dependent lysine transporter (2); *AAS*S = Alpha-aminoadipic semialdehyde synthase.

Supplemental Table A.2 Uterine gene expression (least squares means of $2^{-delta\text{-}CT}$, normalized to 3 reference genes) at 28 days after calving of cows fed or not with rumen-protected lysine during peripartal period.

		Treatments[1]					*P*-value[3]		
	n	PRE-L POST-L	PRE-L POST-C	PRE-C POST-L	PRE-C POST-C	SEM[2]	PRE	POST	PRE × POST
Immune Response[4]	64								
SOD1		0.43	1.53	0.70	0.82	0.74	0.74	0.07	0.46
NOS2		13.9	14.0	13.5	13.2	0.77	0.36	0.89	0.74
IL1β		10.5	10.2	10.9	9.10	1.10	0.72	0.27	0.39
IL6		10.8	10.2	10.4	9.76	0.75	0.53	0.34	0.97
IL8		2.93	3.16	3.11	3.06	0.19	0.81	0.58	0.36
IL10		3.54	3.59	3.64	3.58	0.08	0.58	0.94	0.54
TNFα		9.18	8.72	8.65	8.55	0.34	0.43	0.52	0.69
TLR4		6.85	6.59	6.04	5.73	0.45	0.04	0.46	0.96
GPX		2.20	2.13	1.65	1.27	0.70	0.26	0.71	0.80
DUOX2		3.50	3.74	3.64	3.60	0.15	0.99	0.45	0.27
NFKB1		2.13	1.94	1.88	1.79	0.11	0.04	0.15	0.61
OCLN		6.03	6.21	5.31	6.21	0.58	0.49	0.29	0.49
END2		3.69	3.65	3.65	3.54	0.08	0.26	0.25	0.62
PTGES3		0.96	0.97	0.26	0.48	0.35	0.06	0.72	0.73
APOL3		3.74	3.62	3.68	3.56	0.04	0.04	0.19	0.43
S100A2		1.76	1.49	1.37	1.60	0.21	0.47	0.92	0.20
MUC1		4.50	5.37	4.37	5.72	0.61	0.83	0.04	0.66
MUC4		6.57	8.08	6.70	7.87	0.79	0.95	0.06	0.81
Metabolism [5]	64								
GLUT4		2.96	2.89	3.01	2.93	0.14	0.72	0.56	0.97
IGF-I		27.6	26.6	27.7	27.8	0.91	0.45	0.55	0.51
SLC7A1		2.49	2.33	2.48	2.43	0.13	0.72	0.38	0.63
SLC7A6		3.12	2.94	2.92	2.84	0.08	0.04	0.07	0.48
SLC7A7		9.93	9.27	9.87	9.93	0.43	0.44	0.45	0.35
SLC3A2		7.09	6.82	7.01	7.12	0.46	0.79	0.84	0.64
HGF		2.89	2.70	2.75	2.87	0.09	0.92	0.66	0.07
AASS		3.57	3.52	3.47	3.43	0.07	0.15	0.59	0.94
SAHH		4.12	3.99	4.19	4.11	0.16	0.41	0.42	0.85
AHCY		1.81	1.78	1.51	1.43	0.20	0.08	0.73	0.89
MAT2A		2.81	2.34	1.88	1.96	0.41	0.07	0.57	0.46
EHMT2		2.41	2.41	2.19	2.15	0.13	0.05	0.84	0.90
TSPO		2.60	2.46	2.45	2.41	0.10	0.28	0.32	0.57
CPT1A		3.80	3.76	3.72	3.72	0.05	0.18	0.64	0.70
FGF10		2.70	2.48	2.46	2.75	0.14	0.88	0.79	0.05

[1]Dietary treatments included at top dress with RPL prepartum and postpartum (PRE-L POST-L), with RPL prepartum and without RPL postpartum (PRE-L POST-C), without RPL prepartum and with RPL postpartum (PRE-C POST-L), and without RPL prepartum and postpartum (PRE-C POST-C) in a carrier of 300 g of dried molasses.

[2]Greatest value of standard error of the mean within treatment.

