

**LATE-GESTATION METABOLIC STRESS IN DAIRY CATTLE: ASSOCIATION WITH
COLOSTRUM YIELD AND IMMUNOGLOBULIN CONTENT**

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ABSTRACT

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Despite improvements made in the dairy industry and advances on heifer management, calf morbidity and mortality are still high. Calves rely on colostrum ingestion for acquisition of passive immunity, and provision of insufficient quantity of colostrum may lead these animals to failure of transfer passive immunity, hence leading to higher risks of morbidity and mortality. Many farms do not have sufficient colostrum available at times to feed their calves. Synthesis of colostrum starts few weeks before calving and it is at the time when cows are experiencing augmented metabolic adaptations due to increasing nutrient demands for fetal growth, colostrogenesis, and preparation for the onset of lactogenesis. The study described in *Chapter 2* aimed to compare the metabolic status of dairy cows during the last 6 wk of gestation based on colostrum volume and Ig content across seasons during a year. The results in the latter study suggest that greater availability of antioxidants might support the production of higher volumes of colostrum. Therefore, the study reported in *Chapter 3*, aimed to evaluate whether administering a dietary antioxidant supplement during the last 3 weeks of gestation improves colostrum volume and immunoglobulin content. Our results showed that DATS increased colostrum volume. However, despite finding higher volume of colostrum in treatment group with statistical differences compared to control, the distribution of colostrum volume between groups are relatively the same, as well for IgG, IgM, and IgA.

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KEY TO ABBREVIATIONS

ADG	average daily gain
Alb	albumin
AOP	antioxidant potential
BCS	body condition score
BHBA	beta-hydroxybutyrate acid
BUN	blood urea nitrogen
Ca	calcium
Chol	cholesterol
CON	control
Cu	copper
DATS	dietary antioxidant supplement
DCAD	dietary cation-anion difference
DMI	dry matter intake
ED	energy deficit
FTPI	failure of transfer passive immunity
Glu	glucose
HCP	high colostrum producer

HIA	high immunoglobulin A
HIG	high immunoglobulin G
HIM	high immunoglobulin M
Hp	haptoglobin
Ig	immunoglobulin
IQR	inter quartile range
LCP	low colostrum producer
LIA	low immunoglobulin A
LIG	low immunoglobulin G
LIM	low immunoglobulin M
MEC	mammary epithelial cells
Mg	magnesium
Mn	manganese
MS	metabolic stress
NEFA	non-esterified fatty acid
OS	oxidative stress
OSi	oxidant status index
RFU	relative fluorescence units
RID	radial immunodiffusion

RONS reactive oxygen and nitrogen species

SE standard error

TE trolox equivalent

TP total protein

TPI transfer passive immunity

TRT treatment

VOL volume

Zn zinc

**CHAPTER 1: REVIEW OF LITERATURE: MATERNAL METABOLIC FACTORS,
NUTRITIONAL ADAPTATIONS AND SUPPLEMENTATION STRATEGIES
ASSOCIATED WITH COLOSTROGENESIS**

This chapter will be submitted for publication in a peer-reviewed journal

INTRODUCTION

Despite improvements made in dairy calf health management over the last decades, preweaning morbidity and mortality incidence risks in US herds are still high, at 33.9% and 5%, respectively (Urie et al., 2018). One of the major contributors to preweaning disease occurrence is failure of transfer passive immunity (FTPI), a problem that still has a high prevalence of 13% in US dairy herds (Raboisson et al., 2016). These elevated measures of disease impact the economic stability of dairy operations owing to the expenses of calf mortality, care and long-term poor performance (Urie et al., 2018). Thus, it is feasible to infer that there are still opportunities to optimize the management practices of dairy calves.

The development of calf diseases is linked to the calf's immunological potential, which only at 3 months of age will become equivalent of an adult animal. In fact, calves are born with a naïve immune system that relies on transfer of passive immunity (TPI) (Godden et al., 2019). Therefore, maternal antibodies become necessary in the first weeks of life to provide immunologic protection to calves against pathogen infections. Nevertheless, transfer of immunoglobulins (Ig) from the dam to the fetus does not occur during pregnancy, but rather by TPI through ingestion of colostrum. Ig are absorbed across the small intestine, along with other maternal immunologic substances, cytokines, and immune cells, essential for calves' optimal health and growth (McGuirk and Collins, 2004). Nonetheless, absorption of maternal Ig gradually reduces over time and ceases, approximately, at 24 hours of life (Bush and Staley, 1980), making the immediate provision of colostrum extremely important, preferably right after birth, to avoid FTPI (Godden, 2008).

Currently, feeding newborn calves with 4 L of high-quality colostrum within 6 hours of birth and 2 L at 12 h has been recommended to optimize the transfer of passive immunity and calf health (Hammon et al., 2013, Godden et al., 2019). In fact, it has been shown that, compared to calves receiving only one meal of colostrum, those receiving a second meal within the first 12 h of birth had enhanced ADG and reduced risks for FTPI and preweaning morbidity (Abuelo et al., 2021). However, there is considerable variability in colostrum production among cows (Morin et al., 2001, Gavin et al., 2018, Kessler et al., 2020), making it difficult to dairy farms to harvest the volume of colostrum needed to sustain this feeding regime. Thereby, it may be linked as a cofactor for higher risks of calf morbidity or mortality, besides greater risk for FTPI. Scientific evidence on factors affecting the volume of colostrum being produced is limited. In fact, one study found association between colostrum variability with individual cow factors, such as dry period length, the animal's genetic, and the month of the year in which cows give birth (Gavin et al., 2018). Nevertheless, to the best of our knowledge, no study has elucidated whether the exacerbated physiological adaptations during late gestation are associated with poor colostrogenesis.

Colostrogenesis, rapid fetal growth, metabolic changes of nutrient mobilization and mammary tissue growth are some of the challenges that dairy cows face during the last 3 to 4 weeks of gestation (Lean et al., 2013). Hence, cows' metabolism become overwhelmed by the increased metabolic demands to which may lead them to metabolic stress where inflammatory and oxidative status would also increase (Sordillo and Raphael, 2013, Abuelo et al., 2015). Although the detrimental effects of metabolic stress on the health and production of dairy cattle during the transition period is well described

(Sordillo et al., 2009, Bradford et al., 2015) studies describing its impact on colostrogenesis is scarce. Therefore, this article reviews important aspects of late-gestation nutritional and metabolic factors that might be involved in the process of colostrogenesis.

COLOSTROGENESIS OVERVIEW

The synthesis of colostrum, orchestrated by endocrine changes, starts several weeks before calving and ceases immediately before parturition, when the copious milk production begins (Barrington et al., 2001, Baumrucker et al., 2010). During colostrogenesis, higher concentration of maternal Ig and other immunologic substances are gradually accumulated into mammary secretions, accompanied by its unique nutritional components, such as lactose and casein (Baumrucker et al., 2010, Farmer and Quesnel, 2020). Nonetheless, Ig and nutritional components have different secretory pathways. Thereby, the process of selective Ig accumulation, IgG1 (predominantly), IgG2, IgA and IgM, occur in three distinct mechanisms: a) Local production within the gland by lymphocytes; b) transcytosis across the endothelial and epithelial cell walls, and; c) epithelial membrane receptor-mediated protein transporters (Hine et al., 2019). In contrast, the nutritional components are actively produced and secreted into the mammary gland lumen by the mammary epithelial cells (MEC) (Nguyen and Neville, 1998, Barrington et al., 2001). Collectively, accumulation of Ig and synthesis of colostrum nutrients has been associated with substantial increase of cows' nutrient requirements (Lean et al., 2013). Therefore, this complex process of colostrogenesis may impose higher nutritional demands from dairy cows that, as an effect, may corroborate to the

development of metabolic disorders. Hence, it may lead cows to produce colostrum with lower quality and/or quantity.

Colostrogenesis starts 3 to 4 weeks before calving coinciding with cows experiencing metabolic adaptations (Lean et al., 2013) due to a profound increase in nutrient requirements associated with fetal growth and milk production (Sordillo and Mavangira, 2014). Nonetheless, it has been shown that the enlargement of the uterus compressing the rumen has a negative relationship regarding reduction of dry matter intake (DMI) (Ingvarsen and Andersen, 2000). In fact, the combination of changes in endocrine factors, normal physiological needs, physical and behavioral factors, in addition to the aforementioned factors of the pregnant cow are associated with a reduction of DMI; hence, it is a multifactorial issue that leads cows to a state of energy deficit (ED) (Ingvarsen and Andersen, 2000, Contreras and Sordillo, 2011). Therefore, ED triggers augmented metabolic adaptations to meet the cows' nutritional demands that may lead them to metabolic stress (MS), to which starts several weeks before calving (Grummer, 1993, Sordillo and Raphael, 2013, Sordillo and Mavangira, 2014). MS is caused by a synergistic imbalance between the excessive altered nutrient utilization, inflammatory and oxidative status dysfunction (Abuelo et al., 2015) that can result in substantial damage to tissues and potentiating the reduction of DMI (Contreras and Sordillo, 2011). The detrimental effect metabolic stress on the health and production of dairy cattle during the transition period is well described (Sordillo and Aitken, 2009, Bradford et al., 2015). Nevertheless, to the author's knowledge, no studies have investigated the effects of MS on colostrogenesis. Colostrum is more concentrated in nutrients than milk (Godden,

2008), thereby imposing important nutritional demands in the cow for its production that could lead to metabolic stress.

Maternal nutritional status of dairy cows during late-gestation has a great influence over the fetal growth, health, reproduction and production performance of the dam (Cooke, 2019; Chebel et al., 2018). For instance, animals overconditioned are associated to greater susceptibility to metabolic diseases, whereas underconditioned animals to lower milk production and conception risk during the transition period (Roche et al., 2009). Therefore, it might impact colostrogenesis by either metabolic disorders or insufficient cow's nutrient mobilization. Although other factors affecting the nutrient demands during late gestation have been described, such as heat stress (Seyed Almoosavi et al., 2020), overstocking (Krawczel and Lee, 2019), feed quality (Grummer, 1995), reviewing these subjects goes beyond the purpose of this paper. In addition, while several studies have been published elucidating the factors affecting the transferring of Ig, such as the endocrine modulation, receptor and transcytosis across MEC (Barrington et al., 2001, Nguyen et al., 2001, Baumrucker et al., 2010), the objective of this article is to review important aspects of late-gestation nutritional and metabolic factors that might be involved in the process of colostrogenesis.

Maternal metabolic factors, nutritional adaptations, and supplementation strategies that may influence colostrum yield

Despite cows being managed identically within each herd, they exhibit different magnitudes of metabolic stress (Kessel et al., 2008), which in combination with ED status may lead cows to poor production and health performance (Sordillo and Mavangira, 2014). In an event of cows experiencing severe ED, synthesis of carbohydrates from

musculoskeletal proteins can be activated as a source of energy. Nevertheless, the cow's adaptive mechanism induces lipolysis from adipose tissues for the synthesis of carbohydrates to prevent this degradation against body proteins (Contreras and Sordillo, 2011). However, non-esterified fatty acids (NEFA) are released in the bloodstream from adipose tissue and used as alternative energy substrates to be taken by cells in various tissues, including MEC (Contreras and Sordillo, 2011, Mesilati-Stahy and Argov-Argaman, 2018). Excessive NEFA metabolites accumulation, such as beta-hydroxybutyrate acid (BHBA), often accompanied by triglycerides, develop persistent cellular dysfunction, reduction of size and number of organelles, and damage in parenchymal cells of multiple organs. Thereby, it is possible that greater concentrations of NEFA in the late-pregnant cow might affect the colostrogenesis through MEC damage, where transport of glucose into the cell may be prejudiced. Moreover, induction of programmed cell death or apoptosis, altering structure and function of protein signaling pathways, increasing production of reactive oxygen and nitrogen species (RONS) (Inoguchi et al., 2000, Listenberger et al., 2001), and indirect activation of pro-inflammatory mediators are some of the consequences of excessive NEFA metabolites (Weinberg, 2006, Ringseis et al., 2015).

