The Relationship Between Nutrient Metabolism and Health Measures During the Periparturient Period in Pacific Northwest Dairy Herds

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Summary

During the periparturient period, dairy cows reduce their energy consumption, but maintenance energy demands higher; these induces mobilization of stored nutrients to support fetal development and milk production. Some infectious diseases and metabolic disorders may occur during the periparturient period. The objective of this study was to determine the relationship between serum metabolites of dairy cows and calves with the health status of the periparturient cows. Blood samples from periparturient cows were obtained (farm A, n = 645; farm B, n = 559, respectively) on d-21, d-7, d+1, d+7 and d+14 relative to calving. Blood samples of calves were obtained within the first 4 days of life (farm A, n =429; farm B, n = 428, respectively). Sera were analyzed for α-tocopherol, β-carotene, retinol, haptoglobin (HP), β-hydroxybutyrate (BHB), and glucose (calves only). Additionally, 115 healthy and mastitic or lame cows were randomly selected for milk fatty acid composition and serum type1/type 2 immunity balance analysis. The type 1/type 2 immune balance was evaluated by measuring the relative quantity ratio of immunoglobulin G1 and G2 subclasses (IgG1/IgG2) using a rapid D2Dx immunity test. Health records were categorized based on the occurrence of parturient diseases. Data were analyzed using linear mixed models in SAS with significance declared at $P \leq 0.05$. Results showed that cows with mastitis had greater serum retinol concentration compared with that of healthy cows during postpartum, as well as serum α-tocopherol was affected by time, seasons,

and mastitis interaction (farm A). Furthermore, cows with pneumonia and lameness had lower serum α-tocopherol and retinol compared with that of healthy cows at postpartum. Cows with mastitis had lower serum α-tocopherol and β-carotene (farm B). Retained placenta (RP) cows had lower serum retinol, and RP cows tended to decrease serum retinol in their calves (farm B). Serum HP was lower when cows had pneumonia at d+1, and greater at d+14 (farm A). Greater HP was observed with RP cows at d+1 and pneumonia (farm B). Serum BHB was greater at d+7 and +14 for cows with lameness and RP, as well as greater in pneumonia cows (Farm B). The D2Dx immunity test score was greater at d+14 in diseased cows corresponding to a decreased relative quantity ratio of IgG1/IgG2. Serum glucose in calves was greater when the calves were born from the RP dams. No significant difference in milk fatty acid composition between diseased and healthy cows was observed. In summary, diseases affected the lipid-soluble vitamins status and serum metabolites of periparturient cows, and consequently, calves may experience health issues. Future studies should focus on the milk lipid-soluble vitamins in diseased cows and proceed same project in Oregon commercial dairy farms to gain diseases in nutrients metabolites in the Pacific Northwestern region.

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Dedication

I would like to dedicate this thesis to my dear family.

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LIST OF ABBREVIATIONS

ATP	Adenosine Triphosphate
BCS	Body Condition Score
BHB	β-hydroxybutyrate
BW	Body Weight
Ca	Calcium
DCAD	Dietary Cation Anion Difference
DA	Displaced Abomasum
DIM	Days in Milk
DMI	Dry Matter Intake
GH	Growth Hormone
GnRH	Gonadotropin-Releasing Hormone
HP	Haptoglobin
IGF-1	Insulin-Like Growth Factor 1
IgG	Immunoglobulin G
IL	Interleukin
IMI	Intramammary Infection
LH	Luteinizing Hormone
MHC	Major Histocompatibility Complex
MUFA	Monounsaturated Fatty Acid
NEB	Negative Energy Balance
NEFA	Non-esterified Fatty Acid
PMN	Polymorphonuclear Neutrophil
PTH	Parathyroid Hormone

PUFA	Polyunsaturated Fatty Acid
RBP	Retinol Binding Protein
ROS	Reactive Oxygen Species
RP	Retained placenta
SCC	Somatic Cell Count
SFA	Saturated Fatty Acid

INTRODUCTION

The transition period of dairy cows is defined as three weeks before to three weeks after parturition (Drackley, 1999). During this period, dry matter intake (DMI) of dairy cattle would reduce due to fetal size, hormone changes, and environmental effects (Wankhade et al., 2017). Fetal requirement of nutrients reaches a maximum at three weeks of parturition, but DMI of dams reduces by 10 to 30 percent (Bell, 1995). These events not only reduce DMI and energy consumption on cows but also output energy for milk synthesis at postpartum (Bertoni et al., 2016). The energy requirement to synthesize milk protein, fat, and lactose excessed the energy intake from diet within three weeks of lactation (Bertoni et al., 2016). This situation induce a negative energy balance (NEB), which induces mobilization of stored fat or glycogen for maintenance and lactation (Wankhade et al., 2017). Immunity of the host is a system that defends against foreign pathogens, but impaired immunity in hosts has been found during the periparturient period in dairy cows, and it relates to infectious diseases and metabolic disorders (Esposito et al., 2014). Proper management of nutrients to reduce NEB can improve immunity in dairy cows (Esposito et al., 2014). Immune function is affected by NEB, which can facilitate chronic inflammation (Wankhade et al., 2017). Some metabolic diseases and infectious diseases may happen during the periparturient period, such as mastitis, lameness, ketosis, fatty liver, metritis, retained placenta (RP), and displaced abomasum (Wankhade et al., 2017). These

diseases are costly for treatments, from $7-$325 per case (Bartlett et al., 1995; Esslemont and Kossaibati, 1997; Halasa et al., 2007; Cha et al., 2010). Dairy cows with NEB during the periparturient period have increased lipolysis and reduced insulin/glucagon ratio (Holtenius and Holtenius, 1996); these increase ketogenesis to generate ketone body and raise β-hydroxybutyrate (BHB) in serum (Wankhade et al., 2017). The insulin sensitivity of peripheral tissues declines during the late pregnancy of cows, which would increase ketogenesis and accumulation of ketones leading to ketosis. In addition, serum concentration of α-tocopherol, β-carotene, and retinol have been observed that relates to diseases and would decline relatives to parturition (Strickland et al., 2021). This study determines the relationship of health status on serum nutrient metabolites, inflammation biomarkers, and milk fatty acid composition in Holstein dairy cows and their newborn calves during the periparturient period.

LITERATURE REVIEW

Measurement of energy balance

Energy balance can be measured indirectly or directly to predict the energy requirement of dairy cattle. The energy balance model of lactating dairy cows can be formulated to NE_L Balance (Mcal/d) = NE_L Intake − NE_L for Milk Production − NE_L for Maintenance (Ellis et al., 2006). The NE_L intake can calculate DMI (kg/d) multiply energy content of feeds. The maintenance requirement of NE_L is $0.08 \times BW^{0.75}$ (kg) for mature dairy cows. Net energy for milk production can determine fat corrected milk production (kg/d) multiply by 0.749 Mcal/ kg of milk (NRC, 2001). Energy utilization in cattle measured by the slaughter (directly) can be more accurate, but more expensive compared to indirect prediction. Besides, indirect calorimetry can be used to determine the relationship between oxygen utilization, heat production, carbon dioxide production, methane production, and nitrogen excretion in urine (Nienaber et al., 2009). Indirect calorimetry could measure 1) heat production and gas exchange, 2) energy input from feed to the energy requirement of animals to energy utilization, and 3) predict energy output (Johnson et al., 2003; Myers, 2018). Thus, measurement of energy balance in dairy cows during the periparturient period could explain a relationship between energy intake and energy expenditure to predict the following health issues related to NEB.

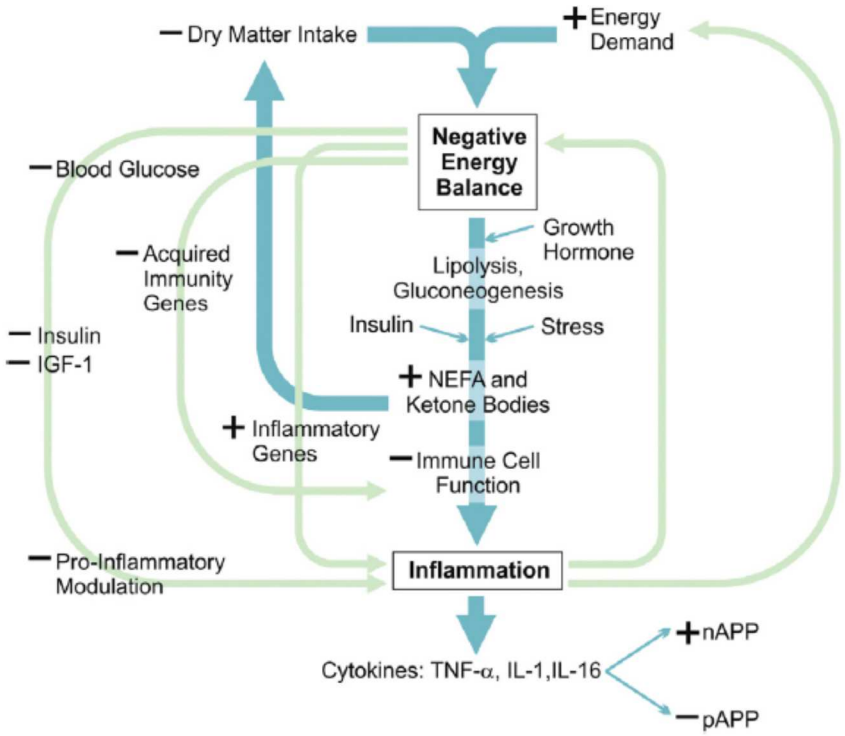
Periparturient period and energy balance

The transition period of dairy cows is defined as three weeks before and after relative to parturition (Drackley, 1999). The animals are challenged with a ration transition, fetal growth, and hormone stimulation for calving and lactation, which could reduce the DMI (Wankhade et al., 2017). On one hand, the energy requirement for fetal excessed energy intake of dams within 3 weeks before calving (Bell, 1995). The energy requirement of milk synthesis excesses energy intake within three weeks after calving (Bertoni et al., 2016). In addition, feed intake is in part controlled by the central nervous system depending on adenosine triphosphate (ATP) level in liver through hepatic vague nerve (Wankhade et al., 2017). When less energy is oxidized to produce a lower level of ATP would increase appetite for food consumption (Wankhade et al., 2017). Dairy cows commonly apply fat mobilization in the form of non-esterified fatty acid (NEFA) to generate ATP in liver, which could suppress feed intake during the periparturient period (Wankhade et al., 2017). On the other hand, physiology of dairy animals during the periparturient period, endocrine is playing an important role in NEB. Multiple hormones alter growth hormone (GH) especially in GH- insulin-growth factor 1(IGF-1) glucose signal pathway (Lucy et al., 2001). During late pregnancy, GH reduces insulin sensitivity in adipose tissues subsequently reduces glucose uptake in adipose tissues (Leury et al., 2003), muscle, and liver tissues (Esposito et al., 2014). Reduction of insulin sensitivity in peripheral tissues reduces

glucose uptake by tissues, however, this allows nutrition facilitated from maternal to placenta (Esposito et al., 2014). Besides, this reduction of glucose utilization by adipose tissue can increase mobilization of stored fat and produce NEFA for milk production during postpartum (Wankhade et al., 2017). Growth hormone level increases during NEB because NEB regulates the liver GH receptor in the GH-IGF axis and reduce IGF-1 level to elevate GH (Lucy et al., 2001). The IGF-1 decline in circulation impairs follicular maturation and steroidogenesis, which delay reproduction function (Knop and Cernescu, 2009). Otherwise, GH can promote lipolysis and gluconeogenesis in early lactation (Esposito et al., 2014; Figure A). Furthermore, leptin is another metabolic hormone that can affect DMI. Leptin affects voluntary feed intake and insulin sensitivity, and its' level declines during late pregnancy and remain at a low level during early lactation (Esposito et al., 2014). Leptin is a cytokine-hormone protein synthesized from white adipose tissues that modulate energy metabolism at the hypothalamus (Leury et al., 2003). Leptin could reduce food intake and increase energy expenditure in lipid oxidation, but leptin declines can initiate voluntary intake (Ahima and Flier, 2000). Besides, leptin plays a direct role to the central nervous system, which controls luteinizing hormone (LH) release (Knop and Cernescu, 2009). Negative energy balance can affect hypothalamus and reduce gonadotropin-releasing hormone (GnRH) pulse frequency, which reduces LH releasing and induce follicle steroids output and anovulation. These events would decrease follicle sensitivity

of gonadotropin. Hypothalamus increases sensitivity of negative feedback of estradiol, which induces a suboptimal GnRH released as well as affects reproduction (Butler et al., 2006). Block et al. (2001) found a positive correlation between plasma insulin, leptin, and glucose as well as a negative correlation between GH, and NEFA on dairy cows during the periparturient period. In another research, it was found that insulin and leptin levels of dairy cow were lower in early lactation compared to late pregnancy (Leury et al., 2003). Insulin infusion elevates plasma leptin and insulin level at late pregnancy period, and reduce plasma NEFA level of cows at late pregnancy (Leury et al., 2003). The elevated plasma GH in early lactation can increase the adaption of liver, adipose tissue, and muscle to promote glucose circulation toward milk synthesis (Block et al., 2001). Thus, reduction of leptin during early lactation could increase the voluntary feed intake of dairy cows.

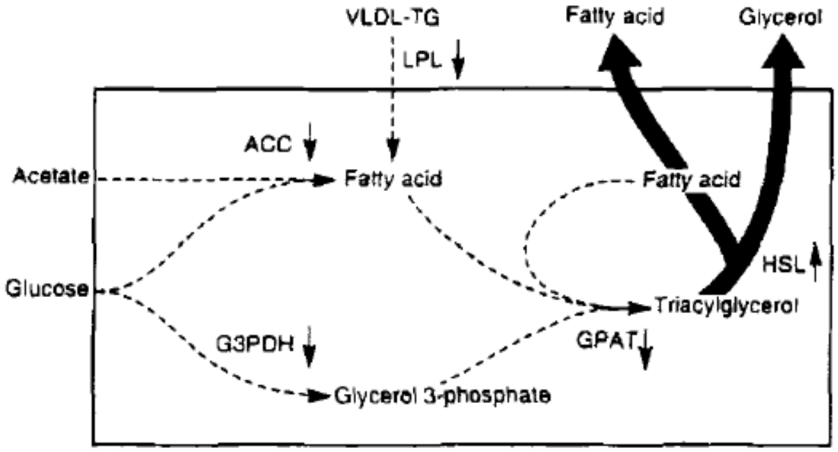
Figure A. Interactions between the immune, endocrine, and metabolic systems in dairy cows during the transition period (adapted from Esposito et al., 2014)



Dairy cows are in NEB during the transition period. Animals could apply energy to reach a balance in homeostasis on daily maintenance and lactation, such as mobilization of stored fat (Figure A). Fat in adipose tissues is stored in a form of triglyceride, which releases upon catabolism glycerol and three fatty acids (FA; Figure B). Transition cows during NEB have an increased lipolysis and reduced insulin/glucagon ratio (Holtenius and Holtenius, 1996). Triglyceride can be mobilized as glycerol and NEFA, which can be processed via lipid-oxidation or ketogenesis and generate acetyl Coenzyme A (Acetyl CoA) or ketone body [acetone, acetoacetate, and BHB; Wankhade et al. (2017)]. Body can use ketones as replacement energy to generate 22 ATP by regulation of insulin, but the accumulation of ketones could

induce ketosis (Dhillon and Gupta, 2018). For this reason, ketones are important replacement energy source for animals to maintain body function and lactation, but accumulation in ketones can become a ketosis in dairy animals.

Figure B Some metabolic pathways of adipocytes and their adaptation to lactation (adapted from Vernon and Pond, 1997)



Cortisol and estrogen increase at gestation after progesterone rapidly declines during the transition period. Alternation in cortisol level will affect DMI and activate mobilization of stored fat (Esposito et al., 2014). Glucocorticoids regulate energy metabolism, which includes raising glucose in circulation from gluconeogenesis by hepatic cells and inhibits peripheral tissues from up taking glucose (Huzzey et al., 2012). Besides, cortisol can increase mobilization of adipose tissues to increase plasma NEFA (Sapolsky et al., 2000), but excess glucocorticoids have been found to associate with insulin resistance (Andrews and Walker, 1999). Plasma cortisol level at week -2 relative to parturition has been found to associate with development of disorders such as RP, DA, subclinical ketosis and high HP concentration or death at postpartum

(Huzzey et al., 2011). The increased fecal cortisol at week -2, which increase 1.4 times the risk of health disorder or death (Huzzey et al., 2011). Thus, cortisol level raising during the transition period would increase energy metabolism, and toward insulin resistance in peripheral tissues to support glucose for milk synthesis but raising level of cortisol may induce health disorders or death at postpartum.

The stresses during the periparturient period may increase level of hormones in the endocrine system such as cortisol, adrenaline, noradrenaline, oxytocin, vasopressin, and endorphin (Hydbring et al., 1999). Glucose is the main energy resource for brain, erythrocytes, and kidneys in mammals (Zierler, 1999). Fetal liver glycogen can become an extended carbohydrate resource for dams during gestation (Vannucchi et al., 2015). These depend on the fetal adrenal activity such as adrenocorticotropic hormone and corticosteroid (Vannucchi et al., 2015). Glucose in neonatal calves after birth would shift from parental glucose through placenta supplied to consumption of lactose through colostrum, but lactose consumption would be lower than glucose requirement, which may develop as hypoglycemia in newborn calves (Hammon et al., 2013). For this reason, neonatal calves would increase catecholamines to increase a mobilization of hepatic and muscle glycogen toward the homeostasis of glucose (Vannucchi et al., 2015). Hypoglycemia in calves can affect postnatal growth or development (Hammon et al., 2013). Besides, pregnancy is considered as a stressor for dams and calves, which can affect the

serum cortisol and glucose in both cows and calves. Jacob et al. (2001) found that the pregnant cows had an increased serum cortisol and glucose levels in cows and their calves compared to the non-pregnant cows.

All in all, plasma GH and cortisol increased during periparturient will increase lipolysis and energy metabolism to supply energy for fetal growth and milk synthesis. However, these will reduce the insulin sensitivity of peripheral tissues to reduce glucose uptake, which is insulin intolerance in peripheral tissues. The lower level of leptin during the periparturient period can improve voluntary intake to enhance energy balance, which may affect reproduction and diseases in the following lactation period on cows.

Periparturient period and immunity system

The immune system has multiple mechanisms for defending against pathogens. The teat end of sphincter muscles and keratin are considered the first barrier defense system in mammary gland (Sordillo and Streicher, 2002). Bacterial invasion and colonization in internal mammary gland can activate innate defenses such as neutrophils, macrophages, natural killer cells, and soluble factors (Sordillo and Streicher, 2002). These innate defense cells can rapidly eliminate pathogens by phagocytosis and release pro-inflammatory cytokines to recruit more leukocytes and lymphocytes to join the inflammation area and assist in eliminating pathogens (Sordillo and Streicher, 2002). Macrophage, dendritic cells, and newly recruited neutrophils can join in

phagocytosis to eliminate the invaded pathogens. An internalization occurs within phagosome during phagocytosis such as reactive oxygen species (ROS) and hydrolytic enzyme to assist breakdown the pathogens (Sordillo, 2016). In addition, macrophages can breakdown pathogens in granules of cells and present protein segment of the pathogen on cell membrane with a major histocompatibility complex (MHC) for lymphocytes. The MHC complex allows lymphocytes to recognize pathogens and initiate an adaptive immune reaction (Sordillo and Streicher, 2002). Adaptive immunity includes T lymphocytes and B lymphocytes. The MHC class II could present pathogen segment to $CD4^+$ T helper cells (Th) to process cell-mediated (Th1) or humoral (Th2) immune response (Sordillo and Streicher, 2002). The MHC class I can be presented on host cells with a pathogenic antigen, which allows $CD8^+$ T cytotoxic cells to recognize the MHC class I to eliminate infected host cells (Sordillo and Streicher, 2002). B lymphocytes can self-recognize antigen by cell surface of receptor and presenting on MHC class II to Th cells and activation of B lymphocytes can secrete antibodies of immunoglobulin (Ig), which includes IgG1, IgG2, IgM, and IgA in bovine. Immunoglobulin G can be an opsonin for improving phagocytosis of neutrophils and macrophages (Sordillo and Streicher, 2002).

There is an increase in incidence of diseases such as ketosis, fatty liver, displaced abomasum (DA), mastitis, RP, metritis, and lameness, as well as immune dysfunction in cows during the periparturient period. Delayed migration of neutrophils or other innate immunity

functions may be related to the diseases. A study observed that polymorphonuclear neutrophils (PMN) were significantly lower in early lactation in blood and milk than that of mid or late lactation (Mehrzad et al., 2001). Lacking capacity of neutrophils to produce ROS had increased incidence of mastitis and metritis (Cai et al., 1994). Imbalance of the immune system will increase chronic inflammation. Grommers et al. (1989) determined PMN activity in low doses of mammary gland infused with *Escherichia coli* in transition cows; PMN lacked chemotactic activity in early lactation. Furthermore, glucocorticoid will increase at the parturition, and glucocorticoid receptors have been found in multiple immune cells, these have relevance to immunity suppression (Anderson et al., 1999; Lippolis et al., 2006; Kelley et al., 2007). Glucocorticoid delayed L-selectin and CD18, which are adhesion molecules to assist neutrophils to migrate from blood to infectious areas (Burton et al., 1995). Besides administration 750 mg of progesterone to steers enhanced neutrophil random migration (Roth et al., 1982). All in all, dairy cows were under immune suppression during the periparturient period and delaying of immunity would be affected by endocrine hormones during the periparturient period, subsequently, these may facilitate diseases.

Periparturient period and acute-phase protein

Body temperature increases during the transition period of dairy cows (>39.5 °C) is linked with inflammation, and release of pro-inflammation cytokine, which would stimulate acute

phase proteins such as haptoglobin (HP; Esposito et al., 2014). Haptoglobin, a protein produced by liver, is a type of acute-phase protein (Heegaard et al., 2000). The inflammation response may increase red blood cells lysis, but hemoglobin can be released from red blood cells to bloodstream (Hod and Spitalnik, 2012). Hemoglobin is mainly working as an oxygen carrier in mammals, and it contains four iron atoms. The free iron ions have highly oxidative capability to form as free radicals to damage the cells. Some pathogens can use free iron ions to grow and multiply (Crawford et al., 2005). For this reason, the free iron ions require transferrin and ferritin for transportation and storage (Ponka et al., 1998). When red blood cells release free hemoglobin during cell lysis, HP can bind free hemoglobin as HP-hemoglobin complex and move free hemoglobin from bloodstream to liver (Sarpong-Kumankomah and Gailer, 2019).