[3]Consists of the main effect of RPL prepartum (PRE), the main effect of RPL postpartum (POS), and their interactions.

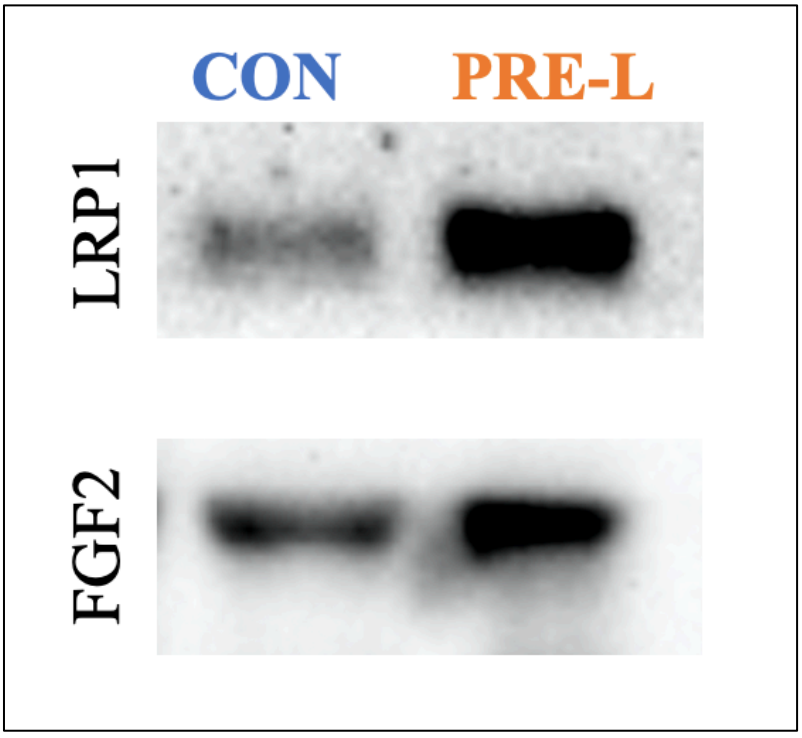
[4] SOD1 = Superoxide dismutase 1; NOS = Nitric oxide synthase; *IL1β* = Interleukin 1 Beta; IL6 = Interleukin-6; IL8 = Interleukin 8; IL10 = Interleukin 10; TNFα = Tumor necrosis factor alpha; GPX = Glutathione peroxidase; DUOX2 = ; *NFKB1* = Nuclear factor kappa B 1; OCLN = Occludin; END2 = Endothelin 2; PTGES3 = Prostaglandin E synthase 2; APOL3 = Apolipoprotein L 3; S100A2 = S100 calcium binding protein; MUC1 = Mucin 1; MUC4 = Mucin 4; TLR4 = Toll-like receptor 4.

[5]GLUT4 = Glucose transporter 4; IGF1 = Insulin-like growth factor I; SLC7A1 = Arginine, lysine, and ornithine transporter; SLC7A6 = Light chain of the second Na+ dependent lysine transporter (1); SLC7A7 = Light chain of the second Na^+ dependent lysine transporter (2); SLC3A2 = Heavy chain of the second Na+ dependent lysine transporter; HGF = Hormone growth factor; *AAS*S = Alpha-aminoadipic semialdehyde synthase; *SAHH* = S-Adenosyl-L-Homocysteine hydrolase;

Supplemental Table A.2 (cont.).
AHCY = adenosylhomocisteinase; MAT2A = methionine adenosyltrasnferase 2 A; *EHMT* = Histone-lysine 9 N-trimethyltransferase; TSPO = Translocator protein; CPT1A = Carnitine palmitohyltransferase 1; FGF10 = Fibroblast growth factor 10.

APPENDIX B. SUPPLEMENTAL MATERIAL FOR CHAPTER 4

Supplemental Figure B.1. Western blot images obtained from Image Lab Software by ChemiDoc after 3 min incubation in Western ECL substrate. Placenta samples derived from cows consuming dietary treatments included in top dress with RPL prepartum (PRE-L) or without RPL prepartum (CON) in a carrier of 300 g of dried molasses. Protein abundance of fibroblast growth factor 2 (FGF2) and LDL-related protein 1 (LRP1) was greater in placental samples from cows in PRE-L than from cows in CON ($P < 0.05$).