Bradford et al. (2015) emphasized the effects of excessive RONS on the endoplasmic reticulum of the cells where RONS can disrupt its membranes and, therefore, alter the normal functions of the cell. In addition, mitochondrial membrane is also affected by RONS, and worsened by the influx of lipids, which can exacerbate the intracellular production of RONS. Consequently, it leads to an oxidative stress (OS) status of the cells when the production of RONS overwhelms the neutralizing capacity of

antioxidants (Halliwell, 2007, Lykkesfeldt and Svendsen, 2007). OS in combination with inflammatory status and altered nutrient utilization, impair liver function, the reproductive performance (Kaufmann et al., 2010) and milk yield of dairy cows (Ospina et al., 2010, Ringseis et al., 2015). Therefore, to the best of our knowledge, no studies have investigated metabolic factors directly affecting the yield in colostrum synthesis. Several studies performed on dairy cattle around calving show that cows experience OS (Sordillo and Aitken, 2009, Abuelo et al., 2013, Abuelo et al., 2015). During this time period, oxidative stress impairs the adaptive capability of immune cell populations and increases the cow's susceptibility to many pathogens. Collectively, it is possible that OS in dairy cattle also affects colostrogenesis through changes in protein and gene expression, and insulin signaling. Nevertheless, limiting prepartum OS by implementing micronutrient antioxidant supplementation may offer a route to minimize the pathological changes associated with various maternal stressors.

In dairy cows, supplementation strategies of antioxidants during late gestation have been described mainly to promote transition cow health, but also with the goal to improve colostrum production, focusing more on quality instead of quantity. Nevertheless, Roshanzamir et al. (2020) found an increase in colostrum yield when dietary trace minerals containing Mn, Zn and Cu, as sulphate, glycine or methionine salts, were added to cows during the entire non-lactating period. In another study, compared to cows fed with a negative dietary cation-anion difference (DCAD) containing cholecalciferol, it has been shown that those fed a negative DCAD diet containing calcidiol at 3 mg per day during the last 3 weeks before parturition had higher colostrum yield (Aragona et al., 2016). The supplementation of nicotinic acid, which is thought to increase nutrients to the

mammary gland by the increase of blood flow, did not result in higher colostrum yield (Aragona et al., 2020), however, resulted in lower fat metabolites in the cows' serum (Aragona et al., 2016). Therefore, the authors inferred that it may play a role on inhibiting the exacerbated breakdown of triglycerides from adipose tissue when cows are experiencing adaptations of the increased metabolic demands.

Vitamin E and selenium are perhaps the most used antioxidants in dairy cattle diets, either alone or in combination. Vitamin E is a potent lipid-soluble, chain-breaking antioxidant that inhibits the propagation of free radicals in membranes and plasma lipoproteins (Traber and Stevens, 2011). The majority of selenium's antioxidant properties are ascribed to its function as a cofactor for selenoproteins, such as GSH-Px; nevertheless, a recent study highlighted selenium's direct role in reversing OS and controlling immunity in dairy cattle at the time of calving (Sordillo, 2013). Therefore, it might play a role in improving colostrogenesis by reducing the negative effects of OS. Vitamin E decreased is a common occurrence in cows at the start of lactation (Politis, 2012). However, the cause for the decreased concentration of α -tocopherol – vitamin E – at the end of pregnancy remains unknown. This may be attributed in part to the use of antioxidants during colostrum synthesis (Goff and Horst, 1997), but also to the inflammatory-like state of the liver of dairy cows during the transition period, which results in decreased hepatic production of various vitamin carrier proteins (Abd Eldaim et al., 2010); therefore, resulting in decreased plasma levels of vitamin E. However, further studies clarifying whether the observed effects of trace minerals and vitamins are due to their role in redox balance (Bauman and Bruce Currie, 1980, Herdt, 2000a, Contreras

and Sordillo, 2011, Roshanzamir et al., 2020) or their biological properties, merit investigation.

CHAPTER 2: CHANGES IN BIOMARKERS OF METABOLIC STRESS DURING LATE GESTATION OF DAIRY COWS ASSOCIATED WITH COLOSTRUM VOLUME AND IMMUNOGLOBULINS CONTENT ACROSS SEASONS

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ABSTRACT

The objective of this study was to compare the metabolic status of dairy cows during the last 6 wk of gestation based on colostrum volume and Ig content across seasons during a year. For this, healthy Holstein cows were randomly selected from 3 commercial herds in Michigan. In each farm, four cohorts of 21 cows (one per season), stratified by parity, were enrolled. Cows were blood sampled weekly during the last 6 wk of gestation, and biomarkers related to nutrient utilization, oxidant status, and inflammation were quantified in serum. Cows were milked within 6h of calving and the volume of colostrum produced was recorded and an aliquot collected. Concentration of IgG was measured by radial immunodiffusion. Cows were grouped into high colostrum producer (HCP) or low (LCP), high IgG (HIG) or low (LIG), high IgM (HIM) or low (LIM) and high IgA (HIA) or low (LIA). For volume category, we arbitrarily defined 6 L of colostrum (4 L for first and 2 L for second feeding of calves) as the cut-off point, whereas for IgG we used the industry standard of $\geq 50\text{g/L}$. To create groups of low and high IgM or IgA, we used the median of these Ig as the cutoff point. Data were analyzed statistically through mixed models with repeated measures including the fixed effects of groups (HCP vs LCP, LIG vs HIG, LIM vs HIM or LIA vs HIA), time, and their interaction; and the random effects of cow, season, lactation number, and farm ($P < 0.05$). Compared to LCP, HCP cows had higher concentrations of antioxidant potential, beta-hydroxybutyrate acid and lower cholesterol and oxidant status index. For IgG group, HIG cows had higher concentrations of albumin, and glucose compared to LIG. For IgA group, HIA cows had higher concentrations of cholesterol, reactive oxygen and nitrogen species, oxidant status index, and total protein, while BHBA, and glucose were lower compared with LIA. For the

IgM group, biomarkers of the colostrum variables were not significantly different between HIM and LIM. Nevertheless, the differences observed did not result in changes in inflammatory status in any of the colostrum variable categories analyzed, suggesting that physiological homeostasis was not disrupted during late gestation in association with the colostrum variables studied. Moreover, our results suggest that greater availability of antioxidants might support the production of higher volumes of colostrum.

INTRODUCTION

Despite improvements in dairy calf health management practices over the last decades, preweaning morbidity and mortality incidence risks in US herds are still high, at 33.9% and 5%, respectively (Urie et al., 2018). One of the major contributors to preweaning disease occurrence is failure of transfer passive immunity (FTPI), a problem that still has a high prevalence of 13% in US dairy herds (Raboisson et al., 2016). These elevated measures of disease impact the economic stability of dairy operations owing to the expenses of calf mortality, care, and long-term poor performance (Urie et al., 2018). Providing inadequate volume and/or quality of colostrum within the first hours of life is the major contributing factor to FTPI in calves (Morin et al., 1997). Traditionally, a volume of colostrum of 10 – 12% of the calves' body weight, given in one single meal, was recommended as a feeding strategy to transfer passive immunity successfully (Godden, 2008). However, feeding 4 L of high-quality colostrum within 6 hours of birth and 2 L at 12 h of life has been recently recommended to optimize the transfer of passive immunity and calf health (Hammon et al., 2013, Godden et al., 2019). In fact, it has been shown that, compared to calves receiving only one meal of colostrum, those receiving a second

meal within the first 12 h of birth had enhanced ADG and reduced risks for FTPI and preweaning morbidity (Abuelo et al., 2021).

However, there is considerable variability in colostrum production among cows (Morin et al., 1997, Gavin et al., 2018, Kessler et al., 2020), making it difficult to harvest the volume of colostrum needed to sustain this feeding regime. Scientific evidence on factors affecting the volume of colostrum being produced is limited. Colostrogenesis starts 3 to 4 weeks before calving coinciding with cows experiencing metabolic adaptations (Lean et al., 2013) due to a profound increase in nutrient requirements associated with fetal growth and milk production (Sordillo and Mavangira, 2014). Significant increases in metabolic demands may lead to metabolic stress where inflammatory and oxidative status would also increase (Abuelo et al., 2015). The detrimental effect of the biomarkers of metabolic stress on the health and production of dairy cattle during the transition period is well described (Sordillo and Aitken, 2009, Bradford et al., 2015). Nevertheless, to the best of our knowledge, no studies demonstrated an association between the different magnitudes of metabolic stress and colostrogenesis. Colostrum is more concentrated in nutrients than milk (Godden, 2008), thereby imposing important nutritional demands in the cow for its production that could lead to metabolic stress. We, therefore, hypothesized that cows producing high volume of colostrum and quality as assessed by immunoglobulin content would exhibit increased metabolic activity during late gestation. Thus, the objective of this observational study was to identify the association between biomarkers of metabolic stress during late gestation and colostrum volume and concentration of IgG, IgA, and IgM.

MATERIALS AND METHODS

Animals, Feed, Farms and Management

All procedures were approved by the Michigan State University Institutional Animal Care and Use Committee (protocol 04/18-065-00) and animals were enrolled with owners' consent. This prospective cohort study was conducted using a convenience sample of three commercial Michigan dairy farms, selected based on proximity to the university and willingness to participate in the study. The study was designed to have one cohort per season for each farm, a total of four cohorts, to account for changes in colostrum production associated with season (Gavin et al., 2018). Sampling occurred during the period between June 2019 and September 2020.

The sample size was calculated using an online calculator (<https://epitools.ausvet.com.au>) to estimate the within-herd prevalence of late-gestation cows with 95% confidence and 80% of power, resulting in 21 animals per cohort (n=252) per farm. Exclusion criteria for enrollment were animals not being apparently healthy based on clinical observation, cows with a successful breeding above 150 days in milk, and body condition score (BCS) under 2 or over 4 on a scale of 1 – 5 (Wildman et al., 1982). Holstein cows were selected using randomization software (<https://www.graphpad.com/quickcalcs/randomSelect1/>) among those expected to calve 6-8 weeks after the start of sampling from a list of cows generated by the herd management software. To reflect common demographics of dairy farms, random selection was stratified by parity groups of cows entering their first, second, or third to fifth lactation. Finally, data from 24 cows had to be excluded from analyses due to cows failing

to be pregnant, abortion, injury, or colostrum samples not been collected or recorded. A final number of 228 cows were therefore analyzed.

Housing and characteristics of management practices of late-gestation cows of the three farms are reported in Table 2.1. Cows had ad libitum access to a total mixed ration and water for the entire dry cow period. A far-off dry cow diet was provided as soon as cows were moved to the late-pregnant pen and switched to a close-up diet targeting at least 21 days prior expecting calving date. Diets were formulated by the farms' nutritionist to meet or exceed NRC (2001) recommendations. Samples of all total mixed ration were collected at 2-weeks intervals from the feed bunk at the time of distribution and sent to an external laboratory for chemical composition analysis. The composition and chemical analysis results of the diets are summarized in Tables 2.2 and 2.3.

Sample Collection

Blood samples were obtained weekly starting 6 wk before expected parturition, taken approximately to the feeding time via puncture of coccygeal vessels using two-10mL evacuated tubes with serum separator (BD Vacutainer; Becton, Dickinson and Company, Franklin Lakes, NJ). Blood samples that were collected within 2 days of actual calving date were not considered as the -1 wk point to avoid changes in blood biomarkers due to the hormonal changes associated with calving, considering the previous week sample instead. Tubes were transported to the laboratory on ice, separated within 1 h by centrifugation at $2,000 \times g$ for 20 min at 4°C, snap-frozen in liquid nitrogen, and stored at -80°C until analysis. All samples were frozen in 4 aliquots to prevent multiple freeze-thaw cycles.

Table 2.1: Housing and management practices for late-gestation cows in the study farms.

	Farm A				Farm B			Farm C		
	Close up cow	Close up heifer	Far off cow	Far off heifer	Close up cows / heifers	Far off cow	Far off heifer	Close up cows/heifers	Far off cow	Far off heifer
Housing										
Style	Free Stall	Free Stall	Free Stall	Free Stall	Bedding	Free Stall	Free Stall	Bedding	Free Stall	Free Stall
Fan	✓	✓	-	-	✓	✓	-	✓	✓	✓
Target duration for prefresh diet length (d)	21				21			21		
Target dry cow length period (d)	60				60			45		

Table 2.2: Ingredient composition of diets.

	Prepartum diet composition							
	Farm A				Farm B			Farm C
	Close up cow	Close up heifer	Far off cow	Far off heifer	Close up	Far off cow	Far off heifer	Dry cow / heifer
Ingredient, % of DM								-
Corn Silage	34.3	40.1	18.2	18.2	53.47	20.91	-	40.5
Sorghum Silage	-	-	-	-	-	8.71	30	-
Beet Pulp Wet	12.9	-	-	-	-	-	-	-
Haylage	-	-	69.2	69.2	-	48.79	54.44	12.8
Wheat Straw	-	28	10.9	10.9	20.58	19.17	-	37.6
Grass hay	21.6	-	-	-	-	-	-	-
Lact TMR Leftove	-	-	-	-	-	-	12.34	-
Mineral premix	31.2	31.9	1.6	1.6	25.95	2.07	3.21	9.1
Salt	-	-	-	-	-	0.35	-	-

Table 2.3: Analyzed composition of diets.