Haptoglobin is a major acute-phase protein in bovine, and it associates to trauma, inflammation, and infection. It can be used to monitor inflammation and health status (Eckersall, 2000). During the early stage of inflammation, pro-inflammatory cytokine (IL-1, IL-6, and TNF-α) secretion can activate monocytes upon pathogen invasion (Eckersall, 2000). When IL-6 binds to IL-6 receptor, phosphorylated transcription factor NF-IL6 will transfer to nucleus, and mediate acute phase protein transcription (Eckersall, 2000). In addition, when IL-1 and TNF- α bind with their receptors, and induce phosphorylation to degrade inhibitor of NF-κB, they could translocate to nucleus and initiate transcription of acute-phase gene (Eckersall, 2000).

The primary functions of HP are binding with hemoglobin, inhibition of bacteria, inhibition of neutrophil respiratory burst activity, immunomodulation, and regulating lipid metabolism in cattle (Petersen et al., 2004). Implementation of HP analysis has found that related to diseases such as pneumonia (Petersen et al., 2004), mastitis (Nazifi et al., 2011), metritis (Huzzey et al., 2009), RP (Huzzey et al., 2011; Pohl et al., 2015), and lameness (Smith et al., 2010). Haptoglobin in serum is less than 0.02 g/L in healthy cattle, and it would increase larger than 2 g/L within two days after infection (Eckersall and Bell, 2010). Nazifi et al. (2011) compared HP concentration of serum in dairy cattle; clinical mastitis cow had greater HP concentration than subclinical mastitis and control group (0.96, 0.59, and 0.09 ± 0.03 g/L, respectively). Excessively 0.8 g/L of HP at d1-7 postpartum would increase a 2.17 times of dystocia and RP (Dubuc et al., 2010). In addition, HP has related in dairy cows with metritis compared to healthy dairy cows. Severe metritis cows had significantly greater HP at d6 postpartum compared with that of healthy cows (1.62 vs. 0.31 ± 0.47 g/L). Mild metritis cows had greater HP at d3 postpartum than the healthy cows (1.06 vs. 0.58 ± 0.15 g/L; Huzzey et al., 2009). Sixty percent of lame cows had greater HP concentration (> 0.01g/L) compared to the healthy cows (< 0.01 g/L), and the sick cows' HP concentration ranged from 0.37 to over 1g/L (Smith et al., 2010). Heegaard et al. (2000) tested HP concentration changing in calves with inoculating bovine respiratory syncytial virus; HP concentration reached the plateau (8-10 g/L) on d 6-7 after

infection with the virus. Furthermore, Alsemgeest et al. (1994) determined inflammation diseases of cows in HP concentration, the cows' inflammation had significantly greater HP concentration compared with that for healthy cows (57.2 ± 7.5 vs N.D. g/L). In addition, ten cows were defined into chronic diseases that had greater HP concentration compared with the acute diseases group (100.3 vs. 21.6 ± 14.5; Alsemgeest et al., 1994). For this reason, HP would be a great biomarker of diseases.

Immunoglobulin G in cows and calves

Immunoglobulins include two light chains and two heavy chains of polypeptide. There are three types of immunoglobulin, such as IgG, IgA, and IgM, which occur in bovine serum and milk (Butler, 1969). The proportion of IgG and IgM in serum and colostrum around 85-90% and less than 10% respectively. Immunoglobulin A is less than IgG and IgM in bovine serum and colostrum (Butler, 1969). There are two subclasses of IgG: IgG1 and IgG2, IgG1 is rich in lacteal and salivary secretion, but IgG2 is greater in serum (Butler, 1969). The IgG1 is the predominant isotype that found in healthy mammary gland, but IgG2 is transported into mammary gland during inflammation (Sordillo and Streicher, 2002). Both subclasses of IgG can opsonize bacteria and induce phagocytosis (Sordillo and Streicher, 2002).

Colostrum is a major passive immunity transfer source from dam to newborn calves and the total IgG concentration of colostrum is used to determine the quality of colostrum. The goal

is to have greater than 50 g/L of IgG in colostrum, and high-quality colostrum should be fed 4 quarts to calves within an hour of birth (USDA, 2010). Various reasons may affect colostrum IgG concentration such as parity and diseases. The first parity dairy cows had lower IgG in colostrum compared to that for third or later lactating of dairy cows (Muller and Ellinger, 1981). Besides, milk IgG concentration could change during intramammary infection (IMI) with lipopolysaccharide. In a previous study, Wellnitz et al. (2013) found that milk IgG1 would increase after IMI, and IgG2 in milk would increase after three hours of IMI. They concluded that mammary gland inflammation could induce more IgG2 transfer from blood to milk (Wellnitz et al., 2013). In another study, it was found that IgG2 increased in milk (37.46 ± 16.98 mg/mL) and serum (15.58 ± 3.73 mg/mL) when mammary glands were infused with *Staphylococcus aureus* (Caffin and Poutrel, 1988). Furthermore, serum IgG concentration decreased from 8 weeks before (36.8 ± 11.6 mg/mL) to d +1 after calving (18.0 ± 9.1 mg/mL; Herr et al., 2011). Immunoglobulin G concentration of serum can transfer from blood circulation to udder. Transformation is related to the inflammation of the udder, which is caused by blood-milk barrier leak (Muller and Ellinger, 1981). Immunoglobulin G concentration of milk might increase because of the time relative to parturition, and IgG in colostrum is a passive immunity for calves. Thus, IgG in blood and milk can be used as biomarkers for inflammation in cows. It also can be used to determine the quality of colostrum for passive immunity in the

calves.

Periparturient period and ketosis- fatty liver complex

Negative energy balance is a common phenomenon in transition dairy cows with high production, but lower intake during this period can induce hypoglycemia and hyperketonemia. Growth hormone and cortisol release would increase energy metabolism and induce mobilization of stored fat to increase NEFA in plasma during the transition period (Sapolsky et al., 2000; Esposito et al., 2014). The raising of NEFA can generate energy through lipid-oxidation or ketogenesis to produce acetyl CoA or ketone body (Wankhade et al., 2017). Non-esterified fatty acids and ketone bodies are important energy metabolites as replacement fuels for dairy cows. There is a limit in capacity of liver to utilize NEFA to produce triglycerides. Triglycerides accumulation in the liver may lead to a shift from generating acetyl CoA in a tricarboxylic acid cycle to ketogenesis pathway (Wankhade et al., 2017). Excessive triglycerides accumulation in the liver causes fatty liver syndrome. An excessive ketone body, which may cause ketosis.

Most lactating animals may suffer from clinical or sub-clinical ketosis because of their NEB, and these can affect milk production and other diseases (González et al., 2011). Clinical ketosis can be determined by signs such as loss of appetite, body condition, and breath ketone smell. A common diagnosis of hyperketonemia is by measuring BHB (Benedet et al., 2019).

The threshold of BHB for hyperketonemia is from ≥ 1.0 to 2.9 mmol/L in blood of dairy cattle and clinical ketosis is categorized as ≥ 1.2 to 3 mmol/l (Benedet et al., 2019). In a previous study, the risk of DA was eight times higher in dairy cows with serum BHB excessed 1.2 mmol/L (LeBlanc et al., 2005; Duffield et al., 2009). The greater BHB could reduce milk yield, protein, whereas increasing milk fat percentage (Duffield et al., 2009). Displaced abomasum is an abomasum enlarged with fluid and gas, which induces migration of abomasum to left or right in the abdominal cavity (Coppock, 1974). Displaced abomasum has been suggested to that relate to NEB, high body condition score, suboptimal feed management during the transition period, and young cows (Cameron et al., 1998). Retained placenta, metritis, increased BHB, and NEFA were found to be associated with DA (LeBlanc et al., 2005). Excessively in serum BHB level (1.4, 1.1, and 1.7 mmol/L) may have a higher risk in metritis, ketosis, and DA (1.7, 10.5, and 6.9 times, respectively; Suthar et al., 2013). Dairy cows that have larger than 1.1 mmol/L of BHB may have a 1.8 times higher risk of lameness (Suthar et al., 2013). Those diseases caused by hyperketonemia can increase the culling rate of dairy cows in the first 60 days in milk (DIM). Roberts et al. (2012) reported that the serum BHB ≥ 0.7 mmol/L at a week before parturition, ≥ 1.6 mmol/L at first week at postpartum, and ≥ 1.6 mmol/L at two weeks postpartum would increase the risk of culling rate during the first 60 DIM. Furthermore, IMI dairy cows infused with higher dosage BHB (1.7 mmol/L) tended to increase serum HP, and a

greater interleukin IL-8 and IL-10 mRNA expression (Zarrin et al., 2014). For this reason, the elevation of BHB during the prepartum period would increase the risk of metabolic disorders and infectious diseases, as well as an effect on acute-phase proteins.

Periparturient period and mastitis

Dairy cattle are more susceptible to mastitis during the periparturient period than other times. Incidence of mastitis is as high as around 33.2% (Esslemont and Kossaibati, 1996). Mastitis is caused by lacking mammary gland protective characteristics of teat canal and defensive system. Keratin is the outer layer to seal streak canal and prevent invasion of pathogens. Milk is not removed from mammary gland during the dry period of lactation, thus the accumulated milk in mammary gland can induce a higher pressure and lead leaking milk on teats end (Pyörälä, 2008). The leakage would induce migration of leukocytes within one week after dry-off. Keratin would seal the teats within 1-2 weeks after dry-off (Dingwell et al., 2004). A previous study showed that milking cows during dry-off period would increase 77% of environmental intramammary infection at calving (Rajala-Schultz et al., 2005). The periparturient period is critical in causing mammary gland infection because colostrogenesis induce keratin leaking and open the teat canal for milk secretion (Oliver and Sordillo, 1988). Some bacteria would induce mastitis, such as *Staphyloccocus aureus, Streptococcus agalactiae, Streptococcus dysgalactiae, Escherichia coli*, and *Streptococcus uberis.* These can be

categorized into two major groups, which are contagious: *Staphyloccocus aureus, Streptococcus agalactiae, Streptococcus dysgalactiae,* and environmental bacteria: *Escherichia coli*, and *Streptococcus uberis* (Kudi et al., 2009). Somatic cells are around 75% of leukocytes and 25% epithelial cells of milk secretion (Sharma et al., 2011). Milk from healthy cows somatic cell count (SCC) is lower than a hundred thousand cells/mL but milk from mastitic cows will increase SCC to millions of cells/mL (Bytyqi et al., 2010). Once bacteria invade mammary gland, leucocytes will release chemo attractive chemicals to recruit more leucocytes to the infection area. Polymorphonuclear neutrophils are an innate immune leukocyte response to mammary gland infection; they can engulf pathogens and recruit leukocytes and lymphocytes, as well as produce ROS to neutralize pathogen in the cells (Sordillo and Streicher, 2002). However, activity of bovine PMN is related to BHB (Hoeben et al., 2000). This activity has been related to metabolic and hormone changes. Besides, leukocytes release cytokines and chemokines, as well as bacterial cell walls, which can stimulate inflammation. These would induce vasodilation, vascular permeability to cause edema, and increase SCC in the milk (Sharma et al., 2011). Different stages of lactation will affect SCC, which has higher than 1×10^7 cells/mL during parturition and reduce to 1×10^5 cells/mL after d 7-10 postpartum (Jensen and Eberhart, 1981). The reason for higher SCC at parturition may be that epithelial cell turnover after resting period occurred in a small amount of colostrum (Sharma et al., 2011). Transition cows will mobilize

stored fat to generate replacement energy during NEB, and a higher BHB level at early lactation is related to an increase in the prevalence of mastitis (Larry Smith et al., 1985; Huszenicza et al., 2004). Ketonemic cows infusion of *Escherichia coli* in mammary gland, all of the cows were observed severe mastitis (Kremer et al., 1993). Mastitis would affect blood glucose concentration in dairy cows. The IMI of lipopolysaccharide could reduce blood glucose in cows after 270 min of infection (Waldron et al., 2006). In another study, infusion of *Streptococcus uberis* to mammary gland under NEB caused serum glucose to be lower compared to that for the positive energy balance cows (Moyes et al., 2009). All in all, NEB in dairy cows during the transition period has fluctuations in ketone body and endocrine hormone, which will cause a delaying of innate immunity and increase the incidence of mastitis. A higher SCC in milk will affect milk quality, subsequently, decrease milk price, and increase expenditure of mastitis treatment and discarded milk.

Periparturient period and dystocia, retained placenta, and metritis

Calving difficulty can be identified by dystocia (Barrier and Haskell, 2011), multiple births (Bicalho et al., 2007), and stillbirth (Heins et al., 2006). Dystocia means a requirement of extra assistance during delivery. Retained placenta can be defined as the fetal membrane presenting more than 24h at postpartum (Markusfeld, 1987). The incidence of dystocia in beef cattle ranged from 35% to 42.2% for twins compared with 20.4% in single (Gregory et al., 1996).

Prevalence of RP was around 3.6% to 16.1 of cows (Markusfeld, 1984; Esslemont and Kossaibati, 1996). Failure of villi in fetal cotyledons separating from crypts of maternal caruncle would cause RP (Sandals et al., 1979). Factors associated with RP include ketosis, DA, higher parity, short gestation, and summer calving (Markusfeld, 1984). In addition, factors associated with dystocia include fetal physical size, birth weight, and small size of dams (Echternkamp and Gregory, 1999). Treatment with corticoid and prostaglandins would reduce RP, while PGE and PGF2α would enhance fetal delivery (Echternkamp and Gregory, 1999). In contrast, progesterone and estrogen have not reduced RP and they might increase dystocia. (Echternkamp and Gregory, 1999). Glucocorticoid has been found to regulate immune cells through membrane receptors and is associated with immunity suppression in the periparturient period of dairy cows (Anderson et al., 1999; Lippolis et al., 2006; Kelley et al., 2007). The immunity suppression reduces the ability of the immunity of dams to recognize fetal antigen and subsequently cause a failure in eject fetal membrane (Mordak and Anthony, 2015). Metritis can be determined in signs of fever, and vaginal discharge (Sheldon et al., 2006; Giuliodori et al., 2013). Metritis commonly along with DA, declining parity, declining ketosis, long gestation, stillbirth, multiple births, and winter calving (Markusfeld, 1984); metritis incidence is around 37.3% (Markusfeld, 1984). Dystocia, RP, and dead calf induce the risk of metritis of 2.85 times higher. Puerperal metritis causes cows to produce less milk by 90 DIM and a longer time for

pregnancy to occur (Giuliodori et al., 2013).

Bellows and Lammoglia (2000) found the newborn calves exposed in 0 °C with severe dystocia of the dam had a significantly increased blood serum glucose (110.1 ± 1.6 mg/dL) and cortisol. They suggested that the newborn calves were delivered from severe dystocia causing reduced efficiency of glucose utilization, which resulted in the blood glucose elevation. The newborn calves from the severe dystocia dams could induce cortisol to increase the glycolysis, which induces a higher level of blood glucose to apply toward the body thermogenic regulation. In another study, mild to severe dystocia had significantly higher blood glucose in the dams and calves compared to the cows treated with oxytocin and calcium (Vannucchi et al., 2015). Dystocia could induce the release of cortisol, which increases the gluconeogenesis of hepatocytes but decreases muscle glucose utilization; both events can increase blood glucose in the calves (Vannucchi et al., 2015). A positive correlation between cortisol level and glucose concentration was found in calves at birth, but a negative correlation between blood glucose of dams and cortisol of calves was found at calving and one hour postpartum in the dystocia group (Vannucchi et al., 2015). This could suggest that the diseases could increase the cortisol and blood glucose level of the dams and impact the cortisol and blood glucose of calves at birth.

All of the effects during periparturient period would affect health status of dairy animals, as well as suppression of immune function, which can facilitate incidence of diseases. Therefore,

all issues would be important with economic impacts on the dairy industry.

Periparturient period and milk fever

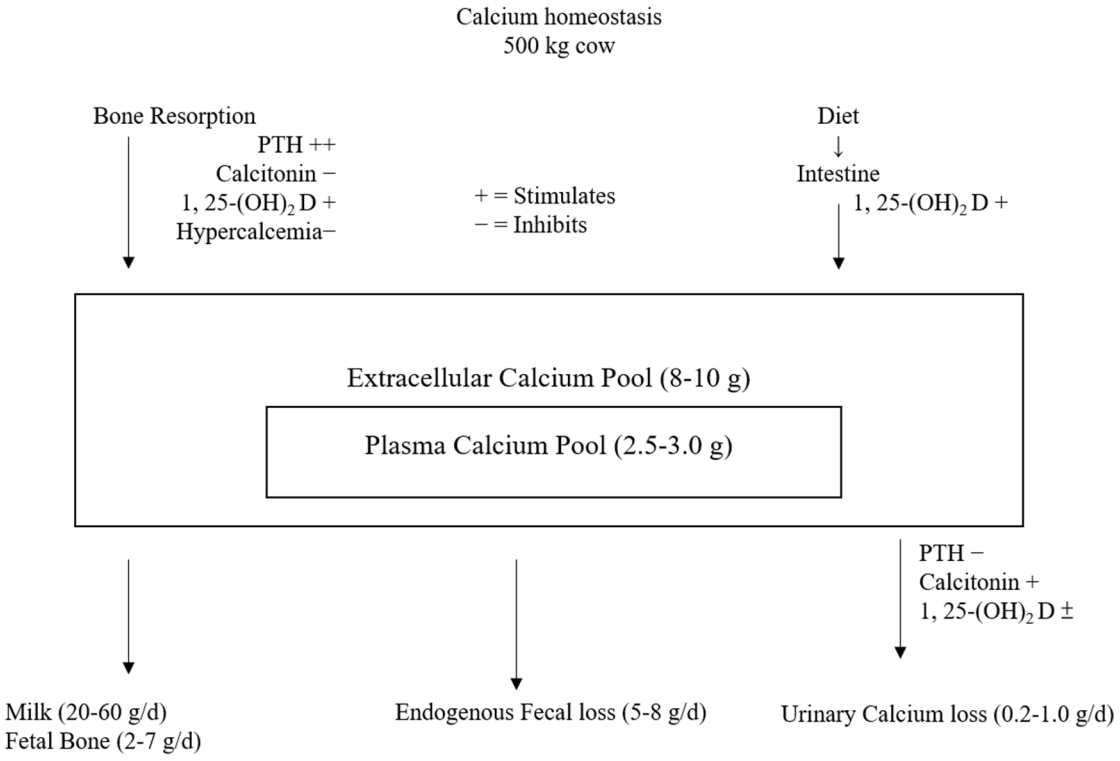
Transition dairy cows are susceptible to milk fever, postpartum hypocalcemia. There are two categories of blood calcium (Ca); sub-clinical (1.4-2.0 mmol/L) and clinical hypocalcemia (< 1.4-2.0 mmol/L; DeGaris and Lean, 2008). Milk fever prevalence is around 7.7 % (Esslemont and Kossaibati, 1996) associated with reproductive disorders (Erb et al., 1985), increasing culling rate (Stevenson and Lean, 1998), and reduce milk production at the first 4-6 weeks postpartum (Rajala-Schultz et al., 1999). Blood calcium of cows is maintained within a range between 2.0 to 2.5 mmol/L and calcitonin could respond a low blood Ca to elevate blood Ca. Parathyroid hormone (PTH) could respond to lower blood Ca level (DeGaris and Lean, 2008). Approximately losing 50% of blood Ca would induce hypocalcemia; however, PTH which releases from the parathyroid gland, can regulate hydroxylated 1,25 dihydroxy-cholecalciferol [$1,25(OH)_2D_3$] in the kidney to reabsorb Ca, as well as increasing bone metabolism to release Ca (DeGaris and Lean, 2008). Another factor that assists Ca absorption in small intestine and bone is vitamin D, which sources come from conversion of 7-dehydrocholesterol to cholecalciferol (vitamin D_3) through photochemical reaction or from plant ingestion of vitamin D_2 (DeGaris and Lean, 2008). Most Ca is stored in bone, and a lower level of plasma Ca induces PTH and subsequently enhance $1,25(OH)_2D_3$, which can induce

absorption of Ca from intestine and mobilization of stored Ca in bone (DeGaris and Lean, 2008). A greater risk of hypocalcemia in older cows associated with a reduction of $1,25(OH)_2D_3$ receptors in small intestine (Horst et al., 1990) or reduced ability to mobilize Ca from bone (Van Mosel et al., 1993). Hypocalcemia would reduce motility in rumen and abomasum leading to increased DA, which can reduce feed intake, subsequently increasing mobilization of stored fat, as well as reducing the contraction of sphincter muscle to facilitate mastitis (Goff, 2008). Metabolic alkalosis would alter the PTH receptors and reduce PTH sensitivity. Failure kidney response to PTH will affect the conversion from 25-hydroxycholecalciferol to $1,25(OH)_2D_3$ to reduce Ca reabsorption from the glomerular filtrate (Goff, 2008). Dietary more cation (potassium, sodium, calcium, and magnesium) than anion (chloride, sulfate, and phosphate) can increase metabolic alkalosis (Goff, 2008). A large amount of positively charged cations go into blood and the blood is required to donate hydrogen ions for electroneutrality; these could increase pH in the blood (Goff, 2008). However, dietary anion salts would allow more hydrogen ions in blood to decrease pH (Goff, 2008). For this reason, dietary higher cation would increase blood pH that could induce metabolic alkalosis, which would alter PTH receptors, and reduce PTH sensitivity. Impaired PTH sensitivity would reduce a regulation of $1,25(OH)_2D_3$, which regulate reabsorption of Ca from the kidney, absorption Ca from the intestine, and mobilize stored Ca from the bone. Subsequently, hypocalcemia in the dairy cow is induced.

There are three practical methods in commercial dairy farms to avoid hypocalcemia. First, oral supplementing of Ca in early parturition. Second, formulate acidified rations with supplementing anionic salts and reduce Ca during the last weeks of gestation. Third, include dietary vitamin D and its metabolites at prepartum (Thilsing-Hansen et al., 2002). The first method provides 40-50 g edible calcium salts such as calcium chloride around parturition that may prevent milk fever (Thilsing-Hansen et al., 2002). When Ca is more than 1mM in intestinal lumen, extracellular Ca increase through passive transportation, with $1,25(OH)_2D_3$ as a limiting factor for this regulation (Goff and Horst, 1993; Thilsing-Hansen et al., 2002). Thus, free Ca absorption will increase when Ca concentration is higher in intestinal lumen. Another practical method to decrease prevalence of milk fever is implementing acidified ration by calculating dietary cation-anion difference (DCAD). The most commonly equation that can predict milk fever risk is $(Na^+ + K^+) – (Cl^- + S^{2-})$ (DeGaris and Lean, 2008). A previous study suggested that ration DCAD should be around -15 mEq/100g DM (Oetzel, 2000), and successful acidified ration implementation can be determined by urinary pH between 5.5 to 6.2 (Horst et al., 1997). Positive DCAD will increase milk fever risk from 0% to 80%, but negative DCAD will keep the incidence of milk fever below 20% (Thilsing-Hansen et al., 2002). However, anionic salts reduce palatability (Oetzel and Barmore, 1993) and consequently reduce DMI, which increases NEB, plasma NEFA, and liver triglyceride (Vandehaar et al., 1999). These events relate to DA

(Cameron et al., 1998), RP (Dubuc et al., 2010), and mastitis (Rezamand et al., 2007) in postpartum. The other way to prevent milk fever is injecting vitamin D metabolites within 2-8 days before parturition (Thilsing-Hansen et al., 2002). Injection of synthetic $1,25(OH)_2D_3$ in seven days before parturition will reduce parturient paresis in the cows fed with a high Ca diet before parturition (Goff et al., 1988). Although injecting synthetic $1,25(OH)_2D_3$ may impair metabolism of 25-hydroxycholecalciferol leading to a lower plasma $1,25(OH)_2D_3$ concentration in parturient paresis cows (Goff et al., 1988). The authors suggested that cows depended on exogenous $1,25(OH)_2D_3$ for reversing hypocalcemia (Goff et al., 1988). All in all, proper management of supplementing oral Ca around calving, adjusting DCAD in the ration, and injecting vitamin D metabolites before calving will be efficient strategies to reduce milk fever in transition dairy cows.