Supplemental Table B.1. Gene symbol and primer sequences of genes analyzed in placental tissue of Holstein cows.

Gene[1]	Forward Sequence	Reverse Sequence
GAPDH	TTGTCTCCTGCGACTTCAACA	TCGTACCAGGAAATGAGCTTGAC
ANGPT1	ATGCTAACAGGAGGCTGGTG	CATGGTTCTGTCCCGCTGTA
ANGPT2	TGACATGGAAACGGGTGGAG	GGGTTCCCGAATCCCACTTT
EHMT2	CTCAGGAGGTGGCTTGTCAG	CTACCACCGTTTCCCACTCC
FGF10	TGGCTACAATGTCAGGGGC	CAGCCTTTTGGGAGGTAGGA
FGF2	AGATGCCACAGAAGCTACCC	CACAAGTCAGCAGTTTCCCG
FGF2R	TGGTGGTGACGGTCATCTTG	GCTGGACTCAGCAGAAACCT
G6PC1	TTCGAGAAGCTGTGGGCATC	AAAACCCACCAGTATGGGCG
GLUT3	CTCGGCCGCGTTCTACTT	CACGGGTCTCAGGAACTTTGA
GLUT5	TGCTGGAGTGAATGAGGACG	CCACCACGAAAATAGCGCAG
HGF	AGCTACAAGGGGACGGTATC	GCTCGAAGGCAAAAAGCTGTG
IGF1	TGCACTTCAGAAGCAATGGG	TGATGGGCATCTTCACCTTC
IGF1R	ACGAGTGGAGAAATCTGCGG	AGTCCTCGGCCTTGGAAATG
IGF2	CGCAGGAGCTGAGACATCAAT	GTAAGCAGCATAGCAGCACG
IGF2R	ACAGCGGGTACGTGTTTGAT	CCGCACACGTTGAACAGAAA
IL1β	ATTCTCTCCAGCCAACCTTCATT	TTCTCGTCACTGTAGTAAGCCATCA
IL6	CCAGAGAAAACCGAAGCTCTCAT	CCTTGCTGCTTTCACACTCATC
IL8	GTGAAGAGAGCTGAGAAGCAAG	CACCAGACCCACACAGAACAT
IL10	ACTTTAAGGGTTACCTGGGTTG	GAAAGCGATGACAGCGCCGC

Supplemental Table B.1 (cont.).

IRS1	CCCTTCGAGAAAGTGGTGAACA	AGCCTGAAGCTCGATGCGATAG
KDM1A	CCGAGGAACCATCTGGTGTG	CTGCTGTGGTCCACTGATGAT
KHK	AGGAGGACACGGACAGCA	CGTCCAGGACAAAATCAGCA
MAT2A	ACTACCACCACCATGAACGG	CAGATCTTATCTGGGTGGCCT
NFKB1	AGGACCAACCAGACCG	TGTCACCAGGCGAGTTAT
NOS3	CAACAGCCCCACGCTGAC	CGCACAGAGTGTCGTAGGTG
P450 (*CYP19A1)*	AGCATGGTGTCCGAAGTTGT	CAGAAAGTAGCTGGGACCTGG
PCK1	CATTGGGAACGCACACTCAC	ATGCCGAGGTTCCCTTTCTC
PGF	ATGAAGTACCGCTCTCTGGAC	TCACCACACCTTGTTTGCTT
RPS6KB1	CCAACCAGGTCTTTCTGGGTT	TGTTCGTGGGCTGCCAATAA
SOD1	TCCACGTCCATCAGTTTGGA	GGTCTCCAACATGCCTCTCTT
S100A4	CCCTCGATGTGATGGTGTCC	CATCCGTCCTTTTCCCCAAGA
SLC7A7	TCAGGGCTGGGGAAGGG	TGCTGGGAGGCCACTTCATA
SLC38A6	CCGGCTGGTTTGTCTCTACT	ATCAGCAGCAAGAAAGTTGC
STAR	TGGCATGGCCACACTCTATG	TGAGAAGTGCTGATGTACCA
STAT5	ACGGTACCTTCTTGTTGCGC	CAGGTTACGGTCAGAGAGTCAAAC
TLR4	TGCGTACAGGTTGTTCCTAACAT	TAGTTAAAGCTCAGGTCCAGCATC
TMLHE	CTCAAGCCTGGCAAGGTACT	CCCAAGAGACGAGCAGTGTT
TNFα	CCAGAGGGAAGAGCAGTCCC	TCGGCTACAACGTGGGCTAC