	Prepartum diet															
	Farm A								Farm B				Farm C			
	Close up cow		Close up heifer		Far off cow		Far off heifer		Close up		Far off cow		Far off heifer		Dry cow / heifer	
DM, %	45.9	± 2.99	46.15	± 5.64	43.03	± 4.89	40.91	± 4.56	48.77	± 2.59	37.85	± 5.8	34.46	± 6.8	61.35	± 3.21
Nutrients, DM basis (±SD)																
Proteins																
CP, %	12.95	± 2.21	13.7	± 0.8	13.74	± 1.38	15.23	± 1.67	13.49	± 1.1	12.43	± 2.9	15.04	± 2.53	11.39	± 1.81
Adjusted Protein, %	12.88	± 2.22	13.7	± 0.8	12.68	± 1.66	14.29	± 2.07	13.49	± 1.1	11.73	± 2.87	14.5	± 2.81	10.59	± 1.95
Soluble Protein, %	10.49	± 15.20	11.85	± 15.42	11.4	± 14.29	14.07	± 18.94	11.54	± 16.15	15.91	± 20.15	19.6	± 21.72	3.73	± 0.81
Ammonia (CPE), %	1.35	± 0.26	0.89	± 0.21	2.43	± 0.35	2.34	± 0.43	2.6	± 0.34	2.68	± 0.78	2.57	± 0.98	0.51	± 0.13
ADICP, %	1.26	± 0.07	1.11	± 0.09	1.98	± 0.29	1.83	± 0.24	1	± 0.13	1.59	± 0.33	1.62	± 0.27	1.42	± 0.09
NDICP, %	2.94	± 0.2	2.55	± 0.2	3.6	± 0.7	3.59	± 0.73	2.18	± 0.25	3.07	± 0.99	3.24	± 0.71	2.52	± 0.33
Fiber																
ADF, %	27.1	± 1.6	29.5	± 2.89	39.3	± 3.89	35.54	± 2.07	26.72	± 2.12	36.74	± 3.77	31.56	± 4.1	35.28	± 2.86
aNDF, %	40.4	± 3.51	45.58	± 3.69	47.3	± 10.39	46.37	± 1.89	41.13	± 3.23	52.81	± 7.53	46.27	± 6.14	51.94	± 4.09
Lignin, %	4.61	± 0.30	4.85	± 0.48	8.74	± 1.34	7.78	± 1.19	4.38	± 0.4	6.6	± 1.3	5.69	± 1.28	6.86	± 0.5
Carbohydrates																
ESC-Sugar, %	5.47	± 1.1	3.81	± 1.32	4.02	± 0.35	3.72	± 0.56	3.67	± 0.7	2.36	± 0.86	2.02	± 1.2	5.45	± 0.66
Starch, %	18.0	± 2.27	19.18	± 4.02	8.1	± 2.01	9.87	± 3.65	23.06	± 1.87	11.09	± 4.79	14.56	± 5.53	10.73	± 1.48
CF, %	2.80	± 0.34	3	± 0.6	3.1	± 0.4	3.3	± 0.43	2.66	± 0.21	3.32	± 0.42	4.09	± 0.22	2.77	± 0.21
Minerals																
Ash, %	8.55	± 0.53	7.4	± 0.74	8.72	± 1.03	9.29	± 1.74	7.97	± 0.67	9.39	± 1	9.1	± 0.54	8.67	± 0.51
Ca, %	1.36	± 0.31	0.72	± 0.1	1.24	± 0.16	1.27	± 0.17	1.03	± 0.27	0.75	± 0.3	0.93	± 0.21	0.69	± 0.13
P, %	0.38	± 0.04	0.36	± 0.02	0.26	± 0.04	0.3	± 0.04	0.4	± 0.04	0.32	± 0.03	0.41	± 0.05	0.36	± 0.07
Mg, %	0.43	± 0.03	0.36	± 0.03	0.31	± 0.06	0.35	± 0.05	0.34	± 0.04	0.27	± 0.05	0.33	± 0.03	0.4	± 0.05
K, %	0.95	± 0.04	1.19	± 0.08	2.01	± 0.16	1.93	± 0.22	1.17	± 0.08	1.98	± 0.45	1.93	± 0.51	1.05	± 0.11
S, %	0.37	± 0.06	0.23	± 0.04	0.16	± 0.03	0.19	± 0.04	0.38	± 0.06	0.19	± 0.02	0.25	± 0.03	0.27	± 0.09
Na, %	0.08	± 0.03	0.06	± 0.02	0.09	± 0.03	0.18	± 0.02	0.11	± 0.02	0.52	± 0.17	0.24	± 0.14	0.13	± 0.01
Cl, %	0.54	± 0.09	0.4	± 0.58	0.47	± 0.1	0.51	± 0.17	0.9	± 0.09	1.11	± 0.31	0.59	± 0.14	0.68	± 0.08
Fe, ppm	398.25	± 109.62	265.91	± 44.15	311.5	± 71.9	441.29	± 138.36	371.71	± 143.33	335.45	± 123.46	393.86	± 130.38	295.25	± 139.43
Mn, ppm	126.92	± 15.87	87.18	± 31.3	47.3	± 10.78	57.71	± 9.72	75.86	± 14.06	49.45	± 7.33	75.14	± 11.05	76	± 11.18
Zn, ppm	118.00	± 20.33	113	± 19.61	36.5	± 9.48	50.14	± 11.58	72.21	± 9.89	45.27	± 7.32	82.43	± 13.85	129.17	± 25.78
Cu, ppm	14.92	± 2.75	14.18	± 1.72	11	± 2.21	13	± 4.65	10.71	± 2.58	11.18	± 2.79	16.29	± 2.43	9.67	± 1.83
Energy & index calculations																
TDN, %	65.93	± 1.93	65.14	± 2.99	57.89	± 1.69	60.26	± 1.6	66.3	± 1.87	59.44	± 2.42	63.93	± 3.56	57.88	± 1.89
Net Energy Lactation, Mcal/kg	1.63	± 0.46	1.61	± 0.44	1.28	± 0.04	1.34	± 0.04	1.61	± 0.44	1.32	± 0.04	1.46	± 0.11	1.30	± 0.04
Net Energy Maintenance, Mcal/kg	1.61	± 0.40	1.57	± 0.37	1.19	± 0.04	1.28	± 0.07	1.59	± 0.37	1.23	± 0.09	1.41	± 0.11	1.26	± 0.09
Net Energy Gain, Mcal/kg	0.95	± 0.22	0.93	± 0.18	0.64	± 0.04	0.71	± 0.04	0.95	± 0.20	0.68	± 0.09	0.82	± 0.11	0.68	± 0.09
ME, Mcal/kg	2.51	± 0.11	2.49	± 0.11	2.49	± 0.88	2.71	± 1.06	2.54	± 0.11	2.47	± 0.82	2.95	± 1.30	2.16	± 0.09
Non Fiber Carbohydrates, %	37.23	± 4.58	32.54	± 4.57	27.72	± 4.88	28.91	± 6.32	36.64	± 3.56	24.57	± 6.38	27.81	± 3.98	27.78	± 2.59
Non Structural Carbohydrates, %	24.14	± 1.44	24.56	± 2.16	11.92	± 2.1	14.18	± 3.35	26.98	± 1.6	14.47	± 3.95	14.22	± 2.98	16.13	± 1.28
DCAD mEq/100g	-10.83	± 5.63	12.97	± 1.46	32.28	± 4.68	30.94	± 3.24	-14.38	± 4.89	28.59	± 7.29	27.51	± 8.46	-3.65	± 5.3

Colostrum was harvested by trained farm personnel within 6 h of calving. The volume of colostrum produced was measured using a graduated bucket (10 Quart Measuring Pail with Handle, United States Plastic Corporation, Lima, OH). A sample of each cow's colostrum was also collected in a 50 mL container and kept frozen at -20°C for further analysis.

Analytical Determinations

Serum samples

Following the manufacturer's instructions, oxidant status was assessed following reported methodologies (Abuelo et al., 2016). The concentrations of RONS in serum were determined using the OxiSelect In Vitro Reactive Oxygen and Nitrogen Species assay kit (Cell BioLabs Inc., San Diego, CA). Briefly, free radicals present in the sample bind to a dichlorodihydrofluorescein probe, converting it to a fluorescing product (2',7'-dichlorodihydrofluorescein). Thus, the fluorescence intensity is proportional to the concentration of RONS in the sample. The fluorescence of dichlorofluorescent dye was determined at excitation wavelengths of 480 nm and emission of 530 nm in a Synergy H1 Hybrid plate reader (Biotek; Winooski, VT, USA). To ensure fluorescence at various concentrations, a standard curve, made by six serial dilutions (0–10,000 nM) of the fluorescence probe 2',7'-dichlorodihydrofluorescein diacetate was included in each plate (Fluotrac Black 96 well plate, Greiner Bio-One, Frickenhausen, Germany). All samples and standards were analyzed in duplicates. Background fluorescence was eliminated by subtracting blank values from sample values. Results are reported as the average relative fluorescence units (RFU) between replicates.

The antioxidant potential (AOP) of serum samples were determined using trolox (synthetic vitamin E analog) equivalents antioxidant capacity, as described previously (Re et al., 1999). Antioxidant components of serum interact, making it difficult to quantify each antioxidant individually. As a result, this method considers the synergism of all antioxidants present in a sample, including albumin, thiols, bilirubin, and superoxide dismutase. In brief, based on the standard curve of 0–25 g/L, a known volume of trolox standard concentration would result in a similar reduction of the radical 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (Sigma-Aldrich, St. Louis, MO). Changes in oxidative balance may occur because of shifts in RONS and/or AOP. Thus, the oxidant status index (OSi) was calculated as the ratio between RONS and AOP, as this better characterizes shifts in redox balance in periparturient cows (Abuelo et al., 2013).

The serum concentration of BHBA, blood urea nitrogen (BUN), calcium (Ca), cholesterol (Chol), glucose (Glu), magnesium (Mg), NEFA, and total protein (TP) were quantified using commercial reagents from Catachem Inc. (Bridgeport, CT) as biomarkers of nutrient utilization. As biomarkers of inflammation, we determined the concentrations of the positive and negative acute phase protein Haptoglobin (Hp) (Phase Haptoglobin Assay TP-801, Tridelata Development Limited, Maynooth, Ireland) and albumin (Alb) (Catachem Inc. Bridgeport, CT), respectively. All biomarkers related to nutrient utilization and inflammation were determined using a small-scale biochemistry analyzer (CataChemWell-T; Catachem Inc.). The analyzer was calibrated every week using the assay manufacturer's calibrators. Reference samples were also analyzed at the time of calibration for two-level quality control.

Colostrum samples

The IgG, IgA, and IgM concentrations of colostrum samples were measured via radial immunodiffusion (RID) (Bovine IgG, IgA and IgM test; Triple J Farms, Bellingham, WA) following manufacturer's instructions (<https://kentlabs.com/iji/triple-j-farms-product-information/rid-plate-procedure/>). The method is based on the precipitation in agarose gel growing in a circle antigen-antibody complexes which develop after 10-20 hours at room temperature and continues to grow until equilibrium is reached. Briefly, the colostrum samples were thawed overnight at 4 °C. Dilutions of each sample were performed in 0.9% NaCl. Samples were diluted at 1:6, 1:9, 1:10 for IgG analyses and at 1:2, 1:4 and 1:5 for IgA and IgM quantification. Standards were included in each plate for reference and ranged from 1.8 to 28.03 (IgG), 0.53 to 3.87 (IgA), and 0.62 to 3.81 (IgM) g/L. The diffusion ring through the agarose gel containing mono-specific antibody after 24 h of incubation at room temperature was measured using a caliper with precision of 0.1 mm (VWR traceable caliper; Radnor, PA). The values of the sample's ring were read off the standard curve to determine Ig concentrations in g/L. Samples falling outside of the standard curve were re-assayed using a higher or lower dilution.