Figure C Calcium metabolism in the dairy cow. Adapted from Goff et al. (1991)



Periparturient period and lameness

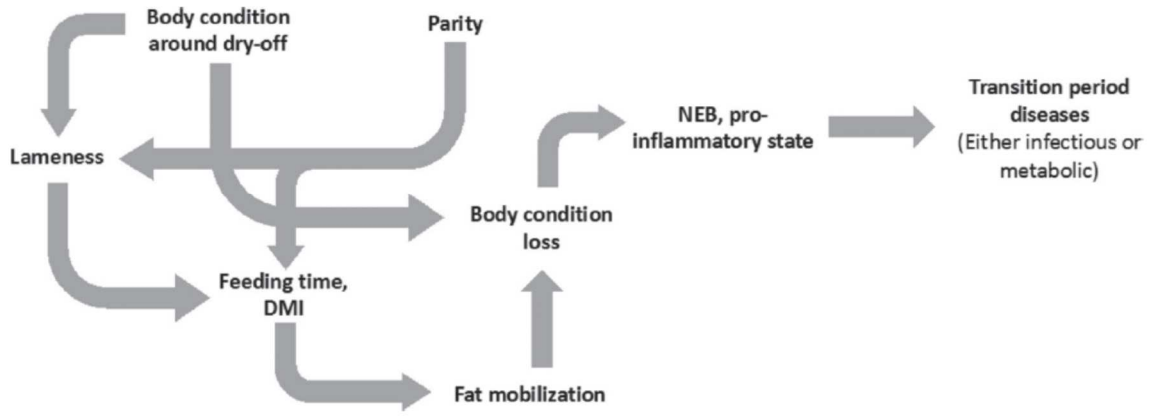
Lameness probably is the second most important economically disease followed by mastitis in dairy cows (Esslemont and Kossaibati, 1996), and it has an important impact on milk production (Rajala-Schultz et al., 1999), reproduction (Peake et al., 2011), and increasing culling rate (Booth et al., 2004). Lameness induces physiological pain (Chapinal et al., 2010) and reduces eating time and DMI (Weigele et al., 2018). Subsequently, NEB and immunity disruption would take place and provide a chance to induce hoof infection (Figure D; Daros et al. 2020). There are four common lesions associated with lameness in dairy cows, such as sole ulcers, foul-in

the foot, white line disease, and digital dermatitis (Archer et al., 2010). Different types of lameness can be categorized in non-infectious and infectious lameness. Non-infectious lameness includes sole ulcers, and white lines and infectious lameness includes digital dermatitis, and food rot (Cha et al., 2010). Esslemont and Kossaibati, (1996) reported incidence of lameness in dairy cattle was 17.4%, and average costs per case of sole ulcer, foot rot, and digital dermatitis were $216.07, $120.70, and $132.96, respectively (Cha et al., 2010). Furthermore, incidence of lameness was related to factors on free-stall design such as uncomfortable stall, concrete floor, and lack of grazing (Cook et al., 2008). Foot lesion determines with a scoring system of one to five scale and score three to five classifies as clinical lameness (Winckler and Willen, 2001). Behavior observation of cows' mobility and resting can determine lameness. Mobility scoring is based on a scale from zero to three; a score of zero indicates normal and a score of three indicates severe lameness, which means cow is unable to walk (Archer et al., 2010). Lame cows had longer resting times and greater plasma BHB compared to the healthy cows during the transition period (Calderon and Cook, 2011). The authors suggested that lameness induces hypersensitivity in cows because of the pain and it facilitates a higher risk of ketosis (Calderon and Cook, 2011) and metritis (Daros et al. 2020). In addition, a negative correlation between the glycemic level and lameness syndrome was observed. Hypoglycemic (1.85 ± 0.06 mmol/L) cows had a 32.14% prevalence of lameness

compared to that for cows with normal glycemic level (2.24 ± 0.62 mmol/L) with 7.14% lameness (Mazreku et al., 2012). Lame cows have a delayed entering to milking parlor than healthy cows and behave restlessly in parlor (Olechnowicz and Jaskowski, 2011; Weigele et al., 2018). Lame cows have a reduced their milk yield ranging from 1.5 to 2.8 kg/day after two weeks of lameness (Rajala-Schultz et al., 1999; Warnick et al., 2001) and reduced 0.8 kg/day milk yield within two weeks of lameness (Warnick et al., 2001). Culling rate increased in lame cows during 121 to 240 DIM, also foot rot, and sole ulcers decreased survival during 61 to 120 DIM (Booth et al., 2004). Besides, lame cows with claw lesions had 40 days delay in conception, greater breeding per conception, and decreased conception rate 0.52 times compared with that for healthy cows (Hernandez et al., 2001). A combination of lameness, subclinical mastitis, and loss of BCS indicated that one of severe disease or two moderate diseases can delay the first luteal phase compared with healthy cows (Peake et al., 2011). One of the severe disease increased calving interval (Peake et al., 2011). Implementation of an earlier detection method, good facility design, and proper management is the key to prevent lameness (Dolecheck and Bewley, 2018). Earlier visual observation or applied precision diary technology in dairy farms to determine lameness, can send cows to hoof trimming or treating place (Dolecheck and Bewley, 2018). All in all, costly expenditures and losses from lameness can be controlled by specific factors in management and facility design and these potential negative effects can be

controlled by prevention strategies leading to precise management.

Figure D Relationship between lameness and transition period diseases. Adapted from Daros et al., (2020).



Lipid soluble vitamins

Lipid soluble vitamins include vitamin A, D, E and K. Vitamin A plays a role in regulation of vision, genes, and cell development (Weiss, 2017). Vitamin A can be derived from β-carotene that serves as a radical-scavenging antioxidant itself against lipid peroxidation (Weiss, 2017). Vitamin D regulates metabolism of calcium and phosphorous, and regulates immune cells (Weiss, 2017). Vitamin E participates in modulating lipid antioxidation, arachidonic acid metabolism, and stabilizing the membrane of cells (Weiss, 2017). Vitamin K regulates blood coagulation and bone formation (Weiss, 2017). Vitamin A, D, and E make up the main dietary requirement of lipid-soluble vitamins, depending on the body synthesis and sun exposure that can affect the requirements (Weiss, 2017).

Vitamin A and carotenoids:

Carotenoids can be classified in two main groups: carotenes and xanthophylls (McDonald et al., 2002). Beta-carotene is a member of carotenes, the most important compound and main precursor of vitamin A (McDonald et al., 2002). The dietary sources of vitamin A are the carotenoid and esterified retinol, which include retinyl palmitate, retinyl propionate, and retinyl acetate (Herdt and Stowe, 1991). In small intestine, retinol esters can be hydrolyzed to retinol and packed into the micelles for transporting to the surface of enterocyte (Herdt and Stowe, 1991). Moreover, retinol in enterocytes of small intestine is converted to retinyl ester by two enzymes: lecithin retinol acyltransferase, and acyl CoA retinol acyltransferase. Retinyl ester is the most abundant form of vitamin A storage in animals and can be packed into chylomicrons (Blomhoff et al., 1991). Also, β-carotene can diffuse into enterocytes and be packed into the chylomicron or convert to retinol in enterocytes (Blomhoff et al., 1991). Once chylomicron is transported to liver, the retinyl ester re-esterifies to retinol for storage (Herdt and Stowe, 1991). Transportation of retinol between liver and other tissues via serum requires retinol-binding protein (RBP), which is a physiological activate form of vitamin A in plasma (Herdt and Stowe, 1991). When retinol is delivered into cells, it can be oxidized to retinoic acid for utilizations (Herdt and Stowe, 1991). The level of serum β-carotene, retinol, and RBP is affected in diseased cows. A previous study observed that subclinical IMI in Jersey cows had a greaterβ-carotene

level compared to infected Holstein, healthy Jersey, and Holstein cows at two weeks before parturition (Rezamand et al., 2007). Whereas, plasma RBP was significantly lower in Jersey and Holstein cows with IMI at the first week of postpartum (Rezamand et al., 2007). Serum β-carotene, and retinol in dairy cows with RP, and mastitis was fluctuating during the periparturient period (LeBlanc et al., 2004). Subsequently, serum retinol increase of 100ng/mL can reduce 60% risk of clinical mastitis during the early lactation period (LeBlanc et al., 2004). Strickland et al. (2021) observed that serum retinol and β-carotene decreased in early lactation. In addition, serum retinol declined in uterine diseased cows during early lactation (Strickland et al., 2021). However, supplementation of retinol and β-carotene may improve reproductive performance in dairy cows. Trojačanec et al. (2012) determined that supplementation of β-carotene and vitamin A reduced chronic fertility impairment in dairy cows. They found that β-carotene and vitamin A supplementation induced follicular size, and luteal tissue growth, also observed a positive correlation between serum concentration of β-carotene and conception rate (Trojačanec et al., 2012). All in all, serum β-carotene and retinol are affected by diseases such as mastitis, RP, and uterine diseases, whereas supplementation of β-carotene and retinol can reduce the mastitis and improve reproductive system.

Vitamin E

Vitamin E may be categorized in two major groups according to saturation of side chain.

Besides, they are separated as α-, β-, γ-, and δ- tocopherol and tocotrienols (McDonald et al., 2002). Beta-, γ-, and δ- tocopherol have only 45%, 13%, and 0.4% of α-tocopherol activity and all tocotrienols have 13% of saturated form activity (McDonald et al., 2002). Vitamin E absorption pathway is similar to that for vitamin A. Supplementation form of vitamin E is tocopherol acetate, which can be hydrolyzed to tocopherol and is absorbed in enterocyte. Passive absorption of tocopherol occurs from the intestine lumen to enterocyte, and tocopherol is packed into chylomicron to be delivered to the liver (Herdt and Stowe, 1991). Liver can release α-tocopherol into blood circulation to peripheral tissues by α-tocopherol transfer protein and coordinate with very-low-density lipoproteins (Baldi, 2005). At the cellular level, α-tocopherol plays a role as a radical scavenger to trap peroxyl radicals and reduce oxidative stress and stabilize cell membrane (Baldi, 2005). Radicals take hydrogen atoms from polyunsaturated fatty acid (PUFA) to form peroxyl radicals and disrupt the structure of cell membrane. However, α-tocopherol can donate a free electron to satisfy free radicals, and become tocopheroxy-radical, which is a low reactive free radical and unable to attract electrons from PUFA (Herdt and Stowe, 1991). In addition, supplementing vitamin E can increase T cells, B cells, IgG1, IgG2, and neutrophil killing activity (Allison and Laven, 2000). However, dairy cows have a significantly decreased plasma α-tocopherol in early lactation compared to that of other times of lactation (Calderón et al., 2007; Strickland et al., 2021). Cortisol raises during

the periparturient period, it can suppress immunity, leading to failure of ejection of fetal membrane (Mordak and Anthony, 2015), and take a place to induce mastitis (Waldron et al., 2006). The most common disease related to vitamin E deficiency is white muscle disease, but the influence of vitamin E on other bovine diseases is shown in other studies (Julien et al., 1976; Harrison et al., 1984; Batra et al., 1992; LeBlanc et al., 2004; Rezamand et al., 2007) such as RP. Julien et al. (1976) found that incidence of RP was decreased by injecting selenium and α-tocopherol. A similar study showed that RP incidence reduced to 0% when cows received selenium and vitamin E (Harrison et al., 1984). A meta-analysis of 53 trials showed that providing vitamin E reduced RP cases by 6.1% (Moghimi-Kandelousi et al., 2020). In addition, mastitic cows had lower vitamin E level in plasma and vitamin E had a positive correlation with erythrocyte of glutathione, which assists to remove free radicals (Atroshi et al., 1987). Supplementing vitamin E decreased milk SCC at d 112 of lactation period (Batra et al., 1992). Plasma vitamin E concentration was negatively related to rate of clinical mastitis (Weiss et al., 1990). Wilde (2006) observed that vitamin E influenced prevention of mastitis during the first weeks of lactation. Lower plasma α-tocopherol was observed in cows with IMI compared to the healthy cows at week 8 of postpartum (Rezamand et al., 2007). Besides, serum α-tocopherol declined at d5 postpartum in mastitic and RP cows (LeBlanc et al., 2004). Hypovitaminosis happens when nutrients such as vitamin E during the periparturient period are insufficient,

subsequently, they may promote lameness (Stokka et al., 1996; Wilde, 2006; Ózsvári, 2017). Serum vitamin E concentration was lower in lame cows compared to that for healthy cows (2.31 ± 0.24 vs 2.44 ± 0.12 μg/mL; Kilic et al., 2007). In addition, injecting 3000 IU of vitamin E at one week before expected calving raised serum α-tocopherol at d7, and d14 after injection (LeBlanc et al., 2002). However, vitamin E injection did not change incidence of lameness, mastitis, DA, and ketosis, but it tended to reduce risk of RP (LeBlanc et al., 2002). Besides, supplementing 550 IU of vitamin E for 40 days had no significant effect on heifers with interstitial pneumonia (Stanford et al., 2007). Although, dietary 500 PPM of α-tocopherol reduced 1000-fold bacterial lung burden in C57BL/6 mice (Bou Ghanem et al., 2015). Additionally, camels with pneumonia had a reduced serum α-tocopherol concentration (Elnisr et al., 2012). All in all, serum α-tocopherol concentration is related to health conditions and supplementation of α-tocopherol during disease status may reduce some of the incidences in animals.

Vitamin A and Vitamin E transfer from cow to calf

Vitamin A and E cannot cross the placenta in a high proportion, so the main sources of vitamins come from colostrum in newborn calves (Quigley and Drewry, 1998). Newborn calves' plasma α-tocopherol, β-carotene, and retinol are relatively low, and received colostrum can raise plasma level of α-tocopherol, β-carotene, and retinol in calves (Zanker et al., 2000). Colostrum

contains a greater amount of vitamin A and E compared to milk in other lactation periods; therefore, supplementation of vitamins to dams during gestation and lactation will improve vitamins level in serum and milk (Debier et al., 2005). Dietary supplementation of vitamin A at prepartum in gestation dairy cows increased plasma retinol, colostrum retinol *plus* retinyl ester level (Puvogel et al., 2008). Furthermore, plasma retinol increased in calves that received this type of colostrum (Puvogel et al., 2008). In addition, a positive correlation was found between retinol and RBP in the dietary supplementation of vitamin A in pre-ruminant calves. Calves fed ≥ 34,000 IU vitamin A had greater retinol and RBP compared to that for calves fed ≤ 1700 IU of vitamin A (Nonnecke et al., 2001). Furthermore, feeding 70 IU/Kg DMI vitamin E during the dry period increased plasma vitamin E level and milk tocopherol level of lactating cows (Weiss et al., 1990). Supplementing vitamin E can increase serum and colostrum vitamin E at parturition in ewes, as well as serum vitamin E in their 3-d old lambs (McDowell et al., 1996). However, milk feeding vitamin A to calves did not affect plasma retinol, serum IgG, and IgM (Franklin et al., 1998). Additionally, feeding vitamin A decreased plasma α-tocopherol level in six weeks old calves (Franklin et al., 1998). All in all, limited blood circulation of α-tocopherol, β-carotene, and retinol in newborn calves may be adjusted by feeding high-quality colostrum, which can be modified by supplementary vitamins in transition ration. Whereas health status of dams may have effects on the quality of colostrum. Thus, supplementing vitamins in transition

dairy cows can improve colostrum quality and reduce postnatal diseases consequently improve calves' welfare and following performance.

Milk fatty acid composition

Bovine milk contains approximately 87% water, 4.6% lactose, 3.4% protein, 4.2% fat 0.8% minerals, and 0.1% vitamins (Månsson, 2008). Milk fat droplets are synthesis from endoplasmic reticulum of epithelial cells in alveoli (Månsson, 2008). Milk fat contains approximately 98% triglycerides, whereas other milk lipid fractions include diacylglycerol, cholesterol, phospholipid, and NEFA (Månsson, 2008). There are three sources of milk FA in triglyceride, which include 1) acetyl-CoA converted from glucose, 2) chylomicron hydrolysis from diet, and 3) *de novo* synthesis in mammary gland (Akers, 2016). Milk FA derives from diets, which are predominant in 18 carbon FA and around 30% C16 FA (palmitate acid; Akers, 2016). Fatty acid from *de novo* synthesis usually is C4-C14 FA, and some C16 FA and are synthesized from acetate and BHB (Akers, 2016). Most dietary fats are in esterified form, which can be hydrolyzed and generate free FA in the rumen (Harvatine et al., 2009). Unsaturated fats in rumen are processed through biohydrogenation to remove double bond (reduction) and shift double bond position (isomerization; Harvatine et al., 2009). Fatty acid composition of milk fat includes 70% of saturated fatty acid (SFA) and has approximately 30 % C16:0, 11% C14:0 (myristic acid), and 12% C18:0 (stearic acid) in total FA (Månsson, 2008). There is 25% of

monounsaturated FA (MUFA) in milk fat predominantly 23.8% of C18:1 (oleic acid) in the total FA (Månsson, 2008). Mastitis affects milk FA composition. Randolph and Erwin, (1974) determined milk FA from mastitic cows that had an increased NEFA, and short-chain FA (C4-C12), and a decreased long-chain FA (C16 and C18). In another research looking into an infusion of *Escherichia coli* endotoxin or *Streptococcus agalactiae* in two-quarters of udder, long-chain saturated FA increased; however, total FA composition in milk was unaffected (Needs and Anderson, 1984). It was shown that cows with subclinical mastitis had a greater milk palmitoleic (C16:1; Needs and Anderson, 1984). Turini et al. (2020) determined the effect of four levels of SCC on milk FA profile. They found that SCC less than ten thousand cells/ml had the highest *de novo* synthesized, and the lowest biohydrogenation compared with that for SCC larger than a hundred thousand cells/ml. All in all, based on these results mastitis may affect milk FA composition.

Summary

Bovine diseases have a big economic impact on the dairy industry. Practical management in commercial dairy farms is related to the diseases. Diseased cows during gestation, and around parturition, not only influence performance but also affect their fetus. Serum metabolites and inflammatory biomarkers can be applied as indicators of diseases in cows and calves. This study will add our knowledge on metabolites, inflammatory biomarkers, milk fatty acid composition, and bovine diseases in cows and their newborn calves. These help to develop improved practical management strategies for the dairy industry.

HYPOTHESIS AND OBJECTIVES

Hypothesis

Diseases in dairy cows during the periparturient period influence serum lipid-soluble vitamins (retinol, α-tocopherol, and β-carotene), serum metabolite (BHB, and HP), IgG, and milk FA composition. In addition, the disease status of cows influence serum lipid-soluble vitamins and glucose status in their newborn calves. Serum IgG concentration can be rapid determined by the D2Dx immunity test kits.

Objectives

The objectives of present study were to determine:

1) If there is an association in serum lipid-soluble vitamins (retinol, α-tocopherol, and β-carotene) in diseased dairy cows during the periparturient period.

2) If serum metabolite (BHB, and HP), IgG, and milk FA composition associates with diseases in periparturient dairy cows.

3) If newborn calves' serum lipid-soluble vitamins (retinol, α-tocopherol, and β-carotene), and glucose relates to dams' diseases.

4) If serum IgG can be rapid test by the D2Dx immunity test kits.

Chapter 1

Relationship between serum metabolites and milk fatty acid with periparturient diseases in Pacific Northwest dairy farms

INTRODUCTION

The transition period in dairy cows is defined as three weeks before to three weeks after parturition (Drackley, 1999). During this period, dry matter intake (DMI) of dairy cattle reduces mainly because of increased fetal size, hormonal changes, and shifts in environmental condition effects such as heat stress (Wankhade et al., 2017). Fetal requirement of nutrients reaches a peak at three weeks before parturition, but DMI of dams reduces by 10 to 30 percent during the same time (Bell, 1995). Feed intake is in part controlled by the central nervous system through adenosine triphosphate (ATP) level in the liver, and lower energy is oxidized to produce less ATP would increase appetite for food consumption (Wankhade et al., 2017). Dairy cows commonly mobilize fat in the form of non-esterified fatty acid (NEFA) to generate ATP in the liver, which suppresses feed intake during the periparturient period (Wankhade et al., 2017). The energy requirement to synthesize milk protein, fat, and lactose exceeds the energy intake from diet within three weeks of lactation (Bertoni et al., 2016). This situation induces negative energy balance (NEB), which induces mobilization of stored fat or glycogen for maintenance and lactation (Wankhade et al., 2017). Dairy cows with NEB during the periparturient period would have an increased lipolysis and reduced insulin/glucagon ratio (Holtenius and Holtenius, 1996); these increase ketogenesis to generate ketones and raise β-hydroxybutyrate (BHB) in serum (Wankhade et al., 2017). The insulin sensitivity of peripheral tissues declines during late

pregnancy of cows, which increases ketogenesis and accumulation of ketones leading to ketosis.

During late pregnancy, growth hormone reduces insulin sensitivity in adipose tissues subsequently reduces glucose uptake in adipose tissues (Leury et al., 2003), muscle, and liver tissues (Esposito et al., 2014). Reduction of insulin sensitivity in peripheral tissues reduces glucose uptake, however, retains higher levels of blood glucose for passage through the placenta to the fetus (Esposito et al., 2014). Glucose in neonatal calves after birth shifts from parental glucose through placenta supplied to consumption of lactose through colostrum, but lactose consumption lower than glucose requirement, which may develop as hypoglycemia in newborn calves (Hammon et al., 2013). Besides, pregnancy is a stressor for dams and calves, which can affect the serum cortisol and glucose in both cows and calves. Jacob et al. (2001) determined that pregnant cows and their calves had greater serum cortisol and glucose compared to the non-pregnant cows. Thus, plasma growth hormone and cortisol raised during periparturient will increase lipolysis and energy metabolism to supply energy for fetal growth and milk synthesis. However, these reduce the insulin sensitivity of peripheral tissues hereby reducing glucose uptake, which can cause insulin intolerance and hyperglycemia.