[1]*GAPDH* = Glyceraldehyde-3-phosphate dehydrogenase; *AASS* = Aminoadipate-semialdehyde synthase; *ANGPT1* = angiopoietin 1; *ANGPT2* = angiopoietin 2; *FGF10* = Fibroblast growth factor 10; *FGF2* = Fibroblast growth factor 2; *FGF2R* = Fibroblast growth factor 2 receptor;

Supplemental Table B.1 (cont.).
G6PC1 = Glucose-6-phosphatase catalytic subunit 1; *GLUT3* = Glucose transporter 3; *GLUT5* = Glucose transporter 5; *HGF* = Hepatocyte growth factor; *IGF1* = Insulin-like growth factor 1; *IGF1R* = Insulin-like growth factor 1 receptor; *IGF2* = Insulin-like growth factor 2; *IGF2R* = Insulin-like growth factor 2 receptor; *IL1β* = Interleukin 1 Beta; *IL6* = Interleukin 6; *IL8* = Interleukin 8; *IL10* = Interleukin 10; *IRS1* = Insulin receptor 1; *KDM1A* = Lysine demethylase 1A; *KHK* = Ketohexokinase; *MAT2A* = Methionine adenosyltransferase 2A; *NFKB1* = Nuclear factor kappa B subunit 1; *NOS3* = Nitric oxide synthase 3; P450 (*CYP19A1)* = Cytochrome P450 family 19 subfamily A member 1; *PCK1* = Phosphoenolpyruvate carboxykinase 1; *PGF* = Placental growth factor; *RPS6KB1* = Ribosomal protein S6 kinase B1; *SOD1* = Superoxide dismutase 1; *S100A4* = S100 calcium binding protein A4; *SLC3A2* = Solute carrier family 3 member 2*; SLC7A7* = Solute carrier family 7 member 7*; SLC38A6* = Solute carrier family 38 member 6; *STAR* = Steroidogenic acute regulatory protein; *STAT5* = Signal transducer and activator of transcription 5A; *TLR4* = Toll-like receptor 4; *TMLHE* = Trimethyl-lysine hydrolase, epsilon; *TNFα* = Tumor necrosis factor alpha.

Supplemental Table B.2. Catalog number and dilution ratio for primary antibodies utilized in western blot analysis.

Antibodies[1]	Company	Catolog number	Dilution ratio
GAPDH	Abcam	ab22555	1:1000
SLC7A7	Abcam	Ab236669	1:1000
TMLHE	Abcam	Ab97842	1:500
FGF2	Santa Cruz Bt	Sc-365106	1:500
IGFBP3	Cell Signaling	25864	1:1000
LRP1	Cell Signaling	64099	1:500
AKT	Cell Signaling	9272	1:1000
mTOR	Cell Signaling	2972S	1:1000
Phospho-mTOR	Cell Signaling	2971S	1:500
JAK2	Cell Signaling	3230S	1:500
Phospho-JAK2	Cell Signaling	3771	1:500

[1]GAPDH = Glyceraldehyde-3-phosphate dehydrogenase; SLC7A7 = Light chain of the second Na^+ dependent Lys transporter; TMLHE = Trimethyllysine dioxygenase; FGF2 = Fibroblast growth factor 2; IGFBP3 = Insulin-like growth factor binding protein 3; LRP1 = Low-density lipoprotein receptor-related protein 1; AKT = Protein kinase B; mTOR = Mechanistic target of rapamycin; phospho-mTOR = Phosphorylated mTOR; JAK2 = Janus-kinase 2; Phospho-JAK2 = Phosphorylated JAK2.