Statistical analyses

Cows were grouped *ex-post* into groups based on their colostrum variables to compare the changes in biomarkers of MS according to the volume and Ig content of their colostrum. Based on the recent recommendation of colostrum volume to sufficiently feed one calf, we considered high colostrum producers (HCP) when cows produced ≥ 6 liters while low colostrum producers (LCP) < 6 liters. Dairy industry standard of colostrum quality is based on IgG concentration, which must be above 50 g/L. Therefore, a variable

was created to dichotomize IgG concentration as either <50 g/L for low IgG (LIG) or ≥50g/L for high IgG (HIG). However, no industry standard of colostrum quality exists for IgM and IgA to date. Thus, we used the median of the IgM and IgA values as the cutoff point to create groups of low and high Ig, LIM or HIM and LIA or HIA respectively. Data were managed in Excel spreadsheets and exported to the statistical software. All statistical analyses were performed with JMP Pro v.15.2 (SAS Institute Inc., Cary, NC) and the criterion for statistical significance was established at $P < 0.05$. Linear mixed models with repeated measures were built for the cows' variables Alb, BHBA, BUN, Ca, Chol, Glu, Hp, Mg, NEFA, TP, RONS, AOP and OSi. Fixed effects included time (sampling weeks -6 to -1 relative to actual calving), groups (HCP vs LCP, LIG vs HIG, LIM vs HIM or LIA vs HIA) and their interaction. Cow, season, lactation number, and farm were used as random effects. For repeated measures, the covariance structures autoregressive 1, compound symmetry, or residual were tested for each variable, and the one with the lowest Akaike information criterion was chosen. Model assumptions were assessed by evaluation of homoscedasticity and normality of residuals. To satisfy these assumptions, some variables were natural log or square root-transformed and the resulting least squares means estimates were subsequently back transformed and presented as geometric means. All P -values given are those controlled for multiple comparisons with Tukey's honestly significant difference test.

RESULTS

Descriptive results of colostrum variables

The distribution of colostrum volume and concentrations of IgG, IgM and IgA by season and lactation group is depicted in Figure 2.1. The mean colostrum yield was 5.47

L (range = 0.5 to 15.32) with a noted variability across seasons (n=225). The mean colostrum yield in summer, winter, fall, and spring were 6.97 (95% CI: 5.79–8.14), 4.16 (3.54–4.77), 5.36 (4.66–6.06) and 5.38 (4.68–6.08) L/cow, respectively. The mean colostrum concentration of IgG (n= 227), IgM (n= 226) and IgA (n= 227) were 118.73 (112.64–124.82) g/L, 4.94 (4.66–5.22) and 6.29 (5.83–6.77), respectively.

Biomarkers of metabolic stress

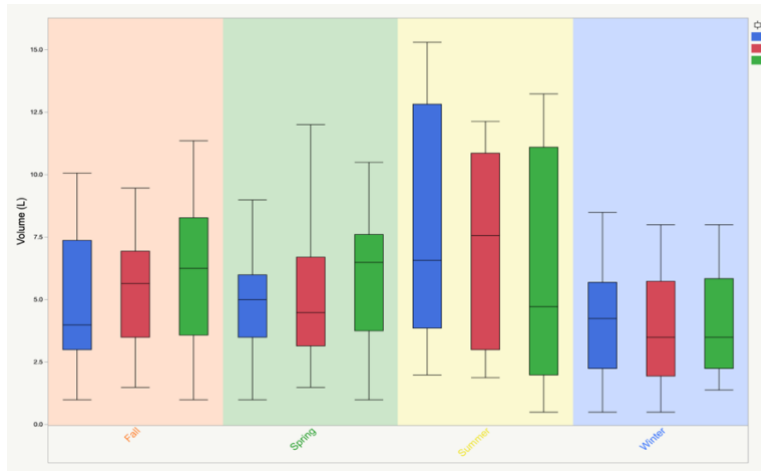
The mean (SE) concentration of the cow biomarkers is presented by group and sampling point for the volume, IgG, IgM, and IgA variables in Tables 2.4, 2.5, 2.6 and 2.7, respectively. Compared to LCP, HCP cows had higher concentrations of AOP, BHBA, and lower Cho and OSi. For IgG group, HIG cows had higher concentrations of Alb, and glucose compared to LIG. For IgA group, HIA cows had higher concentrations of Chol, RONS, OSi, and TP, while BHBA, and Glu were lower compared with LIA. For the IgM group, biomarkers of the colostrum variables were not significantly different between HIM and LIM. No significant differences were found on biomarkers of inflammation for any of the colostrum variable categories analyzed.

DISCUSSION

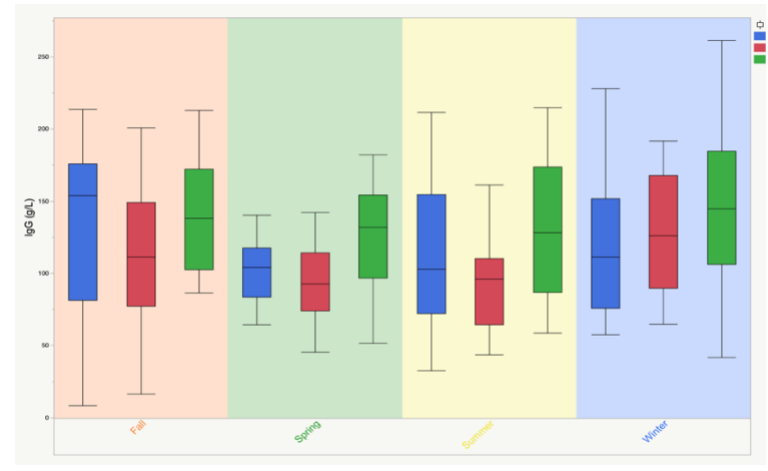
Nutrient utilization

We assessed changes on nutrient utilization of dairy cows, since MS is composed of altered nutrient utilization, inflammation, and oxidative stress (Abuelo et al., 2013). In this study, despite NEFA concentrations not being different between groups and having significant changes nor was elevated, we found that cows producing high volumes of colostrum, exhibited higher concentration of BHBA.

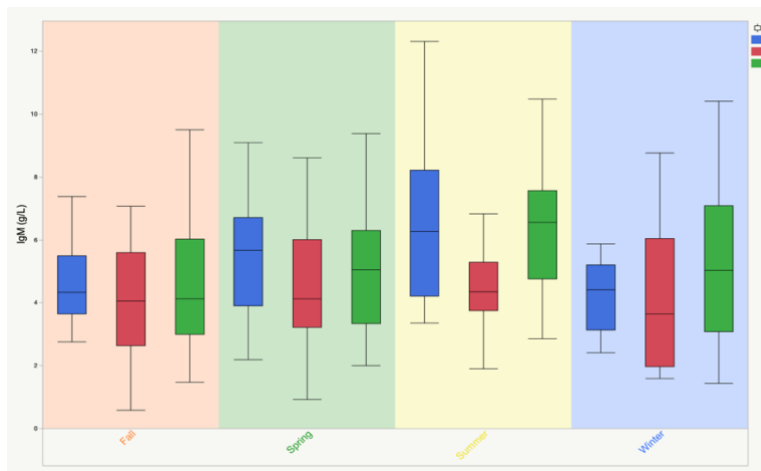
Figure 2.1: Distribution of colostrum (A) volume, (B) IgG concentration, (C) IgM concentration, and (D) IgA concentration per season and lactation group. The whiskers of the Box Plots represent the 95% confidence interval.



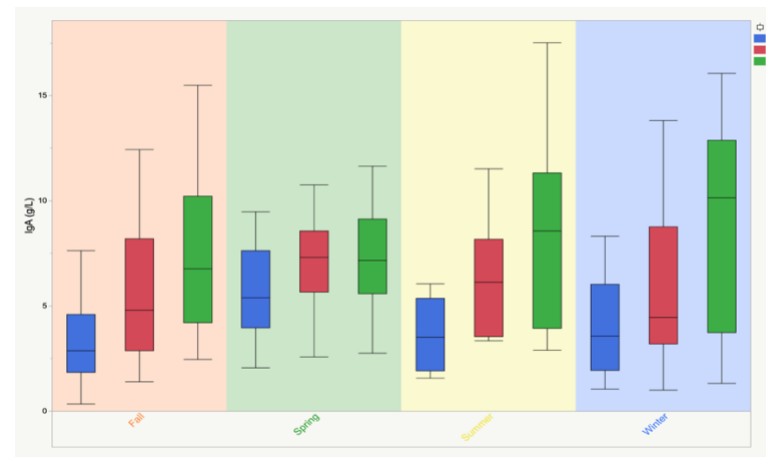
(A)



(B)



(C)



(D)

Table 2.4: Estimated means and significance of the main effects from the generalized linear mixed models of blood biomarkers of late-gestation dairy cows comparing groups of high colostrum producers (HCP, n=86) and low colostrum producers (LCP, n=139).

Outcome (units)	Estimated means of at the different time points												SE	P value		
	Wk -6		Wk -5		Wk -4		Wk -3		Wk -2		Wk -1					
	HCP	LCP	HCP	LCP	HCP	LCP	HCP	LCP	HCP	LCP	HCP	LCP		Group	Time	G×T
NEFA (mmol/L) ¹	0.22	0.24	0.21	0.21	0.2	0.22	0.19	0.22	0.19	0.21	0.23	0.23	1.09	0.491	0.027	0.56
BHBA (mg/dL) ¹	4.23	4.09	4.39	4.05	4.32	4.03	4.51	3.9	4.65	3.78	5.15	4.17	1.03	< 0.001	< 0.001	< 0.001
Cholesterol (mg/dL) ¹	176	198	160	175	143	154	131	139	112	125	98.5	110	1.02	0.002	< 0.001	0.21
Glucose (mg/dL) ¹	73	76.6	74.3	74.8	72.6	73	72.8	73.3	72	74.5	71.7	73.2	1.01	0.222	0.003	0.19
Total protein (g/dL) ¹	7.54	7.41	7.62	7.37	7.45	7.24	7.4	7.23	7.14	7.1	6.97	6.82	1.01	0.153	< 0.001	0.27
BUN (mg/dL)	11.4	10.8	11.7	11	11.7	11.2	11.5	11.9	12	12.3	12.9	12.9	0.37	0.646	< 0.001	0.08
Albumin (g/dL) ¹	4.21	4.13	4.23	4.05	4.09	3.96	4.13	3.98	4.06	3.99	4.04	3.95	1.01	0.051	< 0.001	0.34
Haptoglobin (g/L) ¹	0.5	0.46	0.49	0.48	0.47	0.45	0.5	0.43	0.48	0.44	0.46	0.42	0.05	0.229	0.019	0.21
Calcium (mg/dL)	9.29	9.16	9.27	9.18	9.03	9.02	9.28	9.06	9.1	9.17	9.21	9.11	0.11	0.533	0.016	0.21
Magnesium (mg/dL)	2.87	2.76	3	2.81	3.02	2.8	2.94	2.84	3.01	2.86	2.97	2.89	0.07	0.065	0.323	0.61
AOP (TE/mL) ¹	8.1	7.49	8.42	7.35	8.18	7.23	8.2	7.32	7.97	7.11	7.67	6.77	1.03	0.002	< 0.001	0.69
RONS (RFU) ¹	44.8	49.3	46.8	49.8	48.2	51.5	52.6	55.3	54.9	59.2	53.7	61.7	0.05	0.239	< 0.001	0.57
OSi (arbitrary units) ¹	5.38	6.53	5.46	6.73	5.79	7.07	6.31	7.5	6.78	8.27	6.88	9.05	0.06	0.006	< 0.001	0.58

Generalized linear mixed models with repeated measurements were built for the blood variables as outcomes with the Group, Time, their interaction (G×T) as fixed effects.

Wk-6 through Wk -1 represent time points antepartum. HCP: High colostrum producers; LCP: Low colostrum producers. SE: Standard error.

TE: Trolox equivalent; RFU: Relative fluorescence units; NEFA: non-esterified fatty acids; BHBA: Beta-hydroxybutyrate acid; BUN: Blood urea nitrogen; AOP: Antioxidant potential; RONS: Reactive oxygen and nitrogen species; OSi: Oxidant status index.

Estimated means calculated for the least significant difference.

¹ Natural Log transformed

Table 2.5: Estimated means and significance of the main effects from the generalized linear mixed models of blood biomarkers of late-gestation dairy cows comparing groups of high immunoglobulin G (HIG, n=217) and low immunoglobulin G (LIG, n=10).