Immunity of the host is a system that defends against foreign pathogens, but impaired immunity in hosts has been found during the periparturient period in dairy cows, and it relates to infectious diseases and metabolic disorders (Esposito et al., 2014). Immune function is

affected by NEB, which can facilitate chronic inflammation (Wankhade et al., 2017). Some metabolic diseases and infectious diseases may happen during the periparturient period, such as mastitis, lameness, ketosis, fatty liver, metritis, retained placenta (RP), and displace abomasum (Wankhade et al., 2017). These diseases are costly to treat, from $7-$325 per case (Bartlett et al., 1995; Esslemont and Kossaibati, 1997; Halasa et al., 2007; Cha et al., 2010). Haptoglobin (HP), a protein produced by liver, is an acute-phase protein (Heegaard et al., 2000). The inflammatory response may increase red blood cell lysis to release hemoglobin (Hod and Spitalnik, 2012). Haptoglobin can bind free hemoglobin as HP-hemoglobin complex and move free hemoglobin from bloodstream to liver (Sarpong-Kumankomah and Gailer, 2019). Serum HP concentration had greater in cows with mastitis (Nazifi et al., 2011), RP (Dubuc et al., 2010) dystocia (Dubuc et al., 2010), metritis (Huzzey et al., 2009), and lameness (Smith et al., 2010). Additionally, inflammation can activate B lymphocytes to synthesize and release antibodies of immunoglobulin (Ig), which includes IgG1, IgG2, IgM, and IgA in bovine. Immunoglobulin G can be an opsonin for improving phagocytosis of neutrophils and macrophages (Sordillo and Streicher, 2002). Immunoglobulin G1 is rich in lacteal and salivary secretion, but IgG2 is greater in serum (Butler, 1969). The IgG1 is the predominant isotype found in the healthy mammary gland, but IgG2 is transported into mammary gland during inflammation (Sordillo and Streicher, 2002). Wellnitz et al. (2013) found that milk IgG1 would increase after

intramammary gland infection (IMI), and IgG2 in milk would increase after three hours of IMI. They concluded that mammary gland inflammation could induce more IgG2 transfer from blood to milk (Wellnitz et al., 2013). Thus, IgG in serum and milk can be used as biomarkers for inflammation in cows.

Serum concentration of α-tocopherol, β-carotene, and retinol have been observed that relates to diseases and parturition (Strickland et al., 2021). A meta-analysis of 53 trials showed that providing vitamin E reduced RP cases by 6.1% (Moghimi-Kandelousi et al., 2020). Lower plasma α-tocopherol was observed in cows with IMI compared to the healthy cows at week 8 of postpartum (Rezamand et al., 2007). In addition, subclinical IMI in Jersey cows had a greaterβ-carotene level compared to infected Holstein, healthy Jersey, and Holstein cows at two weeks before parturition (Rezamand et al., 2007). Serum β-carotene, and retinol in dairy cows with RP, and mastitis was fluctuating during the periparturient period (LeBlanc et al., 2004). Subsequently, serum retinol increase of 100 ng/mL can reduce 60% risk of clinical mastitis during the early lactation period (LeBlanc et al., 2004). Overall, serum α-tocopherol concentration is related to health conditions, and supplementation of α-tocopherol, β-carotene, and retinol during disease status may reduce the incidence in animals.

This study intended to determine the relationship of health status on serum metabolites (BHB, HP, and glucose), IgG, serum lipid-soluble vitamin, and milk fatty acid composition in

Holstein dairy cows and their newborn calves during the periparturient period.

METHODS AND MATERIALS

Animal selection and sample collection

This study was approved by the University of Idaho Animal Care and Use Committee (# 2017-52). A total of 1204 cows were enrolled in the study contained both multiparous and primiparous cows. Blood samples were collected from Holstein dairy cows in dairy farms located within the Pacific Northwest region of the United States between March 2018 to November 2018 (farm A, n = 645) and April 2019 to June 2019 (farm B, n=559). Farm A samples were separated in difference seasons: late spring (n = 42), early summer (n = 129), late summer (n= 119), fall (n = 174), and winter (n = 181), respectively. Blood samples were collected on day -21, -7, +1, +4, +7, and +14 relative to parturition (d1) All blood samples were collected by venipuncture of the coccygeal artery or vein using an 18-gauge>>, 2.5-cm blood collection needle. Blood samples were collected into a 10mL vacutainer tube (BD Vacutainer® red top serum collection tubes, Franklin Lakes, NJ, U.S.A.). In addition to cows, blood samples were collected from both male and female calves within 1-3 days after birth from the jugular vein (farm A, n = 429; farm B = 428). All blood samples were placed on ice immediately after collection and stored in 4°C for 24 hours and centrifuged at 1500 × *g* for 10 min at 4°C. The sera were transferred into 1.5 mL centrifuge tubes and immediately stored at -80ºC for later analysis. Cows' health data outputs were collected from 90 days before to 90 days after

parturition from commercial dairy farms. The commercial dairy farms were using DairyComp 305 (farm A) and DHI-Plus (farm B) as dairy management software. Farm A we obtained lame cows (n = 259), mastitic cows (n = 39), and pneumonia cows (n = 11). Farm B we obtained lame cows (n = 28), mastitic cows (n = 51), pneumonia cows (n = 89), and RP cows (n = 49). Milk samples were collected at 1-3 days DIM into 50mL empty centrifuge tubes without preservatives and stored at -20 °C for future analysis.

Lipid soluble vitamins

Serum concentration of α-tocopherol, β-carotene, and retinol were determined by reversed-phase HPLC with a photodiode array detector as we described previously (Tsai et al., 2017). Briefly, 400 μL of serum samples were acidified by 20 μL of 2 N acetic acid, denatured by adding 420 μL acetonitrile, and extracted with 1.5 mL organic solvents mixture (hexane: 2-propanol, 6.5: 1.5; v/v). The samples were vortexed for one minute and centrifugated at 1000 × *g* for 3 minutes and the supernatant was transferred into another tube and dried under a light flow of nitrogen. HPLC mobile phase consisting of 78.2% acetonitrile, 13.0% dichloromethane, 8.7% methanol, and 0.1% n-butanol at a flow rate of 1.5mL/ min was used. Concurrent separation of vitamins was performed on a Waters® C_{18} column (Module 2695; 4.6 × 75 mm, 3.5 μm particle size) and Photodiode Array Detector (PDA) 2998. Waters 2695 and PDA 2998 were controlled by Empower 3 software (Waters®, Milford, MA). Autosampler temperature

was fixed at 4 ºC and column temperature was 50 ºC. Alpha-tocopherol, β-carotene, and retinol were detected at λ = 290, 450, and 325 nm, respectively. Serum concentrations of retinol, α-tocopherol, and β-carotene were eluted at 1.57, 3.10, and 5.56 min, respectively.

IgG1 and IgG2 in the serum of dairy cows

A rapid test of IgG1/IgG2 ratio change during parturition was measured by D2Dx™ immunity test kits in selected serum samples. Total 130 serum samples from randomly chosen diseased (mastitic and/or lame) and healthy cows from farm A and 100 serum samples from farm B were analyzed. The mechanism of this test are explained in Tsai et al., 2021. D2Dx™ immunity test kits (D2Dx-BV-500 Cat# bv05212020) were received from Nano Discovery Inc. (Orlando, FL). Each kit contains a reagent and cuvettes for 500 tests. A handheld reader device, CT-100 from Nano Discovery Inc. was used to read the test result. To perform the test, 50 µL of testing reagent solution was first placed into the cuvette using a micropipette. Then 10 µL of an undiluted bovine blood serum sample was added. After mixing the assay solution for 5 seconds using a mini-vortex mixer, the cuvette was placed in CT-100 and the result was read automatically in 30 seconds. The response of the test was reported directly as an absorbance change of the assay solution over 30 seconds of reaction time. The absorbance change was represented in test score, which inversely relates to the IgG1/IgG2 ratio; the greater test score represents a lower IgG1/IgG2 ratio, and the lower test score represents a greater IgG1/IgG2

ratio.

Haptoglobin

Serum concentration of haptoglobin was determined using an enzymatic assay. Four µL each sample and standard were applied to the assigned well in a 96 wells plate in triplicates (VWR® Cat # 89093-592, Radnor, PA). The Haptoglobin calibrator (Tridelta Development Limited Cat. # TP801-CAL, Kildare, Ireland) was used as the standard. Methemoglobin was prepared by dissolving 300 mg of hemoglobin (Sigma Aldrich, Cat # H2625, St. Louis, MO) in 5 mL of 0.9% sodium chloride solution and added to 3 mL of 10% potassium ferricyanide (III; Sigma Aldrich, Cat # 702587, St. Louis, MO). Following solution was filtrated through a gel Sephadex G-25 column (Sigma Aldrich, Cat # G2580-50G, St. Louis, MO). Chromogen reagent was made by 1.6 mM 4-amino antipyrine,1.0 mM 8-anilino-1-naphthalene sulfonic acid, 0.389 mM DL-dithiothreitol, and 20 mM phenol (Sigma Aldrich, Cat # 06800, Cat # A1028, Cat # 43815, and Cat # P1037, respectively, St. Louis, MO). An 8-channel pipette was used to transfer 100 µL of 0.06 g/L methemoglobin to each well and mixed briefly by hand. Forty-five µL of Chromogen reagent was pipetted to each well using an 8-channel pipette, and the plate was mixed briefly. Finally, 25 µL of H_2O_2 (35.14mM) was added to each well and mixed briefly. Plates were placed into a plate reader (SpectraMax i3x, Molecular Devices San Jose, CA) and the absorbance was determined over 6 min at 390 nm. Inter assay CV was 4.9%, and intra assay CV was 4.2%.

Beta-hydroxybutyrate

Serum concentration of β-hydroxybutyrate (BHB) was determined via an enzymatic colorimetric assay. Twenty-five µL of sample and standard were applied to the assigned well in a 96 wells plate in triplicates (VWR® Cat # 89093-592, Radnor, PA). The standard was purchased from Wako Diagnostics (Cat #41-73791, Fujifilm medical system U.S.A., Inc, Stamford, CT). Assay buffer was prepared by mixing 0.1 M tris, 0.1 M oxalic acid, 2 mM ethylenediaminetetraacetic acid (EDTA), and 2.5 mM β-nicotinamide adenine dinucleotide hydrate (NADH; Sigma Aldrich, Cat # 252859, Cat # 75688, Cat # E9884, and Cat # N7004, respectively, St. Louis, MO). Assay buffer pH was adjusted to 8.5 with 10M sodium hydroxide. On the day of analysis, 100 µL of 3-hydroxybutyrate dehydrogenase (3-HBDH) was transferred to a 1.5 mL microcentrifuge tube and centrifuged at 12,000 × g for 10 minutes. The supernatant was discarded, and the pellet was resuspended in the 15 mL of assay buffer as described above. Serum and standard were mixed with 150 µL reagent (mixture of assay buffer and 3-HBDH) and placed plate in a plate reader (SpectraMax i3x, Molecular Devices San Jose, CA) shaken for 60 seconds at 37°C. Immediately read at 340 nm followed by a reading at 1 and 2 minutes. Inter assay CV was 4.1% and intra assay CV was 2.8%.

Glucose

Serum glucose concentration in calves was determined by an enzymatic assay. Five µL of

samples and standard (Fujifilm Medical Systems U.S.A. Inc., Cat# 997-03001, Lexington, MA) were added to the assigned well in a 96 wells plate (VWR® Cat # 89093-592, Radnor, PA). Solution A was prepared by one capsule of PGO enzyme (Sigma Aldrich, Cat # P7119-10CAP, St. Louis, MO) into 50 mL of dH_2O. Solution B was prepared by mixing 20 g ± 0.01g of dH20 within one vial of o-dianisidine dihydrochloride (Sigma Aldrich, Cat # F5803-50MG, St. Louis, MO). The solution AB mixture was prepared by adding 1.6 mL of solution B to 50 mL of solution A. One hundred and fifty µL of solution AB was added to each well with a multi-channel micropipette. The plate was placed into a plate reader (SpectraMax i3x, Molecular Devices San Jose, CA) and was shaken for 10 seconds. Plates were then incubated at room temperature for 45 minutes and protected from the light. Absorbance was measured that at 450 nm in the plate reader (SpectraMax i3x, Molecular Devices San Jose, CA). Inter assay CV was 3.7%, and intra assay CV was 2.3%.

Fatty acid composition in milk

Lipids in milk were extracted as previously described (Scholte et al., 2017). Briefly, milk fat was extracted by chloroform: methanol (2:1) and sodium chloride to 2 mL of milk and then dried down under a nitrogen system. The lipid was dissolved in 2 mL of 0.5M sodium methoxide and kept in a water bath at 50 ºC for 10 min. Afterward, samples were added 3mL of 5% methanolic hydrochloric acid incubated in the water bath at 80 ℃ for 10 min .

Subsequently, 6% of potassium carbonate and hexane were added to the sample and centrifuged at 500 × g to obtain organic layer. Methylated lipid samples were analyzed by Agilent 7890A gas chromatography system (Agilent Technologies, Santa Clara, CA) equipped with an autosampler, a flame-ionization, and an Agilent HP-88 column (100m x 0.25mm with a 0.20-μm film thickness, Agilent Technologies). The oven temperature was initiated at 120 ºC for 1 minute and gradually increased to 175 ºC by 10 ºC/min held 10 min. Afterward, oven temperature increased to 210 ºC for 5 min and gradually increased to 230 ºC by 5 ºC/min held 5 min. Finally, 230 ºC increased 5 ºC/min to 240 ºC and held for 5 min. Fatty acids were identified in comparison with Supelco 37 Component FA methyl esters mix (Sigma Aldrich, St. Louis, MO) that was used as an external standard.

Statistical analysis

The data obtained from lipid-soluble vitamin (farm A), haptoglobin (farm A), BHB (farm A) were analyzed using repeated measures with linear mixed model in SAS (V. 9.4, SAS Inst. Inc., Cary, NC, U.S.A.). Diseases presence/absence were applied as fixed effect and cows were used as random effect. A repeated measures association between days were used in a compound symmetry correlation structure.

The statistical model was:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \tau_k + (\alpha\beta\tau)_{ijk} + e_{ijkl}$$

Where: Y_{ijk} = observed response of lipid-soluble vitamins, haptoglobin, and BHB concentration

in cows' serum, μ = overall mean, α_i = fixed effect of disease (*i* =healthy, diseased), β_j = fixed effect of time, τ = fixed effect of season, $(\alpha\beta\tau)_{ijkl}$ = disease, time, and season interaction, and e_{ijk} = residual error term assumed to be N (0, σ^2).

The data obtained from lipid-soluble vitamin (farm B), haptoglobin (farm B), BHB (farm B), and pooled both farms' IgG concentration were analyzed using repeated measures with linear mixed model in SAS (V. 9.4, SAS Inst. Inc., Cary, NC, U.S.A.). Diseases presence/absence were applied as fixed effect and cows were used as random effect. A repeated measures association between days were used in a compound symmetry correlation structure.
The statistical model was:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + e_{ijk}$$

Where: Y_{ijk} = observed response of lipid-soluble vitamins, haptoglobin, BHB, and IgG concentration in cows' serum, μ = overall mean, α_i = fixed effect of disease (*i* =healthy, diseased), β_j = fixed effect of time, $(\alpha\beta)_{ij}$ = disease by time interaction, and e_{ijk} = residual error term assumed to be N (0, σ^2).

Lipid-soluble vitamins and serum glucose concentration in calves were analyzed with linear mixed model in SAS (V. 9.4, SAS Inst. Inc., Cary, NC, U.S.A.), considering disease presence/absence in dams as fixed effect and calves as random effect.
The statistical model was:

$$Y_{ij} = \mu + \alpha_i + e_{ij}$$

where the: Y_{ij} = observed response of lipid-soluble vitamin and glucose concentration in calves' serum, μ = overall mean, α_i = fixed effect of disease status of the dam (*i* =healthy, diseased), and e_{ij} = residual error term assumed to be N (0, σ^2).

Milk samples were obtained one time (1-3 DIM) to determine FA composition, and data were analyzed with linear mixed model in SAS (V. 9.4, SAS Inst. Inc., Cary, NC, U.S.A.), considering diseases presence/absence in dams as fixed effect and cows as random effect. The statistical model was:

$$Y_{ij} = \mu + \alpha_i + e_{ij}$$

where the: Y_{ij} = observed response of milk FA composition, μ = overall mean, α_i = fixed effect of disease (*i* =healthy, diseased), and e_{ij} = residual error term assumed to be N (0, σ^2).

The serum lipid-soluble vitamin between cows and calves were conducted a spearman correlation in SAS to represent correlations. However, the results represented correlation does not imply causation. Significance differences were declared when $P \leq 0.05$ and $0.05 < P \leq 0.1$ value considered a trend.

RESULTS AND DISCUSSIONS

Lipid soluble vitamins

In the present study, we determined lipid-soluble vitamin concentration, which included α-tocopherol β-carotene and retinol from cows and calves in commercial dairy farms in the Pacific Northwestern region. In farm A, we observed a greater in serum retinol concentration in dairy cows with mastitis compared with that for healthy cows (36.1 vs. 30.7 ± 2.0 μg/mL, $P = 0.0095$; Figure 1.1). Mastitic cows tended to have an increased serum retinol concentration in their newborn calves (2766.1 vs. 2419.9 ± 191.3 ng/mL, $P = 0.08$; farm A, Table 1.3). In addition, there was an effect of time, season, and time × season for serum α-tocopherol, β-carotene, and retinol in cows (farm A, Table 1.1). Cows' serum lipid soluble vitamins concentration decreased at parturition; however, β-carotene concentration did not increase at postpartum compared with α-tocopherol and retinol. Serum α-tocopherol β-carotene, and retinol in cows had lower concentration in late summer and fall (farm A, Table 1.1). Calves from dams with pneumonia showed a lower concentration of serum retinol compared with that for healthy dams (1893.2 vs. 2463.6 ± 199 ng/mL, $P = 0.005$; farm A, Table 1.3). In the present study, there was a negative association between diseased cows and their newborn calves in serum retinol and β-carotene concentration at d-7 (pneumonia) and d+14 (mastitis; farm A, Table 1.4). There was a positive association between serum α-tocopherol in mastitic cows and

calves at d-7 (farm A, Table 1.4). In farm B, α-tocopherol and retinol were decreased in cows with pneumonia compared with healthy cows (7.59 vs 8.45 ± 0.26 μg/mL, $P < 0.01$; 33.85 vs 38.49 ± 1.01 μg/mL, $P < 0.01$; respectively). Serum α-tocopherol and retinol were reduced in lame cows when compared with healthy cows (7.39 vs 8.37 ± 0.47 μg/mL, $P = 0.05$; 33.51 vs 37.98 ± 1.83 μg/mL, $P = 0.02$; respectively; farm B, Table 1.2). In addition, mastitic cows tended to have a reduced serum α-tocopherol and β-carotene concentration (7.73 vs 8.37 ± 0.36 μg/mL, $P = 0.08$; 19.33 vs 20.90 ± 0.84 μg/mL, $P = 0.07$; respectively; farm B, Table 1.2). There was an interaction between time × RP in serum α-tocopherol ($P<0.0001$; farm B, Figure 1.2) with a greater concentration in RP group at d-7 and lower at d+14. In addition, retinol was reduced (32.43 vs 38.25 ± 1.37 μg/mL, $P < 0.0001$; farm B, Table 1.2) in RP cows, and tended to be reduced in calves serum retinol (1540.5 vs 1739.9 ± 98.9 ng/mL, $P = 0.07$; farm B, Table 1.3). In addition, lame cows in farm B showed a positive association with calves in serum retinol (d-7), and α-tocopherol (d+7 and d+14; Table 1.4). Serum β-carotene in calves had a negative association with mastitis (d-21) and RP cows (d-7; farm B; Table 1.4). In a previous study, concentration of α-tocopherol, β-carotene, and retinol in serum was lower during the early lactation period compared with that of prepartum periods (Strickland et al., 2021). In line with previous studies (Strickland et al., 2021), we report similar findings of decreasing α-tocopherol, β-carotene, and retinol concentration in serum at calving time compared with prepartum period

(Table 1.1). LeBlanc et al. (2004) observed that mastitic cow had a lower serum retinol at d -11, -9, -3, -1, and +5, lower serum β-carotene at d-11, -9, -7, -3, and -1, lower serum α-tocopherol at d-11 relative to parturition (d1). In other research, plasma α-tocopherol reduced in IMI cows at eight weeks of postpartum (Rezamand et al., 2007). Alpha-tocopherol and β-carotene may play a role as antioxidants toward anti-inflammatory in diseased and periparturient cows subsequently had lower concentration in serum. In the current study, cows with mastitis and their newborn calves had a greater serum retinol concentration (farm A; Figure 1.1 and Table 1.3), and serum α-tocopherol and β-carotene were lower in mastitic cows (farm B; Table 1.2) in the present study. Vitamin A concentration was related to the mammary gland epithelial cell proliferation and apoptosis. In addition, supplementation of vitamin A increased numbers of apoptotic epithelial cells at d 1 of dry period in dairy cows (Puvogel et al., 2005). An *in vitro* study demonstrated that antioxidants effects of retinol and α-tocopherol reduced reactive oxygen species, which was stimulated by ochratoxin A response in bovine mammary epithelial cell lines (BME-UV1; Baldi et al., 2004). Supplementation of vitamin E significantly decreased milk SCC at 112 DIM (Batra et al., 1992), and was negatively related to clinical mastitis (Weiss et al., 1990). This may indicate that greater serum concentration of α-tocopherol, β-carotene, and retinol in cows may have related with inflammation. Increased secretion of retinol in colostrum may have resulted from this that subsequently improve retinol in colostrum

and elevated serum retinol in calves. A similar relationship was also found at farm B regarding serum retinol, where cows with RP and their respective calves had a lower level of serum retinol (Table 1.2 and 1.3). In a previous study, cows with RP had lower serum retinol at d+5, lower β-carotene at d-13, +5, and +7. In the present study, serum α-tocopherol level was greater at d-7 and was decreased at d+14 relative to parturition in RP cows (Figure 1.2). Others (LeBlanc et al., 2004) showed that as cows had lower α-tocopherol at d-11, -7, -1, and +5 relative to parturition (LeBlanc et al., 2004). In another study, injection of α-tocopherol (+Se) at prepartum reduced incidence of RP (Julien et al., 1976; Harrison et al., 1984). A recent meta-analysis showed that receiving vitamin E reduced RP cases by 6.1% (Moghimi-Kandelousi et al., 2020).. In general, we can conclude that mobilization of serum retinol, α-tocopherol, and β-carotene might have been related to disease status of cows, which affected serum lipid-soluble vitamins in calves.