APPENDIX C. SUPPLEMENTAL MATERIAL FOR CHAPTER 5

Supplemental Table C.1. Base ingredient composition of prepartum (PRE) and postpartum (POST) diets from all experiments fed to 404 primiparous multiparous Holstein cows.

Ingredient[1]	Experiment 1[1]		Experiment 2[2]				Experiment 3[3]		Experiment 4[4]		Experiment 5[5]	
	PRE	POST	PRE		POST		PRE	POST	PRE	POST	PRE	POST
			Diet 1	Diet 2	Diet 1	Diet 2						
Corn silage	36.4	33.4	37.8	30.3	50.0	34.1	26.6	31.0	32.1	36.6	31.06	39.38
Alfalfa silage	8.34	5.07	6.58	2.75	13.9	5.50	-	-	-	-	-	-
Alfalfa haylage	-	-	-	-	-	-	6.5	7.81	-	-	-	-
Canola meal	-	-	-	-	-	-	-	-	-	11.8	1.45	5.36
Alfalfa hay	4.29	2.98	-	-	-	-	-	-	-	11.3	-	20.95
Wheat middlings	-	-	1.48	1.50	3.00	3.00	-	-	0.83[6]	-	4.10	-
Corn gluten feed	-	-	1.98	2.40	4.00	4.81	-	-	8.25	8.34	6.69	-
Soybean meal, 48% CP	2.57	2.39	-	5.47	-	5.94	7.83	10.1	5.78	-	2.19	-
Wheat straw	15.6	2.98	21.4	21.8	2.17	1.00	26.5	3.25	36.3	2.34	40.25	-
Cottonseed	-	3.58	-	-	3.25	5.00	-	2.17	-	-	-	-
Wet brewers grain	4.29	9.09	-	-	-	-	-	-	-	-	-	-
Dry ground corn grain	12.9	23.9	5.97	9.93	5.57	17.0	12.6	22.2	-	18.8	0.16	15.26
Soybean hulls	4.29	4.18	7.90	9.96	-	7.28	3.46	4.25	6.60	4.32	-	-

[1]Acosta et al., (2017) and Stella et al. (2018).
[2]Glosson (2018).
[3]Batistel et al. (2017).
[4]Glosson et al. (2020) and Ryan et al. (2020).
[5]Fehlberg et al. (2020).

Supplemental Table C.2. Least squares means and associated SEM for dry matter intake (DMI), body weight (BW), body condition score (BCS), milk yield, and polymorphonuclear cells (PMN) percentage of Holstein cows classified according to their vaginal discharge at 4 days in milk (DIM).

Variable	MC[1] at 4 DIM		SEM	*P* - value
	MC ≤ 3	MC > 3		
Prepartum				
DMI, kg/d	12.8	12.3	1.29	0.43
DMI, % BW	1.55	1.48	0.15	0.38
BW, kg	830	827	19.3	0.85
BCS				
Postpartum				
DMI, kg/d	13.7	13.6	0.50	0.53
DMI, % BW	2.22	2.13	0.03	0.03
BW, kg	676	678	6.53	0.20
BCS	3.36	3.35	0.03	0.22
Milk, kg/d	38.0	38.1	0.42	0.92
PMN, %				
15 DIM[2]	29.8	31.4	8.05	0.44
30 DIM[3]	17.0	21.5	4.58	0.35

[1]Vaginal discharge was evaluated using the Metricheck (MC) device and following guidelines reported by LeBlanc and Bicalho (2017).Vaginal discharge examined using a Metricheck device and scored on a scale of 0 to 3: score 0 = clear or translucent mucus; score 1 = mucus containing flecks of white or off-white pus; score 2 = discharge containing ≤ 50% white or off-white mucopurulent material; and score 3 = discharge containing ≥ 50% purulent material, which may be white, yellow or sanguineous (Sheldon et al., 2006). Vaginal discharge smell scored (smell 0 = no odor or smell 3 = fetid odor).
[2]Endometrial cytology performed at 15 DIM for the evaluation of polymorphonuclear cells (PMN) percentage.
[3]Endometrial cytology performed at 30 DIM for the evaluation of polymorphonuclear cells (PMN) percentage.