Outcome (units)	Estimated means of at the different time points												SE	<i>P value</i>		
	Wk -6		Wk -5		Wk -4		Wk -3		Wk -2		Wk -1			Group	Time	G×T
	HIG	LIG	HIG	LIG	HIG	LIG	HIG	LIG	HIG	LIG	HIG	LIG				
NEFA (mmol/L) ¹	0.23	0.13	0.22	0.15	0.21	0.15	0.2	0.18	0.2	0.18	0.23	0.24	1.19	0.304	0.328	0.42
BHBA (mg/dL) ¹	4.12	3.75	4.19	3.52	4.13	4.19	4.13	3.88	4.11	3.9	4.51	5.08	1.07	0.605	0.004	0.25
Cholesterol (mg/dL)	196	198	176	154	154	141	139	129	124	110	109	93.3	8.84	0.369	< 0.001	0.58
Glucose (mg/dL) ¹	75.5	68.7	74.9	66.9	73.4	64.7	73.4	69.6	73.9	66.4	72.9	67.4	1.02	0.013	0.464	0.55
Total protein (g/dL) ¹	7.48	7.2	7.51	7.02	7.35	6.98	7.32	7.13	7.14	6.76	6.91	6.51	1.02	0.165	< 0.001	0.65
BUN (mg/dL)	11	12.1	11.2	12.8	11.3	13.7	11.5	13.8	12.1	13.6	12.8	13.8	0.74	0.089	0.101	0.77
Albumin (g/dL) ¹	4.17	3.84	4.13	3.85	4.02	3.8	4.04	3.88	4.02	3.86	4	3.58	1.01	0.043	0.018	0.17
Haptoglobin (g/L) ¹	0.48	0.56	0.48	0.45	0.46	0.45	0.46	0.54	0.45	0.43	0.44	0.45	0.1	0.816	0.242	0.37
Calcium (mg/dL)	9.23	8.92	9.22	9.09	9.04	8.6	9.14	9.05	9.15	8.87	9.15	9	0.21	0.43	0.263	0.82
Magnesium (mg/dL) ²	2.77	2.87	2.86	2.92	2.86	2.96	2.85	2.86	2.88	3.04	2.91	2.57	0.01	0.941	0.27	0.12
AOP (TE/mL) ¹	7.85	7.09	7.84	7.21	7.67	6.82	7.75	6.85	7.52	6.73	7.21	6.18	1.06	0.213	0.001	0.91
RONS (RFU) ¹	47.7	34.1	48.7	41.4	50.5	37.8	54.3	47.2	57.5	50.3	58.2	57.2	1.11	0.282	< 0.001	0.19
OSi (arbitrary units) ¹	6.01	4.81	6.15	5.73	6.51	5.53	6.94	6.89	7.57	7.47	8	9.26	1.13	0.771	< 0.001	0.16

Generalized linear mixed models with repeated measurements were built for the blood variables as outcomes with the Group, Time, their interaction (G×T) as fixed effects.

Wk-6 through Wk -1 represent time points antepartum. HIG: High immunoglobulin G producers; LIG: Low immunoglobulin G producers. SE: Standard error.

TE: Trolox equivalent; RFU: Relative fluorescence units; NEFA: non-esterified fatty acids; BHBA: Beta-hydroxybutyrate acid; BUN: Blood urea nitrogen; AOP: Antioxidant potential; RONS: Reactive oxygen and nitrogen species; OSi: Oxidant status index

Estimated means calculated for the least significant difference.

¹ Natural Log transformed

² Square root transformed

Table 2.6: Estimated means and significance of the main effects from the generalized linear mixed models of blood biomarkers of late-gestation dairy cows comparing groups of high immunoglobulin M (HIM, n=113) and low immunoglobulin M (LIM, n=113).

Outcome (units)	Estimated means of at the different time points												SE	P value		
	Wk -6		Wk -5		Wk -4		Wk -3		Wk -2		Wk -1			Group	Time	G×T
	HIM	LIM	HIM	LIM	HIM	LIM	HIM	LIM	HIM	LIM	HIM	LIM				
NEFA (mmol/L) ¹	0.23	0.23	0.23	0.2	0.21	0.21	0.21	0.2	0.19	0.21	0.23	0.23	1.08	0.925	0.04	0.48
BHBA (mg/dL) ¹	4.01	4.23	4.11	4.22	4.2	4.09	4.27	4	4.17	4.07	4.47	4.6	1.03	0.966	< 0.001	0.21
Cholesterol (mg/dL) ¹	187	188	167	170	150	147	135	135	118	121	105	105	1.02	0.885	< 0.001	0.46
Glucose (mg/dL) ¹	75.3	74.8	74.3	74.5	73.1	72.6	72.2	73.9	73.1	73.6	71.8	73.2	1.01	0.709	0.004	0.44
Total protein (g/dL) ¹	7.43	7.5	7.5	7.45	7.35	7.3	7.32	7.29	7.13	7.1	6.94	6.83	1.01	0.752	< 0.001	0.56
BUN (mg/dL)	11	11.1	11.4	11.1	11.8	10.9	12.1	11.2	12.3	12	12.9	12.8	0.36	0.315	< 0.001	0.27
Albumin (g/dL) ¹	4.19	4.14	4.14	4.1	4.02	4	4.01	4.05	4.01	4.02	3.99	3.98	1.01	0.841	< 0.001	0.64
Haptoglobin (g/L) ¹	0.46	0.51	0.48	0.48	0.45	0.47	0.48	0.45	0.45	0.45	0.43	0.45	1.05	0.714	0.007	0.1
Calcium (mg/dL)	9.12	9.31	9.13	9.29	8.97	9.08	9.06	9.22	9.08	9.2	9.08	9.2	0.1	0.251	0.025	0.99
Magnesium (mg/dL)	2.81	2.79	2.93	2.85	3.01	2.78	2.96	2.8	2.99	2.86	2.99	2.86	0.07	0.089	0.26	0.39
AOP (TE/mL) ¹	7.97	7.66	7.81	7.84	7.66	7.61	7.76	7.66	7.55	7.43	7.23	7.11	1.03	0.706	< 0.001	0.78
RONs (RFU) ¹	50	44	51.6	45.1	53.4	46.6	56.8	51.2	57.1	56.9	60.6	55.6	1.05	0.156	< 0.001	0.07
OSi (arbitrary units) ¹	6.19	5.71	6.53	5.73	6.88	6.08	7.23	6.65	7.47	7.61	8.28	7.77	1.06	0.323	< 0.001	0.08

Generalized linear mixed models with repeated measurements were built for the blood variables as outcomes with the Group, Time, their interaction (G×T) as fixed effects.

Wk-6 through Wk -1 represent time points antepartum. HIM: High immunoglobulin M producers; LIM: Low immunoglobulin M producers. SE: Standard error.

TE: Trolox equivalent; RFU: Relative fluorescence units; NEFA: non-esterified fatty acids; BHBA: Beta-hydroxybutyrate acid; BUN: Blood urea nitrogen; AOP: Antioxidant potential; RONS: Reactive oxygen and nitrogen species; OSi: Oxidant status index

Estimated means calculated for the least significant difference.

¹ Natural Log transformed

Table 2.7: Estimated means and significance of the main effects from the generalized linear mixed models of blood biomarkers of late-gestation dairy cows comparing groups of high immunoglobulin A (HIA, n=112) and low immunoglobulin A (LIA, n=115).

Outcome (units)	Estimated means of at the different time points												SE	P value		
	Wk -6		Wk -5		Wk -4		Wk -3		Wk -2		Wk -1			Group	Time	G×T
	HIA	LIA	HIA	LIA	HIA	LIA	HIA	LIA	HIA	LIA	HIA	LIA				
NEFA (mmol/L) ¹	0.23	0.24	0.19	0.25	0.19	0.23	0.18	0.23	0.18	0.22	0.23	0.23	1.08	0.096	0.03	0.046
BHBA (mg/dL) ¹	3.89	4.29	4.02	4.25	3.99	4.23	4.15	4.05	3.73	4.47	4.12	4.93	1.03	0.007	< 0.001	<0.001
Cholesterol (mg/dL) ¹	203	169	179	156	154	141	137	131	122	115	107	101	1.02	0.002	<0.001	<0.001
Glucose (mg/dL) ¹	73.8	76.3	73.1	75.7	71	74.8	70.8	75.2	72	74.8	70.8	74.2	1.01	0.005	0.003	0.663
Total protein (g/dL)	7.66	7.24	7.69	7.22	7.46	7.11	7.39	7.13	7.29	6.86	7.01	6.66	0.09	<0.001	< 0.001	0.09
BUN (mg/dL)	11.1	10.9	11.5	11	11.6	11.1	12	11.2	12.6	11.7	12.9	12.7	0.36	0.222	< 0.001	0.682
Albumin (g/dL) ¹	4.18	4.14	4.13	4.11	4.01	4.01	3.99	4.07	4.04	3.99	4.02	3.95	1.01	0.782	< 0.001	0.061
Haptoglobin (g/L) ¹	0.49	0.47	0.49	0.48	0.43	0.49	0.45	0.47	0.44	0.46	0.43	0.44	1.05	0.628	0.011	0.04
Calcium (mg/dL)	9.2	9.23	9.21	9.21	9.02	9.03	9.09	9.19	9.17	9.12	9.18	9.11	0.1	0.967	0.026	0.758
Magnesium (mg/dL) ¹	2.77	2.79	2.82	2.92	2.84	2.89	2.84	2.87	2.88	2.91	2.83	2.96	1.02	0.409	0.189	0.747
AOP (TE/mL) ¹	7.94	7.67	7.71	7.92	7.79	7.47	7.75	7.65	7.59	7.37	7.24	7.08	1.03	0.6274	< 0.001	0.083
RONS (RFU) ¹	54.2	40.8	56	41.8	58.4	42.6	62.4	46.9	65.2	50.1	66.7	50.8	1.05	< 0.001	< 0.001	0.928
OSi (arbitrary units) ¹	6.75	5.29	7.19	5.25	7.42	5.66	7.97	6.09	8.51	6.76	9.12	7.13	1.06	< 0.001	< 0.001	0.739

Generalized linear mixed models with repeated measurements were built for the blood variables as outcomes with the Group, Time, their interaction (G×T) as fixed effects.

Wk-6 through Wk -1 represent time points antepartum. HIA: High immunoglobulin A producers; LIA: Low immunoglobulin A producers. SE: Standard error.

TE: Trolox equivalent; RFU: Relative fluorescence units; NEFA: non-esterified fatty acids; BHBA: Beta-hydroxybutyrate acid; BUN: Blood urea nitrogen; AOP: Antioxidant potential; RONS: Reactive oxygen and nitrogen species; OSi: Oxidant status index

Estimated means calculated for the least significant difference.

¹ Natural Log transformed

On the other hand, lower BHBA concentration was associated with cows producing colostrum with higher IgA concentration. With the mobilization of body fat store-derived fatty acids, liver absorbs NEFA and either processes them through the β -oxidation pathway or re-esterifies them to triglycerides and exports them as very low-density lipoproteins (Goff and Horst, 1997, Ospina et al., 2010). The BHBA is the major ketone body found in blood of cows that can be derived from adipose tissue lipolysis and through β -oxidation pathway, in addition to the feed and microbial activity in the rumen of the cow and, from dietary lipids (Goff and Horst, 1997, Ospina et al., 2010). This happens in response to the cows' higher demand of energy for fetal growth and lactose synthesis (Herdt, 2000b). It appears that HCP cows have been able to develop adaptations to cope with the higher energy demands necessary for colostrogenesis using BHBA as a fuel. Nevertheless, consistently elevated concentrations of BHBA negatively affect immune function, health and milk production during lactation period (McArt et al., 2013). To date, the literature does not provide BHBA thresholds for disease risks that could be depicted to dry cows. Nevertheless, when compared to the threshold of BHBA during lactating period which is ≥ 6.97 mg/dL, the prepartum BHBA concentrations found in this study were largely lower. Another finding related to lipid mobilization was that Chol in HCP cows was lower compared to LCP. Indeed, alterations in lipid metabolite profiles, such as lower concentrations of Chol (Reid et al., 1983); or lower triglycerides, Chol, and apoprotein A-1 (Mazur et al., 1989) have been observed in blood from cows with fatty infiltration of the liver. Therefore, it could be possible that alterations in lipid mobilization may have overwhelmed in somewhat degree the liver function of HCP cows since they showed lower concentration of Chol (Emery et al., 1992, Kessler et al., 2014), but not to the point

where inflammation would occur. Therefore, if some subtle degree of inflammation occurred due to this altered nutrient mobilization, it seems that HCP cows have adapted well to the higher demands imposed by colostrogenesis. This may be true because, albeit the concentration of Alb only trending towards statistical significance ($P = 0.051$), Alb had values numerically higher in HCP cows for the entire period of study. Alb is known as a negative acute-phase protein during inflammation, which a decrease in blood concentrations can result from reduced liver synthesis due to inflammatory status (Bertoni et al., 2008). Hence, inflammation is unlikely to have occurred in HCP or even in LCP cows, but rather supports the idea that HCP cows could better cope with the demands of colostrogenesis compared to LCP cows. On the other hand, HIA cows exhibited high concentration of Chol compared to LIA. Since HIA had higher Chol, and lower BHBA, these cows would have been least likely to experience the deleterious effects of altered nutrient utilization, since it indicates healthier liver function. Hence, it is suggested that LIA cows had their immune system more impacted by the presence of higher BHBA concentration.