IgG concentration

A total 230 samples from farm A and B were used in a rapid test of IgG test score in serum during periparturient period (D2Dx™ immunity test kits). A greater ratio of IgG in test score meant that diseased cows had a lower IgG1/IgG2 ratio only on d+14 of postpartum compared with healthy cows ($P = 0.04$, Figure 1.3). In addition, there was a decreased in IgG test score at prepartum relative to parturition ($P = 0.001$, Figure 1.3). Based on Butler (1969) study, lacteal

and salivary secretion were predominantly IgG1, whereas IgG2 was greater in serum. According to Wellnitz et al. (2013), IMI with lipopolysaccharide increased milk IgG1 after 4 hours and IgG2 after 3 hours compared with that for control group. Mastitis not only increased IgG2 concentration in milk but also increased serum IgG2 concentration. Caffin and Poutrel, (1988) found that serum concentration of IgG2 was increased when mammary gland was infused with *Staphylococcus aureus.* In the present study, it was observed that serum IgG test score increased at d+14 after calving in diseased cows, but not in healthy group. This result may reflect that animals with health problems have more chance to increase serum IgG2 concentration and increased milk IgG2. In addition, serum IgG concentration changed during the periparturient period (Herr et al., 2011; Sasaki et al., 1976). In a previous research, total serum IgG concentration decreased from eight weeks before to one day after calving (Herr et al., 2011), while in another study it was showed that plasma IgG1 concentration declined at parturition compared with IgG2, which did not change (Sasaki et al., 1976). In line with the present study, a similar increase in IgG1/IgG2 score was observed during periparturient period (Sasaki et al., 1976). Concentration of IgG2 in serum did not change, but declining serum concentration of IgG1 increased serum IgG1/IgG2 ratio during this period (Sasaki et al., 1976).

Haptoglobin

In farm A, HP was affected by time, season, and time × season ($P < 0.0001$, Figure 1.4).

The cows had greater HP concentration at parturition compared with the other days relative to parturition (farm A, Figure 1.4). We have observed that greater HP concentration in late spring season (farm A, Figure 1.4). Serum HP concentration was lower at d+1 in pneumonia cows compared with healthy cows (0.23vs. 0.50 ± 0.12 mg/mL, $P = 0.03$; farm A Figure 1.5) and tended to be greater at d+14 of parturition in pneumonia cows ($P < 0.07$; farm A, Figure 1.5). In farm B, cows with lameness tended to have a greater HP concentration at d+14 compared with healthy cows (0.27 vs. 0.17 ± 0.04 mg/mL, $P = 0.06$; Figure 1.6), while cows with RP had greater HP at d+1 (0.44 vs. 0.31 ± 0.03 mg/mL, $P = 0.007$; Figure 1.7) compared with healthy cows. In addition, pneumonia cows had greater serum HP concentration (0.24 vs. 0.21 ± 0.01 mg/mL, $P = 0.04$; farm B, Figure 1.8). In one study, a group of calves that were inoculated with the bovine respiratory syncytial virus had the highest serum HP (8-10 mg/mL) 6-7 days after infection (Heegaard et al., 2000). These results corroborate our finding that pneumonia cows had greater serum HP concentration (farm B; Figure 1.8) and tended to have an increased HP 14 d postpartum compared with that of healthy cows (farm A; Figure 1.5). The inflammation status affected HP mobilization. Alsemgeest et al. (1994) determined variation of inflammation status between diseased cows vs. healthy cows, and chronic vs. acute. They found that HP concentration had greater values in diseased cows, chronically diseased cows compared with healthy cows (57.2 ± 7.5, 100.3 ± 14.5 mg/mL vs n.d., respectively). In the present study, lame

cows had greater HP concentration on d+14 in farm B and RP cows at d+1 in farm B (Figure 1.6 and 1.7). These infectious diseases raised HP concentration compared with that for healthy cows. A previous study showed that HP range in lame cows was from 0.37 and > 1 mg/mL (Smith et al., 2010) compared with the present study, which HP concentration was 0.27 mg/mL. Previous studies reported that RP was associated with metritis during parturition and HP concentration of more than 0.8 mg/mL was associated with increase dystocia and RP at the first week of postpartum (Dubuc et al., 2010). These findings may in part explain results of the present study in that serum HP in RP cows was greater than healthy cows. This may indicate that HP was increased because of retained fetal membrane; however, metritis incidence did not increase with proper management protocols at farms. Overall, HP can be used as a biomarker to determine pneumonia, lameness, and RP at postpartum period.

Beta-hydroxybutyrate

In farm A, a time and seasons interaction was observed ($P < 0.0001$, Figure 1.9). Serum BHB concentration had greater at parturition (d1) in late spring, fall, and winter; however, the BHB concentration had greater at d14 postpartum in early and late summer. Cows with pneumonia cows had a greater BHB in late summer (1545.6 vs. 617.0 ± 235.0 μmol/L, $P = 0.002$; Figure 1.10). In farm B, BHB was greater at d+7 and d+14 in lame cows and RP cows (Figure 1.11 and 1.13). In addition, cows with pneumonia had greater serum BHB concentration

(763.0 vs. 687.0 ± 32.9 μmol/L, P = 0.03; farmB, Figure 1.12). We observed that BHB concentration was greater in lame cows at d+7 and d+14 relative to parturition; serum BHB concentration was greater than 1100 μmol/L (Figure 1.11).A study showed BHB level in serum greater than 1100 μmol/L associated with 1.8 times increase in lameness (Suthar et al., 2013). Furthermore, RP cows had a greater BHB concentrations at d+7 and d+14 in farm B (Figure 1.13). A Improper treatment of retained fetal membrane may increase the risk of metritis in cows. Suthar et al. (2013) noted that serum BHB was 1400 μmol/L in metritis cows, while in the present study RP group had 1200 and 1130 μmol/L serum BHB on d+7 and d+14, relative to parturition. This may indicate that RP associated with BHB subsequently the elevated BHB may increase incidence of metritis. During periparturient period the cortisol level have increased, which suppresses the immune system of dairy cows (Esposito et al., 2014). Polymorphonuclear cells and leukocytes process phagocytosis and scavenge the pathogens at the cellular level; however, the neutrophil function declined at calving and remained low for several weeks (Goff, 2006). Pneumonia is an infectious disease and we found that cows with pneumonia in both farms had greater BHB level (Figure 1.10 and 1.12), especially farm A had a season effect in late summer. This may reveal that heat stress can decrease DMI and subsequently elevated BHB level in animals with pneumonia. Hyperketonemia in cows increases risk of mastitis. Zarrin et al. (2014) observed that hyperketonemia cows had higher

risk in mastitic incidence. This may indicate that those cows had NEB with increasing utilization of stored fat and generated a replacement energy causing hyperketonemia, which also increases mammary gland infection susceptibility. For this reason, hyperketonemia in dairy cows has been linked with metabolic disorders and infectious diseases.

Serum glucose of dairy calves

We determined serum glucose level in newborn calves from two farms and compared it with health status of dams. Serum glucose was greater in calves from farm B when dams had RP (P = 0.03, Figure 1.16) Serum glucose tended to decrease in newborn calves of dams with lameness and mastitis (P = 0.06 and 0.08, respectively; Figure 1.14 and 1.15). In a previous study, dystocia cows and their newborn calves had greater blood glucose (79.3 ± 2.4 mg/dL) compared with that for cows treated with oxytocin and calcium to relieve dystocia stress (Vannucchi et al., 2015). Dystocia induced stress of dams and calves, and increased fetal adrenal activity to raise corticosteroid level, which can increase portioning of fetal muscle and hepatic glycogen toward homeostasis (Vannucchi et al., 2015). Cortisol is a powerful immune suppressor, and it will raise during calving and stress. Additional cortisol stimulated by parturition and stresses can inhibit the ability of immunity on dams to recognize fetal antigen, which causes a failure to eject the fetal membranes in the uterus of cows (Mordak and Anthony,

2015). In the present study, calves from dams with RP, their serum glucose (0.91 ± 0.05 mg/mL vs 0.80 ± 0.02 mg/mL) increased. Stress in dams can increase blood glucose in newborn calves. A negative correlation between serum cortisol in dams and glucose in calves was found at postpartum (Vannucchi et al., 2015) Greater cortisol content in cows' hair was found in cows with two or more diseases (Burnett et al., 2015). Studies about serum glucose in newborn calves of lame and mastitic dams are limited. However an increased in blood cortisol in cows with NEB and diseased cows was observed (Moyes et al., 2009; Burnett et al., 2015). Hypoglycemic cows may have an increase in the incidence of lameness up to 32.14% (Mazreku et al., 2012). IMI with lipopolysaccharide can reduce cows' blood glucose after 270 min post-infection (Waldron et al., 2006). In contrast to the present results, serum glucose in calves from mastitic and lame dams tended to be lower compared with calves of healthy dams. These indicate that serum glucose in calves of diseased dams may affect calves' health and subsequent growth and performance.

Milk fatty acid composition

We are randomly a selected total of 230 samples of milk samples from diseased and healthy cows of both farms to determine milk FA composition. We did not find a significant difference between diseased and healthy cows, but we found farm effect on milk FA composition between two farms (Table 1.5). The C11:0, C15:0, C17:0, C17:1, C18:1 trans, C18:2 trans, C18:3 n6,

C18:3 n3, C20:1, C22:0, C20:3 n3, total N3, and total SFA were greater in milk fatty acid profile (g/100g) in farm A than farm B (Table 1.5 and 1.6). The C14:1, C18:1 cis, C20:0, C20 3: n6, C22:1, total MUFA, total N6, and N6:N3 ratio were greater in milk fatty acid profile (g/100g) in farm B (Table 1.5 and 1.6). Previous studies demonstrated that diseases can affect milk FA composition in cows. Randolph and Erwin (1974) showed mastitic cows' milk had greater short-chain FA (C4-C12) and lower long-chain FA (C16 and C18). Besides, SCC level > 1×10^5 cells/ mL reduced *de novo* synthesis and increased biohydrogenation activities in milk (Turini et al., 2020). In the present study, we did not observe an effect of disease on milk fatty acid profile. This may have happened because diagnosing mastitis and lameness during lactation period was not matched with the time that we took milk samples. Milk samples were obtained one time within 1-4 days after parturition, but our health records continued up to 90 days after parturition, thus the diseases may not directly relate to milk FA composition. Milk composition difference between farms may be influenced by dietary effects. This has been demonstrated when increasing supplementation of fish oil to diet reduced ratio of C4:0 to C18:0 and C18:1 cis in milk FA and increased C18:1 trans, C 18:2 trans, and C20:5 n3 and C22:6 n3 (Shingfield et al., 2006). Another study of dietary supplementing trans FA showed decreasing C16:0 and increased C20:0, MUFA, and milk unsaturated FA profile compared with dietary SFA group (Watts et al., 2013). We did not observe any effect of diseases on milk FA

composition, but different farms implement different ingredients in ration can change milk FA composition.

CONCLUSION

Results from the present study support the notion that disease records obtained during periparturient period are related to serum lipid-soluble vitamin, HP, BHB, IgG in cows. Mastitic cows had greater serum retinol in farm A. In farm B, we observed that serum α-tocopherol and retinol were lower in pneumonia and lame cows as compared with healthy cows. However, cows with mastitis had a lower serum α-tocopherol and β-carotene (farm B). Cows with retained placenta had a lower serum retinol concentration (farm B) as compared with that for healthy cows. In addition, serum HP and BHB were greater in pneumonia cows, lame cows (d+14), and RP cows (d+1, d+7, and d+14) compared with healthy cows. Immunoglobulin G test score was greater in diseased cows at d+14 compared with that for healthy cows. We did not observe that diseased cows had a different milk fatty acid composition compared with that for healthy cows, while milk samples obtained from different locations had different milk fatty acid composition. Additionally, different seasons affected cows' serum lipid-soluble vitamin, BHB, and HP concentration. Haptoglobin was greater in late spring and BHB was greater in late summer in pneumonia cows as compared with those for healthy cows. Retinol and β-carotene were greater in late spring during periparturient period and α-tocopherol was greater in winter postpartum. Furthermore, calves born from diseased dams had different serum glucose and lipid-soluble vitamin concentration as compared with those for calves born from healthy dams. Calves born

from cows that developed RP had a greater serum glucose; however, calves that were born from lame and mastitic cows tended to reduce serum glucose. Besides, mastitic cows showed a negative association with calves' retinol and β-carotene at d+14 while pneumonia cows had a negative association with calves' retinol (d-21 and d-7), α-tocopherol (d+1 and d+14), and β-carotene (d-7 and d+14). These events lead to disruption in nutritional homeostasis in newborn calves and subsequently affect their health and growth performance in the future. To improve animal health status during periparturient period, one can implement proper management by supplementing adequate nutrients in dairy farms. Transition dairy cows are susceptible to infectious diseases and metabolic disorders. Thus, a better understanding of the interactions between dairy cattle nutrition and diseases can lead to more effective management to control diseases during periparturient period. We may be able to use lipid-soluble vitamins, BHB, HP, IgG, and glucose as biomarkers to predict and determine diseases at postpartum to reduce dairy farms' expenditure. Additionally, improvement of nutritional strategies can enhance adequate nutrients in circulation and further adjust prominent roles in ration formulation to improve animal health. The findings of the present study demonstrate that biomarkers may associate with some diseased cows and newborn calves during periparturient period. However, there may not be a simple threshold to predict and prevent diseases. More complex investigations such as cellular and physiological level between biomarkers fluctuation and diseased status are required

further clarification. Further research is warranted to elucidate the mechanistic relationship between nutrient metabolites and diseases in dairy farms. We can also continue to determine serum fatty acid composition and milk lipid-soluble vitamin to gain a better understanding of these biomarkers' fluctuation in serum and milk with diseases during periparturient period. Research can provide an overall picture of how serum metabolites in cows and newborn calves in the Pacific Northwestern region affect and are affected in diseased dairy cows during the periparturient period.

TABLES

Table 1.1 Mean concentration of serum α-tocopherol, β-carotene, and retinol (μg/mL) obtained from Holstein dairy cows in different seasons and times relatives to parturition (d1) (Farm A; n = 645).

Farm A	Time					SEM	*P*-value		
α-tocopherol	-21	-7	1	7	14		Time (T)	Season (S)	T × S
Late Spring	6.96	5.28	2.97	5.00	7.10	0.53	<0.0001	<0.0001	<0.0001
Early Summer	5.81	5.37	3.93	5.68	6.64	0.28	<0.0001	<0.0001	<0.0001
Late Summer	4.97	4.74	3.07	4.75	5.93	0.30	<0.0001	<0.0001	<0.0001
Fall	5.13	4.67	3.02	4.42	6.13	0.22	<0.0001	<0.0001	<0.0001
Winter	6.95	5.83	3.93	5.87	8.35	0.25	<0.0001	<0.0001	<0.0001
β-carotene									
Late Spring	27.71	13.80	8.56	5.46	5.00	0.54	<0.0001	<0.0001	<0.0001
Early Summer	13.96	9.25	6.67	8.52	8.41	0.37	<0.0001	<0.0001	<0.0001
Late Summer	9.83	8.19	5.78	5.12	4.55	0.38	<0.0001	<0.0001	<0.0001
Fall	6.66	7.72	5.58	5.56	6.87	0.29	<0.0001	<0.0001	<0.0001
Winter	11.00	7.18	5.31	6.58	8.58	0.32	<0.0001	<0.0001	<0.0001
Retinol									
Late Spring	55.13	39.69	16.30	26.43	42.06	3.0	<0.0001	<0.0001	<0.0001
Early Summer	42.09	34.41	23.80	29.67	33.92	1.6	<0.0001	<0.0001	<0.0001
Late Summer	31.15	24.40	14.22	25.00	31.15	4.4	<0.0001	<0.0001	<0.0001
Fall	28.85	26.31	14.94	25.32	34.03	1.3	<0.0001	<0.0001	<0.0001
Winter	43.18	35.55	21.38	32.25	39.30	1.4	<0.0001	<0.0001	<0.0001

Table 1.2 Mean concentration of serum α-tocopherol, β-carotene, and retinol (μg/mL) obtained from Holstein dairy cows in all samples (Farm B).

Farm B	Healthy (n = 470)	Pneumonia (n = 89)	SEM	*P* value
α-tocopherol	8.45	7.59	0.26	0.003
Retinol	38.49	33.85	1.01	<0.0001
	Healthy (n = 531)	Lameness (n = 28)		
α-tocopherol	8.37	7.39	0.47	0.05
Retinol	37.98	33.51	1.83	0.02
	Healthy (n = 508)	Mastitis (n = 51)		
α-tocopherol	8.37	7.73	0.36	0.08
β-carotene	20.90	19.33	0.84	0.07
	Healthy (n = 510)	RP (n = 49)		
Retinol	38.25	32.43	1.37	<0.0001

Table 1.3 Mean concentration of serum α-tocopherol, β-carotene, and retinol (ng/mL) obtained from Holstein calves with either diseased or healthy status of their dams.

		Healthy (n = 606)	Mastitis (n = 39)	SEM	*P* value
Farm A	Retinol	2419.85	2766.08	191.3	0.08
		Healthy (n = 634)	Pneumonia (n = 11)		
	Retinol	2463.6	1893.21	199.0	0.005
Farm B		Healthy (n = 510)	RP (n = 49)		
	Retinol	1739.9	1540.5	98.9	0.07

Table 1.4 Association in serum retinol, α-tocopherol, and β-carotene in diseased cows and their newborn calves during periparturient period

Farm A		-21	-7	1	7	14
Retinol	Lameness	0.03	-0.08	0.03	-0.11	-0.06
	Mastitis	-0.17	-0.14	-0.27	-0.08	-0.41
	Pneumonia	-0.61	-0.50	-0.04	0.30	-0.14
α-tocopherol	Lameness	0.08	-0.05	-0.10	-0.05	-0.16
	Mastitis	-0.32	0.46	-0.02	0.12	0.02
	Pneumonia	0.14	-0.12	-0.35	-0.13	-0.26
β-carotene	Lameness	-0.13	0.07	0.06	0.05	-0.25
	Mastitis	-0.35	0.27	0.30	0.04	-0.51
	Pneumonia	-0.25	-0.55	-0.07	-0.06	-0.77
Farm B						
Retinol	Lameness	0.22	0.48	0.22	0.24	0.39
	Mastitis	-0.17	-0.23	0.003	-0.22	-0.20
	Pneumonia	0.04	0.03	0.03	0.02	0.12
	Retained Placenta	-0.04	-0.18	-0.08	0.06	0.15
α-tocopherol	Lameness	0.18	-0.35	-0.04	0.52	0.46
	Mastitis	-0.23	0.34	-0.28	-0.01	0.04
	Pneumonia	0.12	-0.03	0.22	0.18	0.05
	Retained Placenta	0.19	-0.39	-0.03	0.07	0.07
β-carotene	Lameness	-0.31	-0.29	-0.32	-0.20	-0.15
	Mastitis	-0.52	0.10	0.06	0.15	0.36
	Pneumonia	-0.03	0.14	0.03	-0.01	0.05
	Retained Placenta	0.08	-0.73	0.20	-0.18	-0.06

Table 1.5 Fatty acid (g/100 g of fatty acid methyl ester) composition of milk (1-3 DIM) obtained from Pacific Northwestern dairy farms with health status (H: healthy; D: diseases; Farm A: healthy: n = 65, diseases: n= 65; Farm B: healthy: n = 50, diseases: n= 50)

Fatty acid, g/100 g	TRT		Farm		SEM	*P*-Value		
	H	D	A	B		TRT	Farm	TRT × Farm
C11:0	1.55	1.41	1.80	1.16	0.11	0.32	<.0001	0.10
C12:0	3.66	3.55	3.66	3.56	0.07	0.22	0.29	0.67
C13:0	0.82	0.80	0.87	0.75	0.06	0.86	0.17	0.74
C14:0	12.51	12.16	12.39	12.28	0.20	0.21	0.70	0.73
C14:1	4.66	4.37	3.24	5.80	0.17	0.19	<.0001	0.87
C15:0	1.53	1.64	1.88	1.29	0.09	0.34	<.0001	0.67
C15:1	0.07	0.06	0.06	0.07	0.01	0.35	0.39	0.41
C16:0	28.15	28.66	28.62	28.20	0.26	0.14	0.22	0.48
C16:1	2.28	2.34	2.50	2.12	0.09	0.59	0.001	0.16
C17:0	0.24	0.26	0.36	0.13	0.01	0.25	<.0001	0.63
C17:1	0.15	0.16	0.22	0.10	0.01	0.44	<.0001	0.40
C18:0	11.82	11.46	11.86	11.42	0.18	0.13	0.07	0.42
C18:1 TRANS	0.53	0.47	0.89	0.11	0.09	0.61	<.0001	0.59
C18:1 CIS	29.21	29.84	28.79	30.26	0.32	0.13	0.0005	0.92
C18:2 TRANS	0.06	0.06	0.10	0.01	0.01	0.96	<.0001	0.60
C18:2 CIS	2.37	2.28	2.27	2.38	0.05	0.13	0.08	0.84
C18:3 N6	0.01	0.01	0.02	0.00	0.001	0.996	<.0001	0.78
C20:0	0.004	0.003	0.002	0.004	0.001	0.50	0.04	0.31
C18:3 N3	0.07	0.08	0.09	0.06	0.004	0.18	<.0001	0.60
C20:1	0.03	0.03	0.06	0.00	0.002	0.81	<.0001	0.81
C21:0	0.002	0.003	0.002	0.003	0.002	0.64	0.46	0.19
C20:2	0.001	0.008	0.001	0.008	0.005	0.25	0.25	0.15
C22:0	0.07	0.05	0.10	0.01	0.01	0.40	<.0001	0.79
C20:3 N6	0.10	0.08	0.01	0.17	0.02	0.49	<.0001	0.56
C20:3 N3	0.03	0.03	0.07	0.002	0.01	0.89	<.0001	0.97
C22:1	0.05	0.06	0.02	0.08	0.01	0.76	<.0001	0.12
C20:4	0.00	0.001	0.00	0.001	0.001	0.21	0.21	0.21
C23:0	0.007	0.09	0.10	0.001	0.08	0.39	0.33	0.39
C20:5	0.01	0.01	0.01	0.02	0.002	0.87	0.08	0.79
C24:0	0.001	0.000	0.002	0.000	0.000	0.29	0.03	0.29
C22:6	0.007	0.008	0.006	0.009	0.002	0.55	0.25	0.12

Table 1.6 Summary of fatty acid (g/100 g of fatty acid methyl ester) composition of milk obtained from Pacific Northwestern dairy farms with health status (H: healthy; D: diseases; Farm A: healthy: n = 65, diseases: n= 65; Farm B: healthy: n = 50, diseases: n= 50)

Fatty acid, g/100 g	TRT		Farm		SEM	*P*-Value		
	H	D	A	B		TRT	Farm	TRT × Farm
Σ MUFA	36.98	37.34	35.79	38.53	0.34	0.43	<.0001	0.80
Σ N6	2.54	2.43	2.40	2.57	0.05	0.13	0.01	0.92
Σ N3	0.13	0.14	0.18	0.09	0.01	0.42	<.0001	0.66
N6:N3	24.39	22.14	15.72	30.80	1.41	0.23	<.0001	0.51
Σ PUFA	2.66	2.57	2.57	2.66	0.05	0.18	0.26	0.99
De novo	16.17	15.71	16.05	15.84	0.23	0.13	0.50	0.86
Σ Saturated	60.35	60.10	61.64	58.81	0.33	0.56	<.0001	0.80

MUFA: Monounsaturated fatty acid. N6: omega-6 fatty acid. N3: omega-3 fatty acid. PUFA: Polyunsaturated fatty acid.