Moreover, our study demonstrated that cows with LIA had a higher concentration of BHBA compared to HIA cows. The fact that BHBA has detrimental effects on the immune system (Erdmann et al., 2018), might explain why cows produce less IgA. IgA is produced by B cells, secreted in the cows' blood, and transported into the mammary gland by a polymeric immunoglobulin receptor (Johansen et al., 2000). It has been shown that ketone bodies impair the activity of bovine leukocytes in vitro, and these results suggest that this can have a negative effect on the in vivo immune response (Targowski and Klucinski, 1983, Kluciński et al., 1988). Indeed, a study on pregnancy toxemic goats have

demonstrated a significant decrease, not only in serum IgA concentration but also IgG and IgM, when BHBA was increased (Hefnawy et al., 2010), which for IgA concentration aligns with our findings. Nevertheless, since we did not assess the dam's serum Ig concentration or its B cell function, this limits our ability to evaluate variations in the dams' circulating blood Ig concentrations as a potential cause of colostral Ig concentration differences.

In the same study of Hefnawy et al. (2010), there was a positive correlation between Glu and other Ig concentration, whereas in our study we found that cows have a negative correlation between Glu and IgA concentration and positive correlation with IgG concentration. In humans, research has shown that colostrum of hyperglycemic mothers had lower levels of both IgA and IgG (França et al., 2012). Nevertheless, concentration of Glu across all groups of the colostrum variables were similar and within normal limits (Ingraham and Kappel, 1988). Therefore, it is unlikely that variation on Glu concentration affected the transport of Ig through the mammary epithelial cells during this period, but rather it may be more associated with the BHBA concentration affecting lymphocyte functions (Targowski and Klucinski, 1983).

Inflammatory status

We observed no differences in the inflammatory status of cows based on colostrum volume or Ig content, suggesting that physiological homeostasis was not disrupted during late gestation in association with the colostrum variables studied. This is likely due to these biomarkers of inflammation increasing just after parturition (Burfeind et al., 2014, Pohl et al., 2015). Although elevated inflammatory markers postpartum have been associated with adverse health events (Burfeind et al., 2014), they might not have much

overall impact in the process of colostrogenesis. In fact, haptoglobin was relatively lower for all colostrum variables in comparison with values reported for lactating animals (Pohl et al., 2015) while Alb was relatively higher, which was expected because we were looking at pre-calving animals and usually inflammation is exacerbated post-calving (Ceciliani et al., 2012).

Oxidant status

Oxidative stress plays a major role around the time of calving limiting the production efficiency of dairy cattle (Castillo et al., 2005, Sordillo and Aitken, 2009, Abuelo et al., 2015). During this time, oxidative stress impairs functional capabilities of immune cell populations, increases the cow's vulnerability to several diseases (Sordillo and Aitken, 2009) and contribute to dysregulated inflammatory responses and dyslipidemia (Sordillo et al., 2009). Changes in oxidative balance may occur as a result of an increase in RONS, a decrease in AOP, or both parameters changing (Abuelo et al., 2013). Thus, an OSi, as the ratio of pro-oxidant to total antioxidant defenses, provides a more reliable representation of the cow's oxidant status than RONS or AOP concentrations alone. Therefore, it is possible that higher AOP might have supported HCP cows to produce more colostrum, rendering AOP a potential limiting factor in colostrum volume production for cows – which warrants further investigation. Hence, it shifted HCP cows' metabolism towards to a more antioxidant status, resulting in lower OSi.

On the other hand, HIA cows had higher concentration of RONS compared to LIA, and higher OSi. Thus, this index shows a shift towards a higher pro-oxidant status in HIA. However, given that there were no differences in biomarkers of inflammation, we speculate that cell damage, if present, might not have been extensive, which culminated

in higher production of IgA in HIA cows. It is important to note that there were no differences in the redox balance among the groups of IgG and IgM, asserting the complexity of the metabolism of dairy cows. Nevertheless, it is necessary to consider not just the changes in oxidant status but the potential macromolecule damage that can occur as a result of excessive free radical production. Since we only assessed redox balance in this study, quantifying and comparing with the concentration of markers of lipid peroxidation, such as isoprostanes (Niki, 2014, Mavangira et al., 2016), requires further investigation to evaluate oxidative damage. Attempting to promote antioxidant protection against increasing RONS, antioxidant supplements are often added to periparturient cow diets (Abuelo et al., 2015). Therefore, controlling prepartum oxidative stress through micronutrient antioxidant supplementation might help ameliorate the phenotypical changes associated with different maternal stressors given the central role of oxidative stress. Given the relationship observed between lower AOP and colostrum volume, increasing antioxidant capacity in late-gestation cows could potentially enhance the production of higher volumes of colostrum.

Study Limitations

Since this study was observational, we could not determine cause-effect relationships, but rather we were only able to show associations. However, this is the first ever study to investigate changes in biomarkers of MS in association with colostrum variables. Additionally, using the industry standard of colostrum quality to create groups based on IgG concentration resulted in unbalanced groups sizes (218 vs. 10 cows in HIG and LIG), which might have influenced our ability to detect differences due to sample size bias. However, these finding highlights that most cows (95%) are able to produce

colostrum meeting the quality standard of >50g/L IgG. Thus, reinforcing that the volume of colostrum produced is the potential bottleneck for optimal colostrum feeding regimes in dairy farms.

CONCLUSIONS

This study evaluated, for the first time, the metabolic status of dairy cows during the last 6 wk of gestation based on the volume and Ig concentration of colostrum. Despite cows being managed identically, this study highlighted that biomarkers of MS indeed changed throughout the dry cow period, providing an insight to which biomarkers might affect colostrogenesis. Also, it could be observed that cows producing high volume of colostrum and quality as assessed by immunoglobulin content exhibited increased metabolic activity during late gestation. While certain biomarkers shifted during the late gestation period, the changes were not always comparable in magnitude or length to those seen during the lactating period, but indeed produced an effect on colostrum volume and quality. Moreover, our results suggest that greater availability of antioxidants might support the production of higher volumes of colostrum, which warrants further investigation.

**CHAPTER 3: EFFECT OF A DIETARY ANTIOXIDANT SUPPLEMENT GIVEN
TO LATE GESTATION DAIRY COWS ON COLOSTRUM QUANTITY AND QUALITY.**

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ABSTRACT

Our previous study reported lower antioxidant capacity during late gestation in cows producing lower colostrum volume. Therefore, the objective of this study was to evaluate whether administering a dietary antioxidant supplement (DATS) containing 25-hydroxyvitamin D₃ (20mg/cow/d), vitamin E (300IU/cow/d) and β -carotene (400mg/cow/d) during the last 3 wks of gestation improves colostrum volume and immunoglobulin content. The study was conducted in 2 commercial Michigan dairy herds. In each farm, 100 Holstein cows were assigned to either control (CON) or treatment (TRT) groups (50 cows/group), stratified by parity. Cows in CON received their regular diet formulated to meet their requirements whereas cows in TRT group received DATS in addition to the regular diet of each farm. Cows were milked within 6h of calving and the volume of colostrum produced was recorded and an aliquot collected. Concentrations of IgG, IgA and IgM were measured by radial immunodiffusion. Data were analyzed statistically using the Wilcoxon or Fisher's exact test. The median (IQR) of colostrum volume was slightly higher in TRT compared to CON (6.0(4.5) vs 4.5(2.8) L, $P=0.02$). However, the IgG concentration in TRT was lower than in CON (90.3 (45.6) vs 103.7 (40.3) g/L, $P=0.01$). Despite the lower concentration of IgG, the proportion of samples meeting the IgG concentration industry standard of ≥ 50 g/L did not differ between groups ($P=0.66$). Also, there were no differences in IgA (TRT 3.6 (2.8) vs CON 4.1 (4.1) g/L, $P=0.11$), and IgM (TRT 4.0 (2.7) vs CON 3.5 (3.0) g/L, $P=0.61$) concentrations between groups. Collectively, our results showed that DATS increased colostrum volume without markedly reducing its quality as assessed by immunoglobulin content.

INTRODUCTION

Provision of an adequate volume of high quality colostrum soon after birth is essential for calves' optimal health and growth (McGuirk and Collins, 2004). Moreover, feeding additional colostrum helps mature the gastrointestinal tract and enhances digestion efficiency (Bühler et al., 1998, Blättler et al., 2001). Indeed, compared to calves receiving only one meal right after birth, those receiving a second meal within the first 12 h of birth had enhanced ADG and reduced risks for failure of transfer passive immunity and preweaning morbidity (Abuelo et al., 2021).

Nevertheless, the considerable variability in colostrum production among cows (Morin et al., 1997, Gavin et al., 2018, Kessler et al., 2020) may limit the ability of producers to harvest the colostrum volume needed to achieve optimal calf performance. Colostrogenesis starts 3 to 4 weeks before calving, coinciding with cows experiencing intense metabolic adaptations due to a profound increase in nutrient requirements associated with fetal growth and milk production (Bell, 1995, Lean et al., 2013, Sordillo and Mavangira, 2014). Significant increases in metabolic demands may lead some cows to MS, in which their inflammatory and oxidative status would also increase (Abuelo et al., 2015). The detrimental effect of MS on dairy cattle health and production during the transition period is well described (Sordillo and Aitken, 2009, Bradford et al., 2015). Furthermore, MS is known to impair milk yield and immune cell function in periparturient cattle, including the B cells responsible for Ig production (Trevisi et al., 2012, Abuelo et al., 2015). Therefore, it is possible that different magnitudes in the metabolic activity that can lead to MS may also limit colostrogenesis. In fact, our observational study found that cows producing high volumes of colostrum or Ig is associated with changes in biomarkers

of MS pre-calving (Chapter 2 of this dissertation). Nevertheless, the extent to which alleviating MS during late gestation improves colostrogenesis remains unexplored.

Supplementation of trace minerals (Overton and Yasui, 2014) and vitamins (Allison and Laven, 2000, Oliveira et al., 2015, Martinez et al., 2018) just above requirement thresholds has been found to increase the health and efficiency of animals. While the majority of commercial herds supplement with the higher amounts of vitamin E (Allison and Laven, 2000) and selenium during the close-up period (Overton and Yasui, 2014), oxidative stress is still observed in these animals (Kuhn et al., 2018). The use of additional vitamins, such as vitamin A, D and E, have been shown to reduce oxidative stress in periparturient cattle and enhance postparturient health status and productivity (Abuelo et al., 2015). Little is known about the potential benefits that antioxidants may exert on colostrogenesis. In fact, our observational study found that cows producing low volume of colostrum exhibited lower total antioxidant potential (Chapter 2 of this dissertation). Therefore, we hypothesized that cows receiving dietary antioxidant supplement (DATS) containing 25-hydroxyvitamin D3, vitamin E and β -carotene during late gestation would produce more colostrum with a higher Ig concentration. The overall aim of this study was to explore a causal effect relationship of administering a DATS during the last 3 wks of gestation on colostrum volume and Ig content by improving the antioxidant status of the dairy cows.