FIGURES

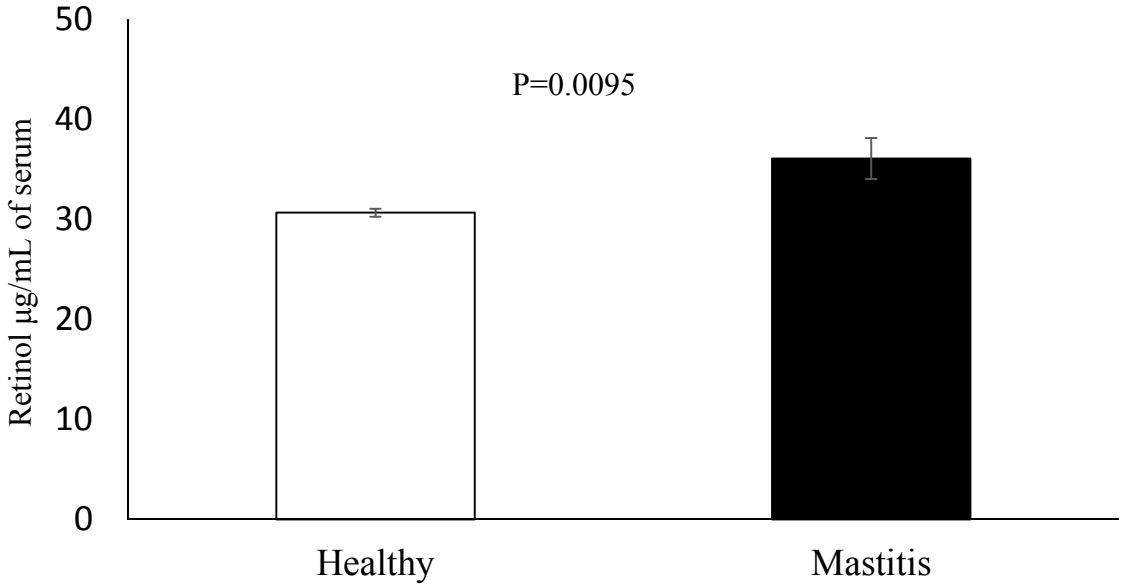


Figure 1.1 Mean concentration of serum retinol (µg/mL) obtained from Holstein dairy cows with either mastitis or healthy status (n= 39 vs 606, respectively; Farm A).

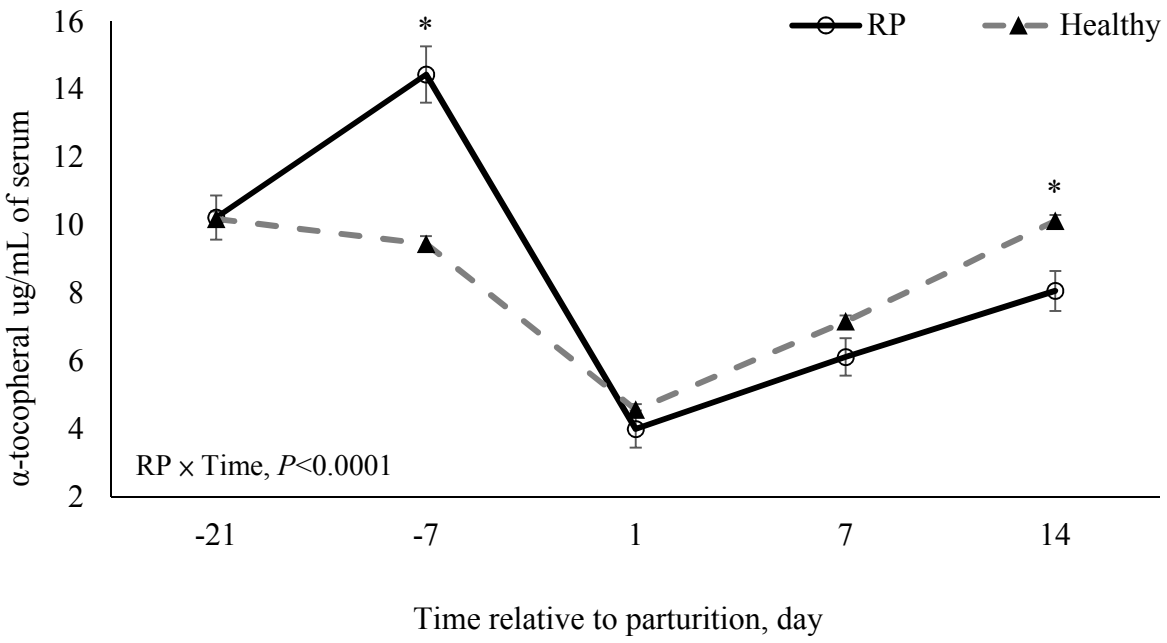


Figure 1.2 Mean concentration of serum α-tocopherol (μg/mL) obtained from Holstein dairy cows with either the retained placenta or healthy status (n= 49 vs 510, respectively; Farm B).

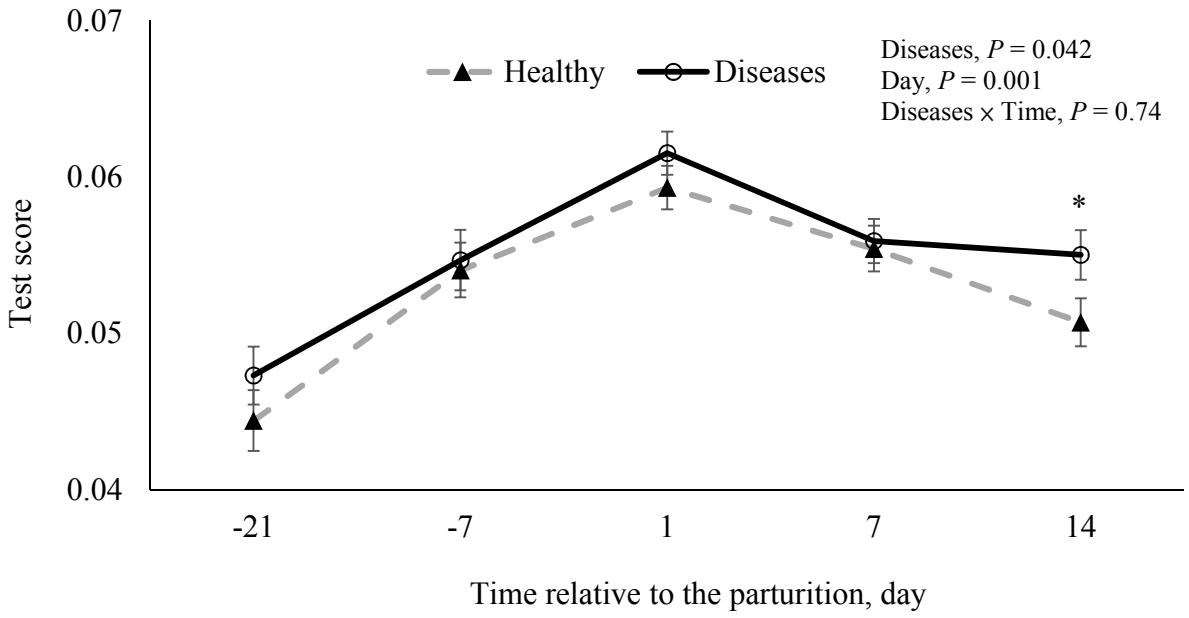


Figure 1.3 D2Dx™ test response of 230 cows the IgG1/IgG2 test score[1] of healthy (n =115) and diseases (n = 115) groups over days relative to parturition (diseases P = 0.042; day P = 0.001)

[1]Test score inversely relates to the IgG1/IgG2 ratio; the greater test score represents a lower IgG1/IgG2 ratio, and the lower test score represents a greater IgG1/IgG2 ratio.

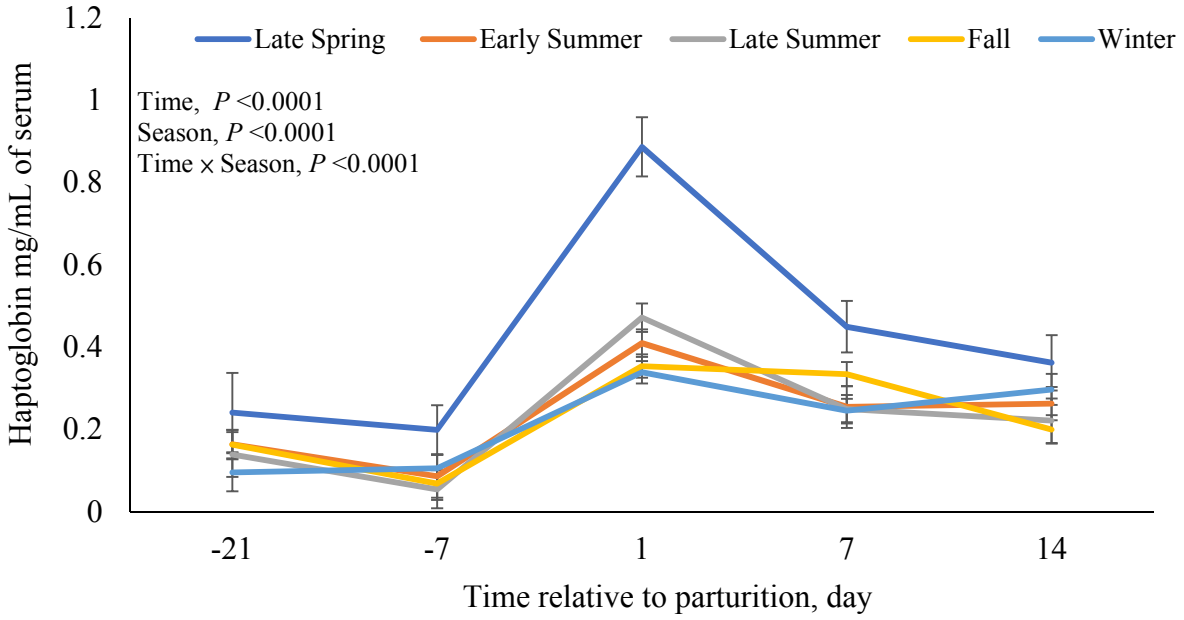


Figure 1.4 Mean concentration of haptoglobin from Holstein dairy cows (Farm A) relative to sampling seasons and days relative to parturition (late spring, n = 42; early summer, n = 129; late summer, n = 119; fall, n =174; winter, n = 181, respectively).

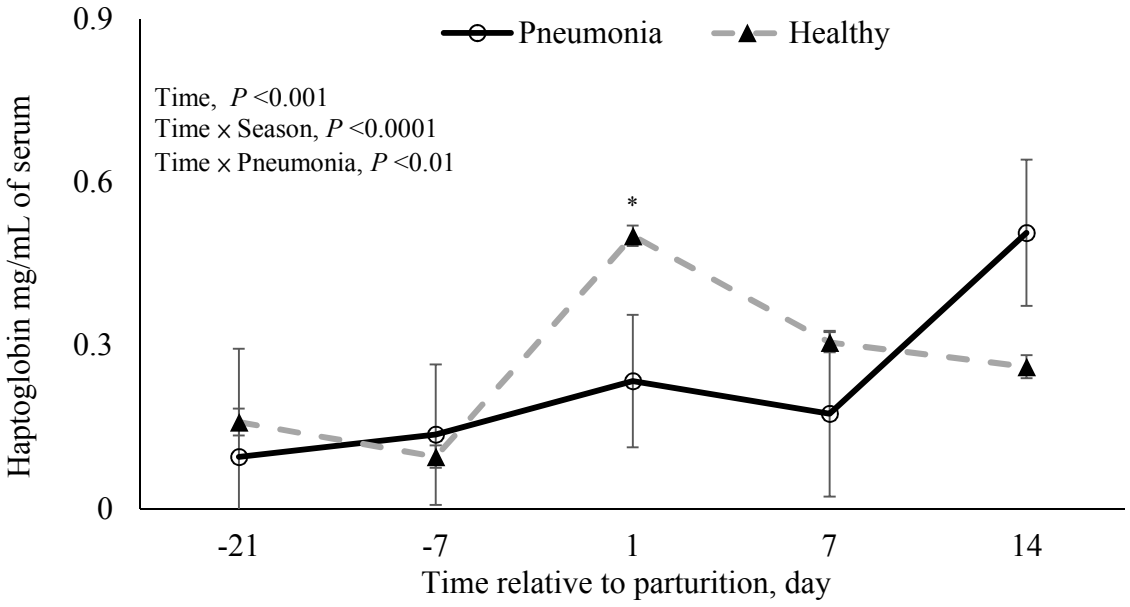


Figure 1.5 Mean concentration of haptoglobin from Holstein dairy cows (Farm A) with pneumonia (n=11).

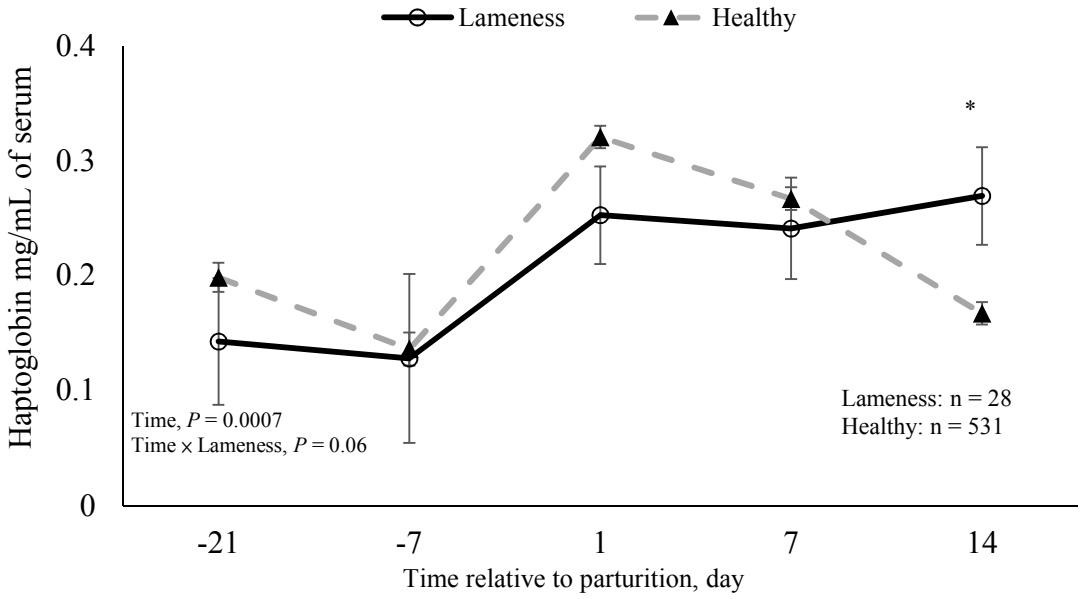


Figure 1.6 Mean concentration of haptoglobin (mg/mL) from Holstein dairy cows (Farm B) with lameness and days relative to parturition.

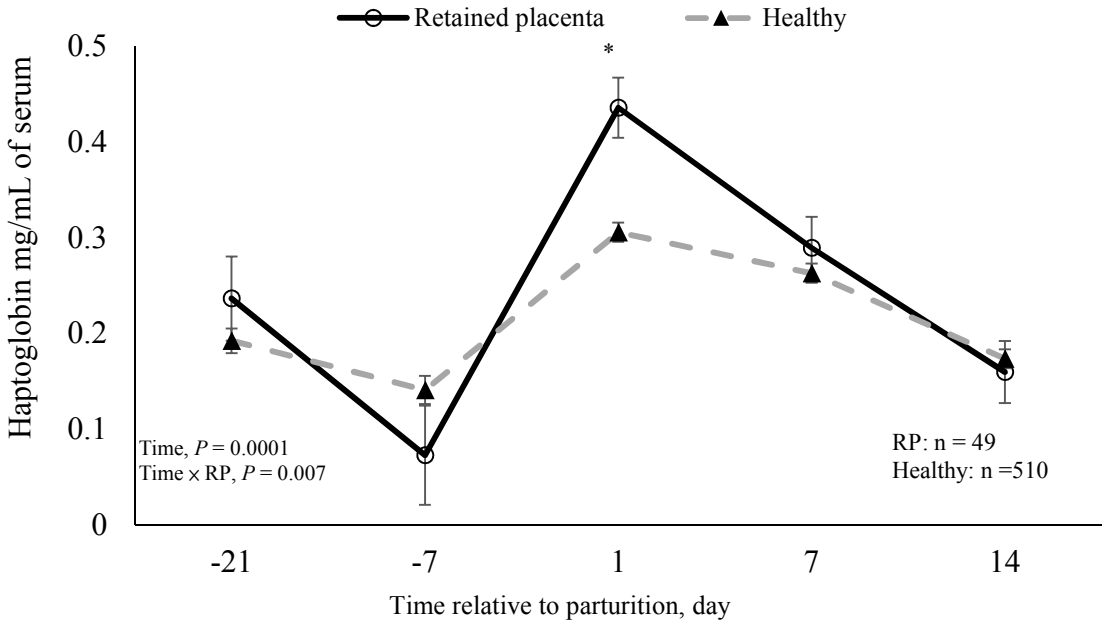


Figure 1.7 Mean concentration of haptoglobin (mg/mL) from Holstein dairy cows (Farm B) with retained placenta and days relative to parturition.

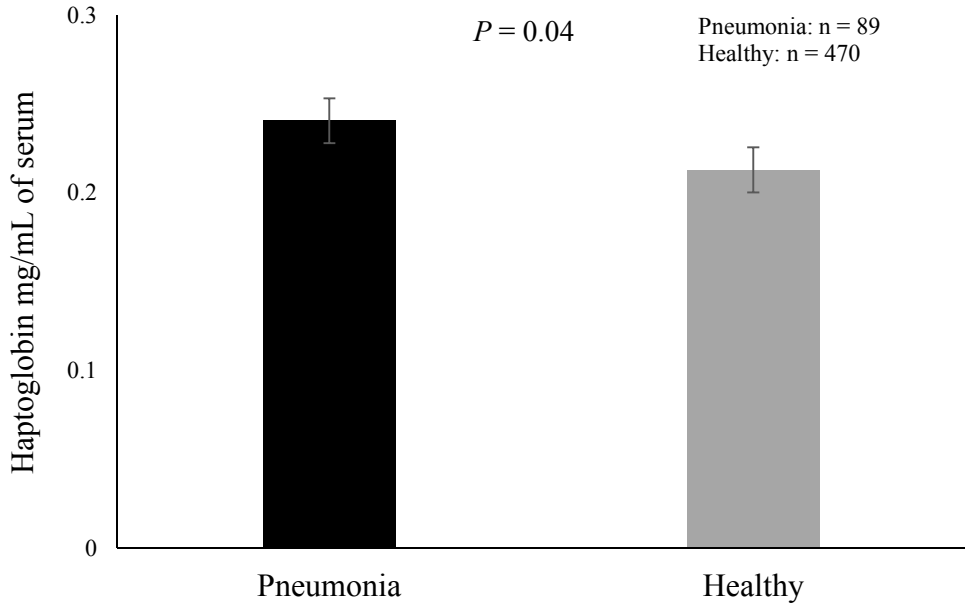


Figure 1.8 Mean concentration of haptoglobin (mg/mL) from Holstein dairy cows (Farm B) with pneumonia vs. healthy cows.

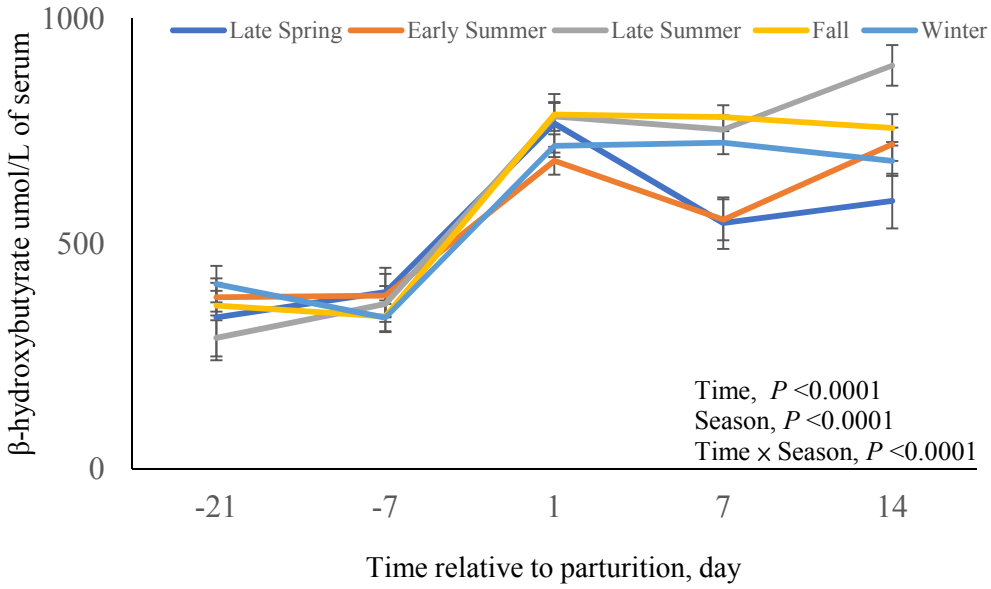


Figure 1.9 Mean concentration of β-hydroxybutyrate (umol/L) from Holstein dairy cows (Farm A) relative to sampling seasons and days relative to parturition (late spring, n = 42; early summer, n = 129; late summer, n = 119; fall, n =174; winter, n = 181, respectively).

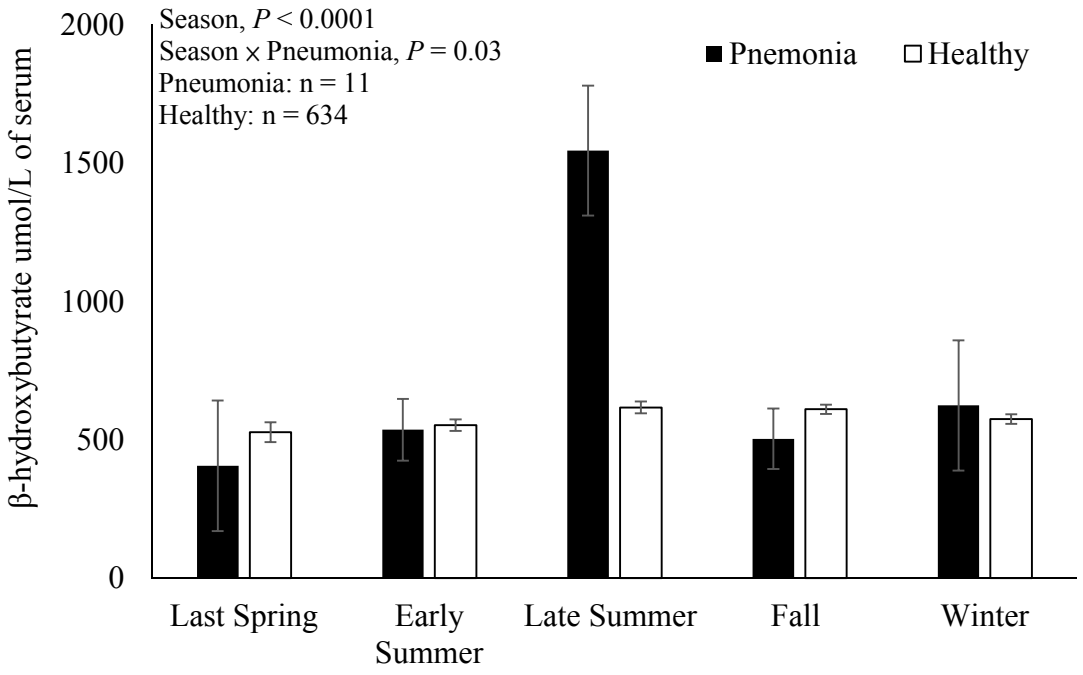


Figure 1.10 Mean concentration of β-hydroxybutyrate (umol/L) from Holstein dairy cows (Farm A) with pneumonia in different seasons (late spring n = 42, early summer n = 129, late summer n = 119, fall n =174, and winter n = 181)..

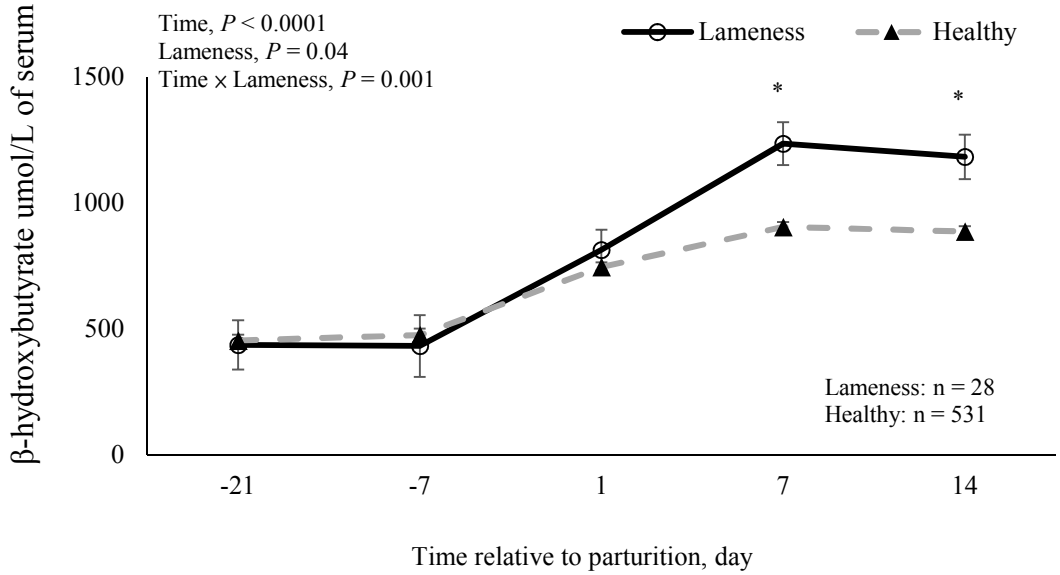


Figure 1.11 Mean concentration of β-hydroxybutyrate (umol/L) from Holstein dairy cows (Farm B) with lameness and days relative to parturition.

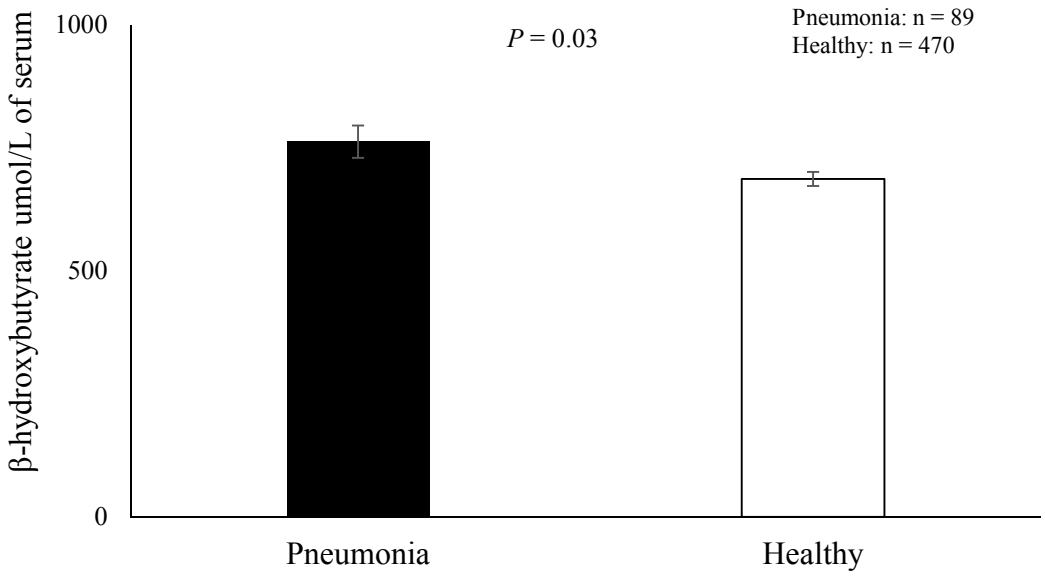


Figure 1.12 Mean concentration of β-hydroxybutyrate (umol/L) from Holstein dairy cows (Farm B) with pneumonia.

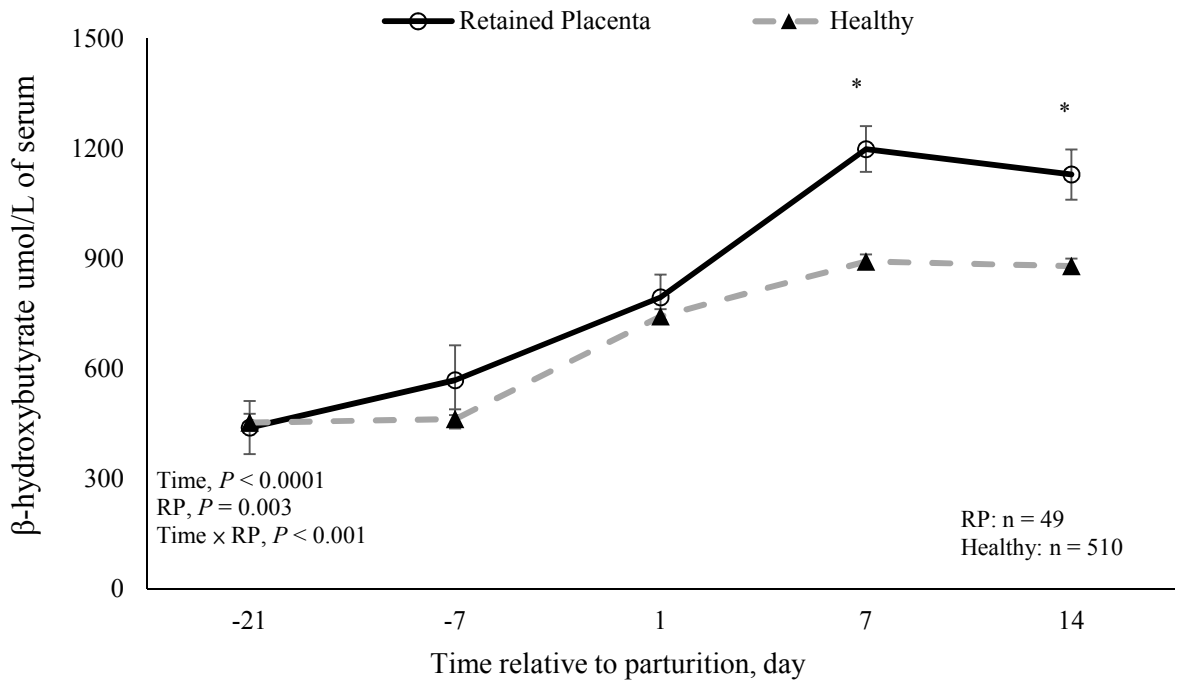


Figure 1.13 Mean concentration of β-hydroxybutyrate (umol/L) from Holstein dairy cows (Farm B) with retained placenta and days relative to parturition.

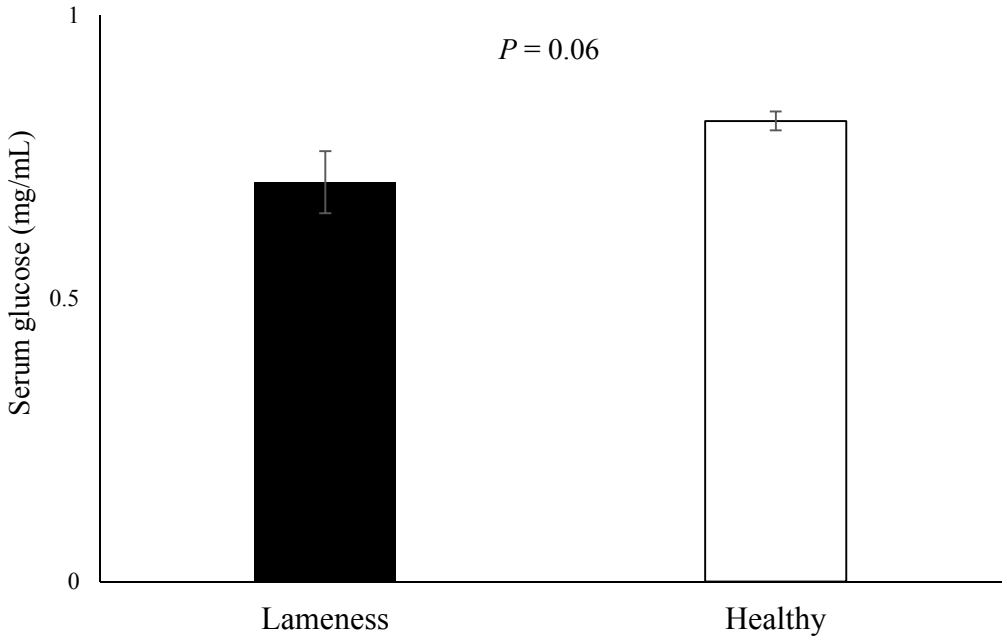


Figure 1.14 Serum glucose concentration (mg/mL) in calves (Farm B) from lameness dams (n= 28).

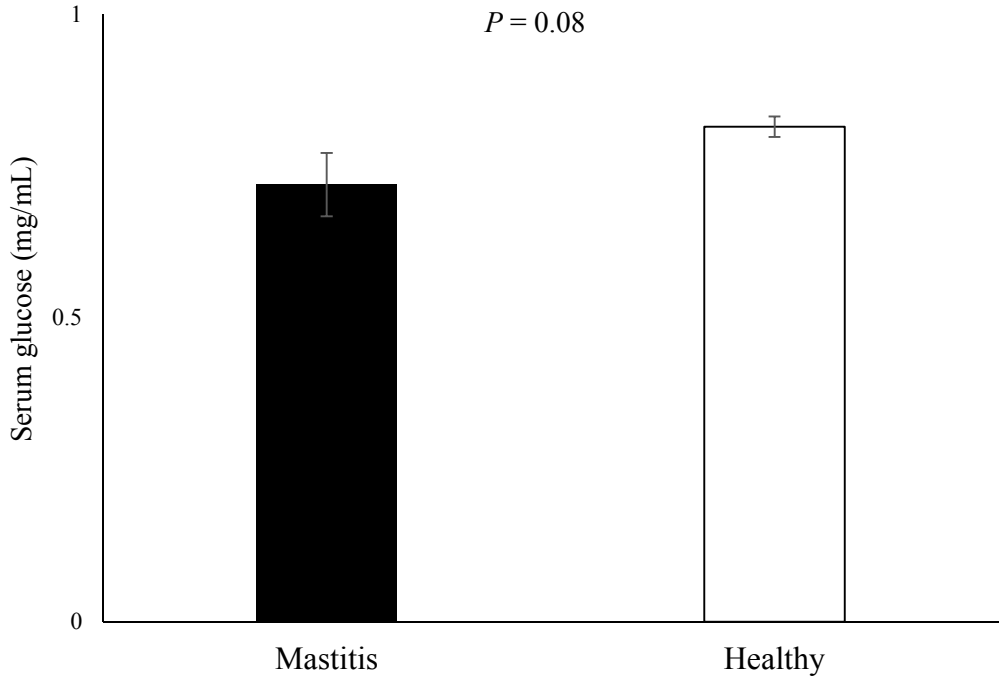


Figure 1.15 Serum glucose concentration (mg/mL) in calves (Farm B) from mastitic dams (n= 51).

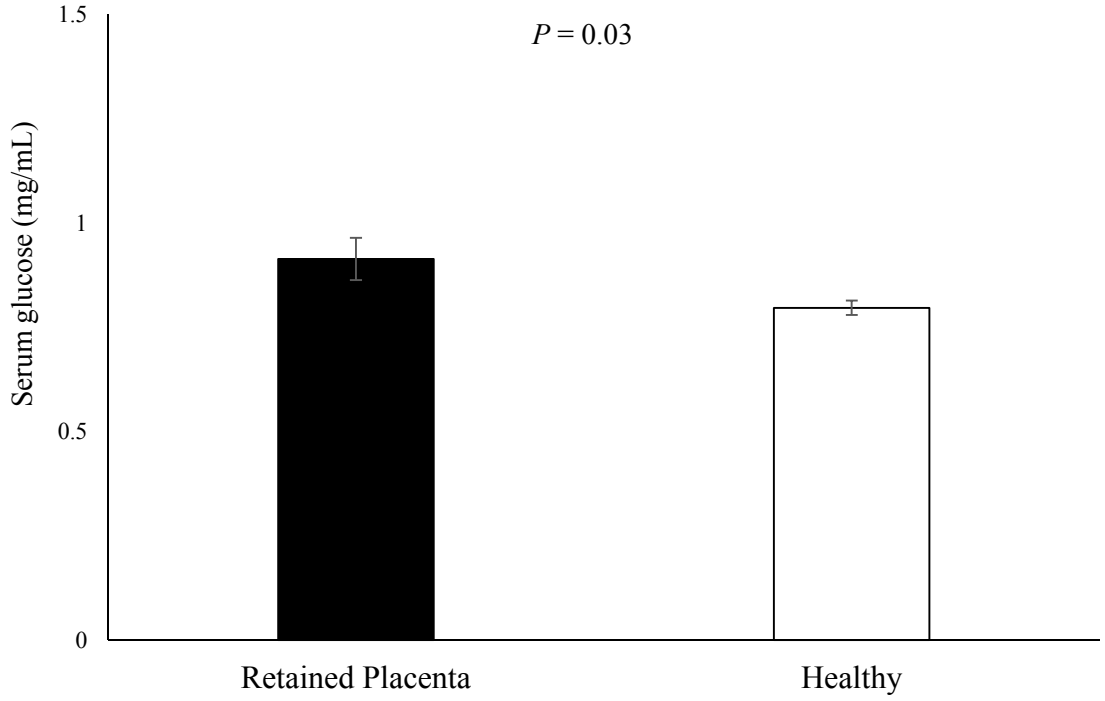


Figure 1.16 Serum glucose concentration (mg/mL) in calves (Farm B) from retained placenta dams (n = 49).

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Chapter 2

A rapid blood test to monitor the immune status change of dairy cows and to evaluate their disease risk during the periparturient period

"A rapid blood test to monitor the immune status change of dairy cows and to evaluate their disease risk during the periparturient period" *Sensors International*, vol 2, 2021, pp 1-6.

Abstract

Dairy cows are at the highest risk of developing clinical and subclinical diseases and disorders in the first few weeks following parturition. During the periparturient period, from approximately 30 days before calving to 30 days postpartum, the immune system of the dairy cows undergoes a multitude of changes to prepare for parturition, colostrum production, and lactation. One such change is the transfer of a large amount of immunoglobulin G proteins, especially subclass IgG1 to colostrum, leading to a reduction of IgG1 in the blood. As IgG1 and IgG2 need to maintain a balance to protect animals from intracellular and extracellular pathogens, a disruption of this balance compromises the immune protection of the animals. Rapid tests that can detect these immunological changes may be potentially useful for predicting the risk of dairy cows developing infectious diseases and other adverse health conditions following parturition. The objective of this study was to determine a new rapid test to detect certain immune status changes in the blood serum of dairy cows. This test uses a nanoparticle probe to evaluate the relative quantity of IgG1 and IgG2 in a sample. The nanoparticle probes are aggregated together upon interaction with bovine IgG2, while bovine IgG1 inhibits such interactions. The nanoparticle aggregates are detected by monitoring the color change of the assay solution using a handheld device. We tested the serum samples from 230 dairy cows collected during the periparturient period, from 14-7 days before calving to 7-

14 days postpartum. Results show that the test detected an immune status change associated with IgG1/IgG2 relative quantity change around the time of parturition. Data analysis using the mixed linear model in SAS (Statistical Analysis System) revealed a difference (*P*-value = 0.042) in their test responses between healthy cows and cows with mastitis and/or lameness. The new rapid test we report here can be used to detect and monitor certain immune status changes in dairy cows during the periparturient period. The test results may be potentially used to evaluate and predict the health risk of the dairy cows following parturition.

Keywords: Dairy cows, periparturient period, rapid test, mastitis, IgG

Background

Dairy cows are at the highest risk of developing clinical and subclinical diseases and disorders in the first few weeks postpartum [1-4]. It is believed that more than 50% of cows suffer from at least one or more disorders during this period [1-4]. Mastitis, lameness, retained placenta, metritis, ketosis, and hypocalcemia are among the most common diseases in dairy cattle. The median prevalence of clinical mastitis is around 20-25 cases per 100 cows per year [5]. In some countries and regions, the overall mastitis incidence in cow can be as high as 60% or more [6]. Milk production loss in combination with treatment costs and culling related to mastitis constitute approximately $116-$325 /case [7]. Lameness is a condition associated with lesions of the hind limb, which is caused by pathogens invading the hooves. Research shows that lameness in dairy cows adversely affects milk production, reproductive performance, longevity, and the general well-being of the animal. The prevalence of lameness among most dairy herds varies from 20-40% [8, 9]. Lameness is the third largest cause of economic loss in the dairy cattle industry, after mastitis, and reproductive disorders.

Around the time of calving, the body of a dairy cow goes through makes many biological and physiological changes including adjustments to prepare for calving, colostrum production, and lactation [2, 10, 11]. The immune system of the dairy cow undergoes numerous changes to help the cow successfully deliver and protect the cow from potential infections after calving.

One such change is the transfer of immunoglobulin G proteins, especially subclass IgG1, to the mammary gland for colostrum synthesis and secretion, leading to a reduction of IgG1 in the maternal blood [12-14]. As the level of IgG1 and IgG2 should maintain an optimal balance to protect animals from intracellular and extracellular pathogens, a disruption of this balance compromises the immune protection of the periparturient dairy cows [12-14].

Another change related to the immune system around the time of parturition is the elevated inflammatory responses during the periparturient period [15]. Inflammation is the first immune response of an organism when facing a microbial infection or a tissue injury. Studies have shown that a certain degree of inflammation is necessary to initiate calving, assist placental expulsion and protect the dams from postpartum microbial invasion [16]. However, the excessive and persistent postpartum pro-inflammatory response has been linked to increased disease risk and decreased milk production [17, 18]. Metabolic changes accompanied by a pro-inflammatory state alter the homeostasis of the immune system, exposing dairy cows to increased disease risk in early lactation [19].

Considering the critical role of immune functions in the overall health, production, and reproduction performance of dairy cows, simple and convenient tests that can allow rapid evaluation of the immune status and conditions of dairy cows will provide a valuable tool for dairy farm management. Although laboratory tools and tests are available to measure and

evaluate different cellular and molecular components of the immune system, these are typically not suitable for on-site farm testing. Here we report the use of a newly developed, D2Dx™ immunity test, to monitor the immune status change in dairy cows during the transition period [20, 21]. This is a single-step test requiring only the mixing of a testing reagent with a small volume of un-diluted and un-treated blood serum or plasma providing a test result within 30 seconds. This quick test can be conveniently performed on-site or in-house at small local veterinary clinics thereby avoiding having to send samples out for offsite processing and waiting for test results to be returned.

The principle of the test is illustrated in Figure 2.1. This test uses a gold nanoparticle probe to evaluate the relative quantity of IgG1 and IgG2 in a blood sample. The nanoparticle is designed to interact with bovine IgG1 and IgG2 through two different modes: upon interacting with IgG2, the nanoparticle forms large aggregates; with IgG1, the protein will bind to the nanoparticle surface to form a protein layer, however, will not cause nanoparticle aggregate formation. The nanoparticle aggregates are detected by monitoring the color change of the assay solution using a handheld reader device, CT-100 from Nano Discovery Inc. The color change is due to the surface plasmon resonance shift in aggregated gold nanoparticles, a phenomenon that has been well studied and established for biosensing and diagnostic test applications [22-25]. In the presence of both IgG1 and IgG2, as in the case of bovine blood samples, the two

proteins will compete to interact with the nanoparticles through the two different binding modes. The degree of color change is reflective of the relative quantities of IgG1 and IgG2 in a sample. The objective of this study was to determine a new rapid test to detect certain immune status changes in the blood serum of dairy cows.

Materials and Methods

Bovine blood sample source

This study was approved by the University of Idaho Animal Care and Use Committee (# 2017-52). Between March 2018 to May 2019, blood samples were collected from dairy cows in two commercial dairy farms located in the Pacific Northwest region of the United States. A total of 230 cows were enrolled. Blood samples were collected from each cow from coccygeal vein on day -14, -7, +1, +7, and +14, relative to parturition (d1) using BD Vacutainer® serum collection tubes (Franklin Lakes, NJ, U.S.A.). All blood samples were kept in ice cooler and stored in 4°C for 24h. The blood samples were centrifuged at 1500 × *g* for 10 min at 4°C and sera were collected into 1.5 mL centrifuge tubes and stored at -80ºC immediately. The health records were collected from dairy farms within 90 days before to 90 days after calving.

For the current study, we tested the serial draw blood samples (day -14, -7, +1, +7, +14, relative to parturition) from 230 cows randomly selected from a study pool a total of 1203 cows. Among 230 cows, half of them (115) had no documented clinical diseases (healthy group),

while the other half (115) had documented mastitis and/or lameness conditions (diseased group). Within diseased group, more than 78% of cows had mastitis. We did not select other diseased cows to enroll this study.