MATERIALS AND METHODS

Animals, Feed, Farms and Management

Experimental procedures were approved by the Michigan State University Institutional Animal Care and Use Committee (protocol 20/19-004-27) and animals were enrolled with owners' consent. This pen-level supplementation trial was conducted in two commercial Michigan dairy farms that were selected based on proximity to the university and willingness to participate in the study. The study was designed as a before-after group comparison, where CON group was enrolled first and TRT group right after. Sampling occurred during the period between January and April 2020. The sample size was estimated based on the variability seen in late-gestation adult cattle on biomarkers of oxidant status in a previous study (Abuelo et al., 2013) for a power of 80%, a precision of 2%, a type I error rate of 5%, and an attrition rate of 3%, using an online calculator (<https://epitools.ausvet.com.au>). Based on these calculations, 100 Holstein cows were selected in each farm (50cows/group/farm) for a total of 200 cows. From a list of cows generated by the herd management software, Holstein cows expected to calve 6-8 weeks after the start of sampling for each group were randomly selected using randomization software (<https://www.graphpad.com/quickcalcs/randomSelect1/>). To reflect common demographics of dairy farms, random selection of cows to groups was stratified by parity groups of cows entering their first, second, or third to fifth lactation. Exclusion criteria for enrollment were animals not being apparently healthy based on clinical observation, cows with a successful breeding above 150 days in milk, and body condition score (BCS) under 2 or over 4 on a scale of 1 – 5 (Wildman et al., 1982). Cows had ad libitum access to a total mixed ration and water for the entire dry cow period. Cows in CON received their

regular diet formulated to meet their requirements until actual calving date, whereas cows in TRT group received DATS in addition to the regular diet of each farm, starting 3 weeks prior to the expected calving date. Formulation of the regular diet was performed by the owner's preference large animal nutritionist, balanced to meet NRC (2001) dry cow diet recommendations, in which vitamin A $\geq$ 80300 IU/day, vitamin D $\geq$ 21900 IU/day, and vitamin E $\geq$ 1168 IU/day. The DATS used in this study contained additional 25-hydroxyvitamin D3 (20mg/cow/d), vitamin E (300IU/cow/d) and β -carotene (400mg/cow/d) and was provided as an in-kind contribution by the manufacturer (DSM Nutritional Products, Parsippany, NJ). Samples of total mixed ration were collected at biweekly intervals from the feed bunk at the time of distribution and sent to an external laboratory. The ingredient components and analyzed composition of the different prepartum diets of both farms are summarized in Table 3.1 and 3.2, respectively. Housing and characteristics of management practices of late-gestation cows of the two farms are reported in Table 3.3.

Sample Collection

Blood samples were obtained weekly starting 6 wk before expected parturition, taken approximately to the feeding time via puncture of coccygeal vessels using two-10mL evacuated tubes with serum separator (BD Vacutainer; Becton, Dickinson and Company, Franklin Lakes, NJ). Blood samples that were collected within 2 days of actual calving date were not considered as the -1 wk point to avoid changes in blood biomarkers due to the hormonal changes associated with calving, considering the previous week sample instead.

Table 3.1: Ingredient composition of diets.

	Prepartum diet composition						
	Farm A				Farm B		
	Close up cow	Close up heifer	Far off cow	Far off heifer	Close up	Far off cow	Far off heifer
Ingredient, % of DM							
Corn Silage	34.3	40.1	18.2	18.2	53.47	20.91	-
Sorghum Silage	-	-	-	-	-	8.71	30
Beet Pulp Wet	12.9	-	-	-	-	-	-
Haylage	-	-	69.2	69.2	-	48.79	54.44
Wheat Straw	-	28	10.9	10.9	20.58	19.17	-
Grass hay	21.6	-	-	-	-	-	-
Lact TMR Leftover	-	-	-	-	-	-	12.34
Mineral premix	31.2	31.9	1.6	1.6	25.95	2.07	3.21
Salt	-	-	-	-	-	0.35	-

Table 3.2: Analyzed composition of diets.

	Prepartum diet								
	Farm A						Farm B		
	Close up cow		Close up heifer		Far off cow		Far off heifer		
DM, %	44.6	± 3.38	45.2	± 8.27	44.5	± 4.62	44.0	± 0.92	48.8 ± 1.68
Nutrients, DM basis (±SD)									
Proteins									
CP, %	14.0	± 0.72	13.8	± 1.08	13.6	± 1.00	14.3	± 0.00	13.1 ± 0.73
Adjusted Protein, %	14.0	± 0.72	13.8	± 1.08	12.5	± 1.03	13.4	± 0.07	11.3 ± 0.73
Soluble Protein, %	3.8	± 0.63	4.5	± 0.96	7.2	± 0.67	7.4	± 0.07	5.2 ± 0.30
Ammonia (CPE), %	1.5	± 0.18	0.8	± 0.24	2.5	± 0.32	2.5	± 0.27	2.5 ± 0.24
ADICP, %	1.2	± 0.06	1.1	± 0.03	1.9	± 0.15	1.6	± 0.11	1.0 ± 0.10
NDICP, %	2.9	± 0.21	2.4	± 0.20	3.2	± 0.37	2.7	± 0.08	2.0 ± 0.15
Fiber									
ADF, %	26.0	± 1.06	29.9	± 0.90	38.3	± 1.20	33.4	± 1.56	26.3 ± 1.94
aNDF, %	38.0	± 1.71	45.8	± 1.27	41.7	± 15.55	44.3	± 0.49	40.4 ± 2.05
Lignin, %	4.3	± 0.22	4.9	± 0.10	8.6	± 0.61	7.1	± 0.64	4.5 ± 0.37
Carbohydrates									
ESC-Sugar, %	5.0	± 1.08	4.0	± 0.89	4.2	± 0.42	3.9	± 0.21	4.0 ± 0.34
Starch, %	19.8	± 1.26	19.9	± 0.25	8.6	± 2.11	13.5	± 0.28	23.7 ± 1.59
CF, %	2.6	± 0.29	3.0	± 0.88	3.0	± 0.34	3.3	± 0.25	2.6 ± 0.20
Minerals									
Ash, %	8.7	± 0.18	7.5	± 0.08	8.7	± 0.77	9.6	± 0.32	7.7 ± 0.49
Ca, %	1.6	± 0.19	0.8	± 0.09	1.2	± 0.14	1.1	± 0.12	0.9 ± 0.20
P, %	0.4	± 0.04	0.3	± 0.01	0.3	± 0.02	0.3	± 0.01	0.4 ± 0.05
Mg, %	0.5	± 0.02	0.3	± 0.01	0.3	± 0.07	0.4	± 0.02	0.3 ± 0.02
K, %	0.9	± 0.06	1.1	± 0.01	2.1	± 0.08	1.9	± 0.04	1.1 ± 0.03
S, %	0.4	± 0.05	0.2	± 0.02	0.2	± 0.02	0.2	± 0.01	0.3 ± 0.04
Na, %	0.1	± 0.02	0.1	± 0.02	0.1	± 0.04	0.2	± 0.04	0.1 ± 0.01
Cl, %	0.6	± 0.08	0.2	± 0.01	0.5	± 0.05	0.5	± 0.10	0.9 ± 0.07
Fe, ppm	421.8	± 76.40	257.8	± 9.54	320.8	± 98.06	425.0	± 67.88	346.3 ± 76.63
Mn, ppm	134.6	± 6.23	97.8	± 6.08	48.5	± 10.91	65.5	± 9.19	79.7 ± 10.93
Zn, ppm	115.6	± 9.21	105.8	± 9.50	39.8	± 14.34	55.0	± 2.83	69.5 ± 8.83
Cu, ppm	14.4	± 1.95	13.3	± 0.96	10.0	± 1.63	11.0	± 1.41	11.2 ± 3.97
Energy & index calculations									
TDN, %	66.9	± 0.91	64.7	± 0.90	58.4	± 0.94	61.3	± 1.56	66.6 ± 1.71
Net Energy Lactation, Mcal/kg	1.5	± 0.03	1.5	± 0.02	1.3	± 0.02	1.3	± 0.02	1.5 ± 0.04
Net Energy Maintenance, Mcal/kg	1.5	± 0.03	1.3	± 0.02	1.1	± 0.04	1.3	± 0.04	1.5 ± 0.04
Net Energy Gain, Mcal/kg	0.9	± 0.03	0.9	± 0.04	0.7	± 0.02	0.7	± 0.04	0.9 ± 0.04
ME, Mcal/kg	2.6	± 0.12	2.4	± 0.00	2.2	± 0.00	2.4	± 0.15	2.6 ± 0.11
Non Fiber Carbohydrates, %	39.6	± 1.06	32.7	± 0.51	28.7	± 2.23	31.3	± 0.57	38.2 ± 2.23
Non Structural Carbohydrates, %	24.7	± 1.07	23.8	± 0.65	12.7	± 2.40	17.4	± 0.49	27.7 ± 1.44
DCAD mEq/100g	-15.4	± 2.69	13.2	± 1.36	33.4	± 3.77	31.2	± 2.76	-11.8 ± 2.27
									29.4 ± 9.16
									24.7 ± 1.34

Table 3.3: Housing and management practices for late-gestation cows in the study farms.

	Farm A				Farm B		
	Close up cow	Close up heifer	Far off cow	Far off heifer	Close up cows / heifers	Far off cow	Far off heifer
Housing							
Style	Free Stall	Free Stall	Free Stall	Free Stall	Bedding	Free Stall	Free Stall
Fan	✓	✓	-	-	✓	✓	-
Target duration for prefresh diet length (d)	21				21		
Target dry cow length period (d)	60				60		

Tubes were transported to the laboratory on ice, separated within 1 h by centrifugation at $2,000 \times g$ for 20 min at 4°C , snap-frozen in liquid nitrogen, and stored at -80°C until analysis. All samples were frozen in 4 aliquots to prevent multiple freeze-thaw cycles. Colostrum was harvested by trained farm personnel within 6 h of calving. The volume of colostrum produced was measured using a graduated bucket (10 Quart Measuring Pail with Handle, United States Plastic Corporation, Lima, OH). A sample of each cow's colostrum was also collected in a 50 mL container and kept frozen at -20°C for further analysis.

Analytical Determinations

Serum samples

Following the manufacturer's instructions, oxidant status was assessed following reported methodologies (Abuelo et al., 2016). The concentrations of RONS in serum were determined using the OxiSelect In Vitro Reactive Oxygen and Nitrogen Species assay kit (Cell BioLabs Inc., San Diego, CA). Briefly, free radicals present in the sample bind to a dichlorodihydrofluorescein probe, converting it to a fluorescing product (2',7'-dichlorodihydrofluorescein). Thus, the fluorescence intensity is proportional to the concentration of RONS in the sample. The fluorescence of dichlorofluorescent dye was determined at excitation wavelengths of 480 nm and emission of 530 nm in a Synergy H1 Hybrid plate reader (Biotek; Winooski, VT, USA). To ensure fluorescence at various concentrations, a standard curve, made by six serial dilutions (0–10,000 nM) of the fluorescence probe 2',7'-dichlorodihydrofluorescein diacetate was included in each plate (Fluotrac Black 96 well plate, Greiner Bio-One, Frickenhausen, Germany). All samples and standards were analyzed in duplicates. Background fluorescence was eliminated by

subtracting blank values from sample values. Results are reported as the average RFU between replicates. The AOP of serum samples were determined using trolox (synthetic vitamin E analog) equivalents antioxidant capacity, as described previously (Re et al., 1999). Antioxidant components of serum interact, making it difficult to quantify each antioxidant individually. As a result, this method considers the synergism of all antioxidants present in a sample, including albumin, thiols, bilirubin, and superoxide dismutase. In brief, based on the standard curve of 0–25 mg/mL, a known volume of trolox standard concentration would result in a similar reduction of the radical 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (Sigma-Aldrich, St. Louis, MO). Changes in oxidative balance may occur because of shifts in RONS and/or AOP. Thus, the OSi was calculated as the ratio between RONS and AOP, as this better characterizes shifts in redox balance in periparturient cows (Abuelo et al., 2013).

The serum concentration of BHBA, BUN, Ca, Chol, Glu, Mg, NEFA, TP were quantified using commercial reagents from Catachem Inc. (Bridgeport, CT) as biomarkers of nutrient utilization. As biomarkers of inflammation, we determined the concentrations of the positive and negative acute phase protein Hp (Phase Haptoglobin Assay TP-801, Tridelata Development Limited, Maynooth, Ireland) and Alb (Catachem Inc. Bridgeport, CT), respectively. All biomarkers related to nutrient utilization and inflammation were determined using a small-scale biochemistry analyzer (CataChemWell-T; Catachem Inc.). The analyzer was calibrated every week using the assay manufacturer's calibrators. Reference samples were also analyzed at the time of calibration for two-level quality control.