D2Dx™ immunity test

D2Dx™ immunity test kits (catalog D2Dx-BV-500, lot number bv05212020) were received from Nano Discovery Inc. (Orlando, Florida). Each kit contains the reagent and cuvettes for 500 tests. A handheld reader device, CT-100 from Nano Discovery Inc. was used to read the test result. To perform the test, 50 μL of testing reagent solution was first placed into the cuvette using a micropipette. Then 10 μL of bovine blood serum sample was added. After mixing the assay solution for 5 seconds using a mini-vortex mixer, the cuvette was placed in CT-100, and the result was read automatically in 30 seconds. The response of the test was reported directly as the absorbance change of the assay solution over 30 seconds of reaction time.

D2Dx™ immunity test of bovine IgG1 and IgG2 protein in pure buffer solution

We purchased purified bovine IgG1 and IgG2 from BioRad (IgG1, catalog pep003, 1 mg/mL; IgG2, catalog pep004, 1 mg/mL, Hercules, California) to illustrate and confirm the effectiveness of D2Dx™ immunity test kit (catalog D2Dx-BV-500). IgG1/IgG2 mixtures were made at the following volume ratios 5/1, 2/1, 1/1, 1/2, 1/5, and 1/10 (IgG1/IgG2, v/v). The

testing of this purified IgG1, IgG2, and IgG1/IgG2 mixture solutions were conducted using the same protocol as used for bovine serum testing as stated above. The test result was reported directly as the absorbance change of the assay solution over 30 seconds of reaction time.

ELISA assay of bovine IgG1 and IgG2 in bovine blood serum samples

We randomly selected total 39 bovine serum to determine IgG1 and IgG2 by commercial ELISA kits from Bethyl Laboratories (Montgomery, Texas), catalog E11-116 for bovine IgG1 (lot E11-116-190912), and catalog E11-117 for bovine IgG2 (lot E11-117-190814) to confirm D2Dx™ immunity test kit activity. The ELISA assay plates were measured by a SpectroStar Nano plate reader from BMG LabTech (Cary, North Carolina).

Statistical analysis

The data obtained from days relative to parturition (period) were analyzed as a repeated measures linear mixed model in SAS (V. 9.4, SAS Inst. Inc., Cary, NC, U.S.A.) with disease presence/absence and day and their interaction as fixed effects and cows as a random effect. A repeated measures association between days was additionally accounted for through a compound symmetry correlation structure. The statistical model was $Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + e_{ijk}$ where the Y_{ijk} = the response of IgG concentration, μ = overall mean, α_i = fixed effect of disease (*i* =control, disease (mastitis or lameness)), β_j = fixed effect of time, $(\alpha\beta)_{ij}$ = disease-time interaction, and e_{ijk} = residual error term assumed to be N (0, σ^2). In addition, the data

obtained from prepartum, calving, and postpartum were analyzed by Proc TTEST in SAS to compare prepartum vs. calving, prepartum vs postpartum, and calving vs. postpartum. The statistical model was: $Y_{ij} = \mu + R_i + e_{ij}$ where the μ = overall mean, Ri= fixed effect of diseases (*i* =healthy, diseased), and e_{ij} = residual error term assumed to be N (0, σ^2). The significance was declared at $P < 0.05$.

Results

Detection of bovine IgG1/IgG2 relative quantity using the D2Dx™ immunity test

The IgG1/IgG2 quantity was confirmed in D2Dx™ immunity test (Figure 2.2) Upon reaction with pure bovine IgG2, there was a clear color change of the assay solution and gave greater absorbance change. While upon mixing with bovine IgG1, there is no color change, and gave lower absorbance change. When IgG1 and IgG2 are mixed, the absorbance change increased with an increasing amount of IgG2, that is, a decreasing IgG test score.

D2Dx™ immunity test of bovine blood samples

Figures 2.3 Panel A and Panel B are the immunity test results of 230 cows on day -14, -7, +1, +7, and +14 relative to parturition (A-healthy group; B-diseased group). The model-based marginal means of the two cow groups at different days are shown in Figure 2.3 Panel C. Overall, there was the time effect on test score ($P = 0.001$): from pre-calving to calving, there is an increase in the test score; and from calving to postpartum, the test score decline from a peak

value to levels seen in pre-calving period. There is not a significant between health status and time interaction ($P = 0.73$).

All of the days relative to parturition has difference in the healthy group ($P \leq 0.01$), but diseased group was no significant difference at d7 vs d14 ($P = 0.30$). These confirmed that both healthy cows and diseased cows show similar time-dependent test score changes during the periparturient period. This can indicate that a decrease of IgG1 level in blood plasma during pre-calving period leads to a decreased IgG1/IgG2 ratio; therefore, an increased D2Dx™ immunity test score; and then a return of plasma IgG1 level following parturition leads to an increased IgG1/IgG2 ratio and, subsequently, a decreased immunity test response.

During the study period, we also observed a difference between the healthy and diseased groups in IgG test score. The diseased cow group has a greater average test score than the healthy cow ($P = 0.04$). This observation indicates that diseased cows may have a lower IgG1/IgG2 ratio than the healthy cows during the entire periparturient period.

ELISA analysis of IgG1 and IgG2 level in selected cows during the study period

To further confirm that the changes detected by the D2Dx™ immunity test were results associated with bovine IgG1/IgG2 ratio change in cows around the time of parturition, an ELISA analysis of bovine IgG1 and IgG2 in selected blood samples was conducted. Total of 39

serum samples from 13 randomly chosen cows that represent pre-calving (-14 and -7), calving (day 1), and post-calving (day 7 and 14) were analyzed. Figures 2.4 Panel A and Panel B are the results (absorbance at 450 nm) of bovine IgG1 and IgG2 analysis, respectively. From pre-calving to calving, there was a clear decrease of IgG1 level in serum, and the level raised following parturition. There was a difference between pre-calving to calving and calving to post-calving ($P < 0.0001$). These data confirmed what has already been reported by other studies [12-14]. On the other hand, the level of IgG2 remained almost unchanged from pre-calving to calving and post-calving. As a result, the ratio of IgG1/IgG2 decreased from pre-calving to calving, and then returned to pre-calving status following parturition. A lower IgG1/IgG2 ratio gives a greater D2Dx™ immunity test score. The D2Dx™ immunity test response increase observed from pre-calving to calving, and then the decline from calving to postpartum reflects this IgG1/IgG2 ratio change in the periparturient cows.

Discussions

D2Dx™ immunity test is a simple, single-step test that may be performed on-site at the farm or in local veterinary clinics. The test is also rapid; taking less than 1 minute to perform and to obtain results. As confirmed by the testing of pure bovine IgG1, IgG2, and their mixture solutions, the D2Dx™ immunity test responds to IgG1/IgG2 ratio change in pure buffer solution.

In bovine blood, the concentration of IgG1 and IgG2 is typically around 10-20 mg/mL, and the two IgG subclasses are maintained approximately at a 1/1 ratio [26]. Several weeks before calving, IgG1 is selectively secreted to the colostrum. The concentration of IgG1 in colostrum is enhanced by 10-fold and can often reach more than 50 mg/mL [27, 28]. This selective secretion of IgG1 to colostrum leads to a decline of IgG1 concentration in blood, for example, according to the study by Sasaki et al., from 14 to ~5 mg/mL [14]. At the same time, IgG2 level do not find a significant change in blood around the periparturient period [14]. This means the IgG1/IgG2 ratio in the blood decreases during colostrogenesis. As the D2Dx™ immunity test can detect IgG test score variations, we hypothesized that this test may be able to detect the relative IgG1/IgG2 quantity change in dairy cows during the periparturient period.

From the testing of close to 1000 serum samples collected from 230 dairy cows, we detected an immune status change in the periparturient cows around the prepartum period, and this change is associated with the IgG1/IgG2 relatively quantity change during this time. Except for a few cases, almost all cows, regardless of their health conditions, follow the same trend: their immunity test responses increased from 1-2 weeks before calving to the day of calving, and then started to decline back following parturition. This trend is an exact reflection of immune homeostasis disruption known to occur in dairy cows and other reproductive animals during parturition [2, 29, 30].

As parturition leads to a disruption of homeostasis in the animals, naturally it only makes sense that the animal body will attempt to recover and return to homeostasis after parturition. If the body fails to restore this balance on time, the animal may be exposed to an increased risk of infectious diseases and other health problems. Studies have found that a certain degree of inflammation is necessary to induce birth and to protect the cows from a bacterial infection in the first few days postpartum [1, 2, 3, 5, 16]. Dairy cows had higher susceptible to diseases during the periparturient period that more likely to experience transition diseases such as metritis and mastitis [15]. The D2Dx™ immunity test results obtained in the present study support this hypothesis. Overall, we found cows with mastitis and/or lameness to have more elevated test responses than cows without clinical diseases during the four weeks of the periparturient period. More notably, the postpartum test response of diseased cows remained at elevated levels than the healthy cows, suggesting that the diseased cows may have had more difficulty recovering their homeostasis following parturition.

The $D2D_X^{TM}$ is not a traditional bioassay that measures the absolute quantity of a specific target protein. Even though the test was designed to detect bovine IgG1/IgG2 relative quantity change, the test result is not an absolute quantity measurement of the IgG1/IgG2 ratio. In our previously published study [21], we explained in detail the mechanism of this test. Briefly, the test uses a nanoparticle “pseudo” pathogen to probe the humoral immune status of a blood

sample. When the nanoparticle probe is mixed with a blood sample, IgG (including bovine IgG1 and IgG2) and IgM proteins will bind with the nanoparticle. Once IgG and IgM are bound to the nanoparticle, the entire structure will be recognized by the complement system as an antibody-coated pathogen particle, and a cascade of reactions and interactions will follow between the blood proteins and the IgG or IgM-bound nanoparticle. As demonstrated in previous studies [21], the D2Dx™ immunity test response is determined by the collective effect of IgG, IgM, complements, and possibly other proteins in the blood. In our current study, we further confirmed that a change of IgG1/IgG2 ratio in the blood will lead to a change of the D2Dx™ immunity test response. To use the D2Dx™ immunity test for decision-making purposes, and to evaluate the immune status and predict the risk level of individual cows, a clinical cutoff value should be established from testing of a sufficiently large number of healthy and diseased animals. The clinical sensitivity and specificity of the test for specific applications shall be determined from such studies.

Limitations of the current study must be considered. First, this study was conducted on frozen serum samples. This test remains to be validated using fresh blood samples if it were to be used as an on-farm test. Second, this study did not record any subclinical disorders or diseases. It is believed that in the first few weeks of lactation, more than 50% of cows may suffer from at least one subclinical disease or disorder [15]. This means that a significant

number of cows classified as “healthy” cows may have had some sub-clinical diseases or disorders. If we consider this factor, the test response difference between the real healthy cows and diseased cows could be even larger and more significant than what we have observed. More extensive studies over a longer period should be conducted to make further evaluations. If the D2Dx™ immunity test can detect cows with subclinical diseases and disorders, the test can add major benefit to dairy cow health monitoring and management.

Conclusion

We demonstrate in this study that the D2Dx™ immunity test can detect IgG1/IgG2-associated immune status change in dairy cows around the periparturient period. The test shows strong potential for monitoring the immune health and evaluating the disease risk of dairy cows following calving. The D2Dx™ immunity test is a very simple and fast test, with results obtained in less than one minute. The test can be easily performed on-site on the farm. Farm owners and veterinary clinicians may use the results to make informed and prompt management and treatment decisions. Although our current study was focused on dairy cows, the test may be potentially applied to other agricultural animals such as goats, sheep, pigs, and horses.

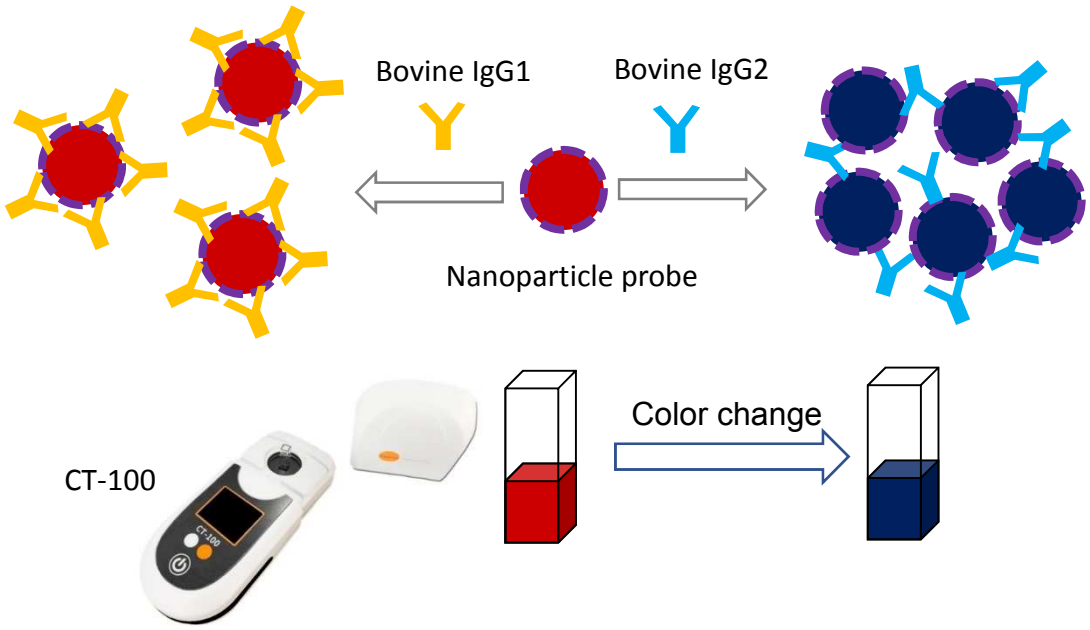

Figure 2.1. Illustration of the principle behind the D2Dx™ immunity test. The interaction of bovine IgG2 with the nanoparticle probe causes a nanoparticle aggregate formation and a color change from red to blue, while the interaction of bovine IgG1 with the nanoparticle probe leads to adsorption of the protein on the nanoparticle surface, but not aggregate formation, and no color change. In the presence of both IgG1 and IgG2, IgG1 and IgG2 will compete to bind with the nanoparticle probe through the two different binding modes. The color change of the assay solution from red to blue is detected using a handheld reader device, CT-100.

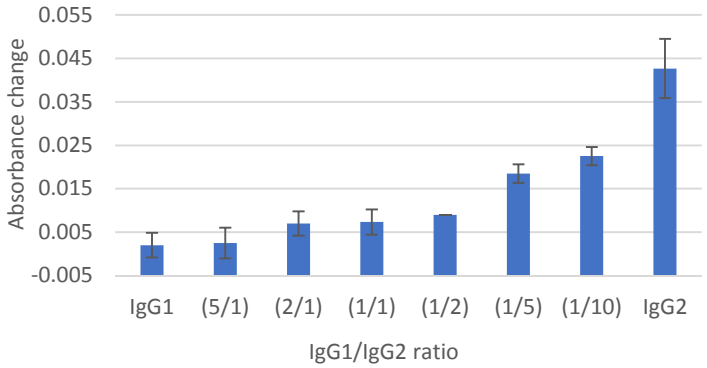

Figure 2.2. D2Dx™ test response of bovine IgG1, IgG2, and their mixtures. Both IgG1 and IgG2 have the same concentration of 1.0 mg/mL. The IgG1/IgG2 ratios indicated in the graph represent both v/v and w/w ratios. Error bars represent the standard deviation from at least two measurements.

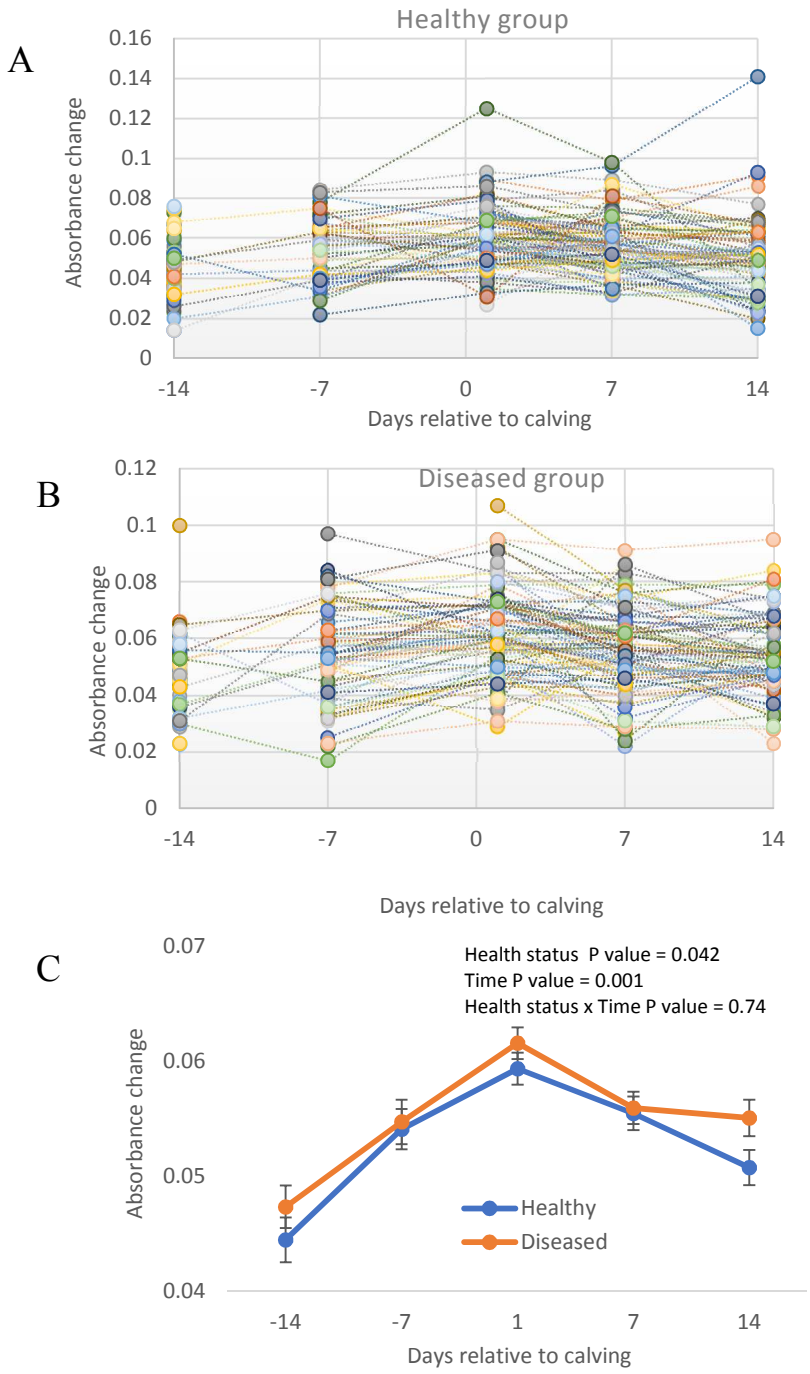

Figure 2.3. D2Dx™ test response of 230 cows during the periparturient period. Panel A-healthy group (n=115); Panel B-diseased group (n=115); Panel C- least-square means of healthy and diseased groups (health status $P = 0.042$; time $P = 0.001$).

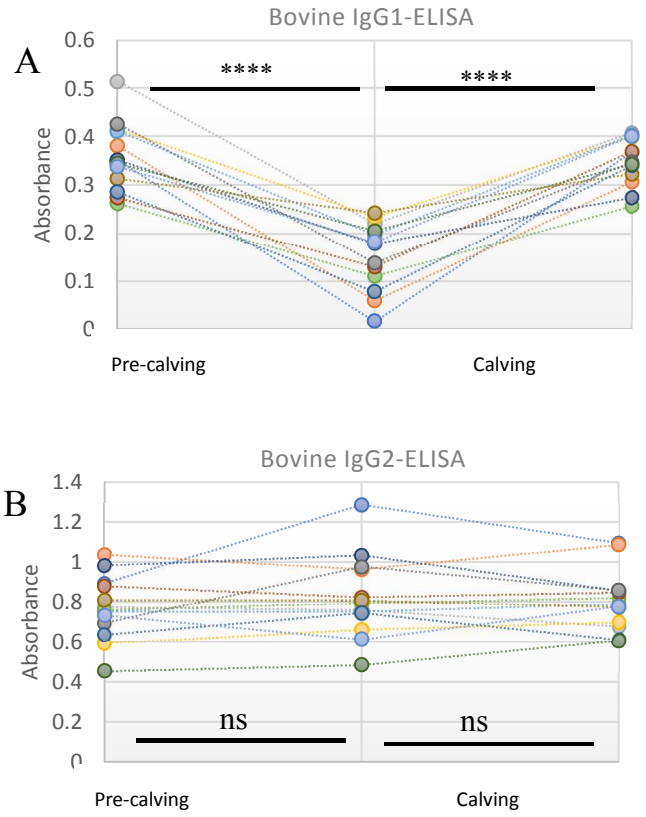

Figure 2.4. ELISA analysis of bovine IgG1 (Panel A) and IgG2 (Panel B) in 39 blood serum samples. The differences for IgG1 levels between pre-calving and calving; and between calving and post-calving, are both significant ($P < 0.0001$, ****). The differences for IgG2 levels between pre-calving and calving; and between calving and post-calving are both not significant (ns; $P > 0.05$).

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APPENDIX

University of Idaho
Office of Research Assurances
Institutional Animal Care and Use Committee
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Date: March 01, 2019
To: Pedram Rezamand
From: University of Idaho Institutional Animal Care and Use Committee
Re: Protocol IACUC-2017-52 *Relationship between nutrient metabolism during transition period and health measures in the Pacific Northwest dairy herds*

Your requested amendment of the animal care and use protocol listed above was reviewed and approved by the Institutional Animal Care and Use Committee on 03/01/2019.

This amendment request, 004392, was submitted for review on: 02/26/2019
The original approval date for this protocol was: 01/10/2018
This approval will remain in effect until: 12/04/2019
The protocol may be continued by annual updates until: 01/10/2021

Currently approved internal personnel on this protocol are: Ahmadzadeh, Amin; Bennett, Madeline; Hiltz, Rebecca; Hung, Hao-Che; McCurdy, Dana; Rezamand, Pedram; Rogers, Bridgette; Shao, Yijing; Tsai, Chia-Yu; Weber, Tanya

Currently approved external personnel on this protocol are: Dr. Scott T. Kieser; Paul A Mensinger

Federal laws and guidelines require that institutional animal care and use committees review ongoing projects annually. For the first two years after initial approval of the protocol you will be asked to submit an annual update form describing any changes in procedures or personnel. The committee may, at its discretion, extend approval for the project in yearly increments until the third anniversary of the original approval of the project. At that time, the protocol must be replaced by an entirely new submission.

Craig McGowan, IACUC Chair