Colostrum samples

The IgG, IgA, and IgM concentrations of colostrum samples were measured via RID (Bovine IgG, IgA and IgM test; Triple J Farms, Bellingham, WA) following manufacturer's instructions (<https://kentlabs.com/iji/triple-j-farms-product-information/rid-plate-procedure/>). The method is based on the precipitation in agarose gel growing in a circle antigen-antibody complexes which develop after 10-20 hours at room temperature and continues to grow until equilibrium is reached. Briefly, the colostrum samples were thawed overnight at 4 °C. Dilutions of each sample were performed in 0.9% NaCl. Samples were diluted at 1:6, 1:9, 1:10 for IgG analyses and at 1:2, 1:4 and 1:5 for IgA and IgM quantification. Standards were included in each plate for reference and ranged from 1.8 to 28.03 (IgG), 0.53 to 3.87 (IgA), and 0.62 to 3.81 (IgM) g/L. The diffusion ring through the agarose gel containing mono-specific antibody after 24 h of incubation at room temperature was measured using a caliper with precision of 0.1 mm (VWR traceable caliper; Radnor, PA). The values of the sample's ring were read off the standard curve to determine Ig concentrations in g/L. Samples falling outside of the standard curve were re-assayed using a higher or lower dilution.

Statistical analyses

All statistical analyses were performed with JMP Pro v.15.2 (SAS Institute Inc., Cary, NC) and the criterion for statistical significance was established at $P < 0.05$ and statistical trends were declared between $\alpha = 0.05$ and 0.1. For the descriptive statistical analysis, four groups of Wilcoxon test were fitted to the data. For the first, second, third, and fourth groups, the independent variables of interest were volume (VOL), IgG, IgM and IgA, respectively. We used Fisher's exact test for the groups categorized in HCP vs

LCP and HIG vs LIG as dependent variables. For both models, the independent variables were CON and TRT.

In order to analyze the shifts of each biomarker within treatments, linear mixed models with repeated measures were built for the cows' variables Alb, BHBA, BUN, Ca, Chol, Glu, Hp, Mg, NEFA, TP, RONS, AOP and OSi. Fixed effects included time (sampling weeks -6 to -1 relative to actual calving), group CON vs TRT and their interaction. Cow, lactation number, and farm were used as random effects. For repeated measures, the covariance structures autoregressive 1, compound symmetry, or unequal variances were tested for each variable, and the one with the lowest Akaike information criterion was chosen. Model assumptions were assessed by evaluation of homoscedasticity and normality of residuals. To satisfy these assumptions, some variables were natural log or square root-transformed and the resulting least squares means estimates were subsequently back transformed and presented as geometric means. All *P*-values given are those controlled for multiple comparisons with Tukey's honestly significant difference test.

RESULTS

The median (IQR) of colostrum volume was higher in TRT compared to CON (6.0(4.5) vs 4.5(2.8) L, *P*=0.02). However, the IgG concentration in TRT was lower than in CON (90.3 (45.6) vs 103.7 (40.3) g/L, *P*=0.01). The distribution of the colostrum volume and IgG, IgA, and IgM are demonstrated in Figure 3.1. Despite the lower concentration of IgG, the proportion of samples meeting the IgG concentration industry standard of ≥ 50 g/L did not differ between groups (TRT: HIG = 94.8% vs LIG = 5.2%; CON: 96.7% vs 3.3%; *P*=0.66). Also, there were no differences in IgA (TRT 3.6 (2.8) vs CON 4.1 (4.1)

g/L, $P=0.11$), and IgM (TRT 4.0 (2.7) vs CON 3.5 (3.0) g/L, $P=0.61$) concentrations between groups. The mean (SE) concentration of the cow biomarkers is presented by sampling point for the and TRT vs CON group (Table 3.4).

DISCUSSION

Colostrum volume and quality performance

The present experiment revealed that supplementing prepartum diets with DATS increase yields of colostrum in dairy cows (Figure 1-A). Despite finding higher volume of colostrum in TRT group with statistical differences compared to CON, the distribution of colostrum volume between groups are relatively the same, as well for IgG, IgM, and IgA. It has been reported that when cows were supplemented with β -carotene (Oldham et al., 1991), calcidiol (Martinez et al., 2018), or vitamin E (Wichtel et al., 2004, Moeini et al., 2009) as a strategy to improve cows health and milk performance, indeed increase milk yield was observed. Nevertheless, limited information is known if the effects that antioxidants have on enhancing milk production would exert the same on colostrogenesis. For this study, nonetheless, perhaps the supplementation rate of antioxidants used was not sufficient to produce larger amounts of colostrum and/or enhance Ig synthesis; or it might be that the supplementation already existent on the farm in the cows' diet suffice their requirements, as the levels of antioxidants provided already exceeded NRC recommendations.

Figure 3.1: Distribution of colostrum (A) volume, (B) IgG concentration, (C) IgA concentration, and (D) IgM concentration by treatment groups DATS vs CON. The whiskers of the Box Plots represent the 95% confidence interval.

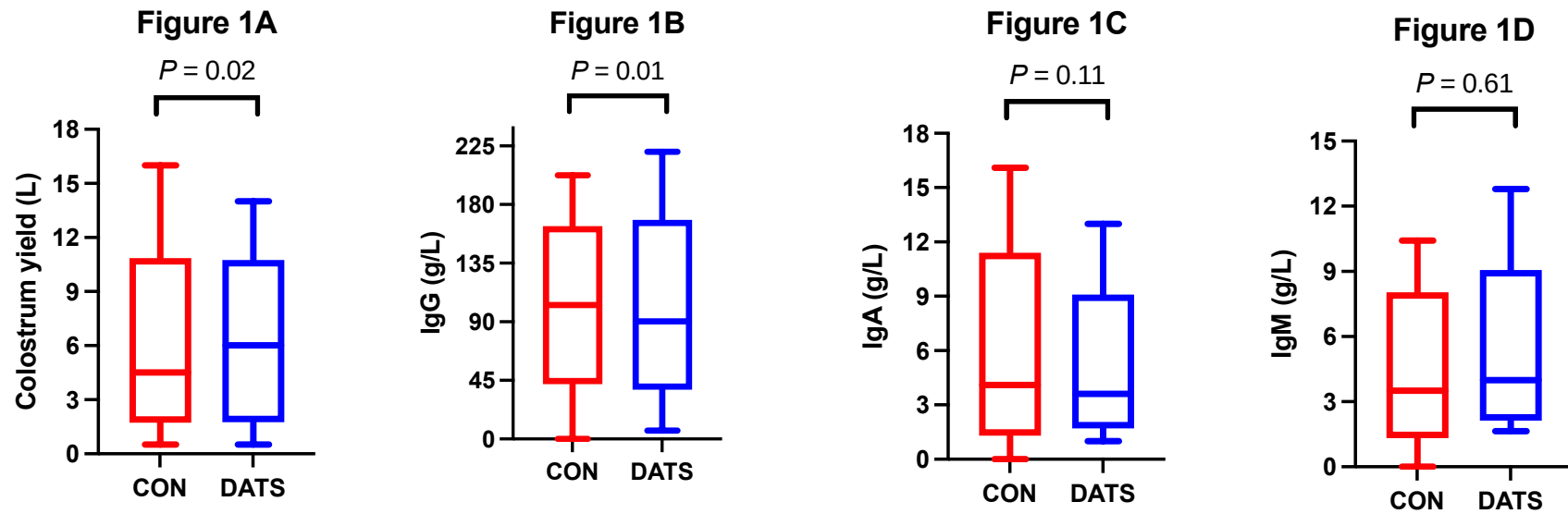


Table 3.4: Estimated means and significance of the main effects from the generalized linear mixed models of blood biomarkers of late-gestation dairy cows comparing groups of control diet (CON, n=93) and treatment diet (TRT, n=97).

Outcome (units)	Estimated means of the different time points															
	Wk -6		Wk -5		Wk -4		Wk -3		Wk -2		Wk -1		P value			
	CON	TRT	CON	TRT	CON	TRT	CON	TRT	CON	TRT	CON	TRT	SE	Group	Time	G×T
NEFA (mmol/L) ¹	0.28	0.23	0.28	0.24	0.28	0.23	0.27	0.24	0.25	0.23	0.28	0.25	0.07	0.068	0.259	0.936
BHBA (mg/dL) ¹	4.11	4.24	4.26	4.19	4.05	4.09	3.91	4.37	4	4.43	4.57	4.48	0.03	0.246	0.002	0.038
Cholesterol (mg/dL) ¹	180.6	180.9	164.3	163.1	146.2	141.4	134.5	126.7	119.5	112.6	103.1	95.1	0.03	0.287	< 0.001	0.084
Glucose (mg/dL) ¹	84.63	84.74	83.06	84.32	82.38	81.37	83.24	81.36	81.47	79.86	78.05	77.58	0.02	0.579	< 0.001	0.206
Total protein (g/dL)	7.93	7.42	7.88	7.55	7.78	7.41	7.76	7.25	7.5	7.16	7.21	6.79	0.13	0.009	< 0.001	0.448
BUN (mg/dL) ¹	10.97	12.07	11.19	12.81	11.27	13.72	11.54	13.75	12.11	13.6	12.82	13.81	0.74	< 0.001	< 0.001	< 0.001
Albumin (g/dL) ¹	4.3	4.19	4.23	4.08	4.11	4.01	4.16	4.03	4.13	4.05	4.09	4.01	0.01	0.192	< 0.001	0.792
Haptoglobin (g/L) ²	0.31	0.37	0.34	0.38	0.35	0.39	0.34	0.41	0.36	0.41	0.34	0.4	0.02	0.025	0.115	0.673
Calcium (mg/dL) ¹	8.9	9.05	8.97	9.25	8.86	9.28	9	9.35	9.03	9.33	9.04	9.38	0.01	0.036	0.004	0.546
Magnesium (mg/dL)	2.75	2.61	2.71	2.67	2.73	2.74	2.74	2.82	2.73	2.86	2.84	2.89	0.07	0.86	< 0.001	0.006
AOP (TE/μL) ¹	8.01	7.62	8.57	7.5	8.22	7.37	8.06	7.36	7.81	7.07	7.54	6.85	0.02	< 0.001	< 0.001	0.529
RONS (RFU) ¹	65.98	121.5	69.82	130.7	69.4	137.3	73.81	140.7	76.02	140.2	75.84	130.8	0.05	< 0.001	< 0.001	0.113
OSi (arbitrary units) ¹	8.28	15.92	8.16	17.42	8.44	18.63	9.16	19.14	9.74	19.84	10.05	19.11	0.05	< 0.001	< 0.001	0.113

Generalized linear mixed models with repeated measurements were built for the blood variables as outcomes with the Group, Time, their interaction (G×T) as fixed effects.

Wk -6 through Wk -1 represent time points antepartum. CON: Control group; TRT: Treatment group. SE: Standard error.

TE: Trolox equivalent; RFU: Relative fluorescence units; NEFA: non-esterified fatty acids; BHBA: Beta-hydroxybutyrate acid; BUN: Blood urea nitrogen; AOP: Antioxidant potential; RONS: Reactive oxygen and nitrogen species; OSi: Oxidant status index.

Estimated means calculated for the least significant difference.

¹ Natural log-transformed

² Square root transformed

We observed in our study that TRT group had lower concentration of serum TP compared to CON throughout the whole period of study. Although the mechanism for this response cannot be elucidated based on our data, it might be suggestive of higher utilization of proteins due to the increase in demands of colostrum protein production (Dixon et al., 1961). This finding was accompanied by the significant higher serum concentration of BUN in TRT group. It has been reported that rises in BUN are associated with high-protein feed intake in dairy cows (Butler, 1998, McCormick et al., 1999). Because cows were fed ad libitum, we were unable to determine any differences in DMI that could potentially explain this. Tsunoda et al. (2017) reported that BUN concentration is likely to have increased as a result of increasing proportion of soluble proteins in the feed. Increased soluble protein concentration have been shown to increase ruminal ammonia concentrations (Ludwick et al., 1972, Broderick and Reynal, 2009), which results in increased ammonia absorption by the ruminal wall. Ammonia is converted to urea in the liver (Hammond, 1997), resulting in elevated BUN. Therefore, it is possible that BUN activated polymorphonuclear leukocytes that resulted in higher concentration of RONS in the TRT group.

Moreover, cows in TRT group had higher concentration of haptoglobin, which is a positive acute-phase protein that in an event of inflammation higher concentrations can be found in the blood (Bionaz et al., 2007, Ceciliani et al., 2012). Most studies have concentrated on early postpartum haptoglobin concentrations (Huzzey et al., 2009, Pohl et al., 2015) and, to the author's knowledge, the only study reporting association between haptoglobin and colostrogenesis is described in this dissertation thesis on chapter 2. Nevertheless, tissue damage may induce the release of these liver acute phase proteins

(Cray, 2012), which this elevated concentration of Hp might have occurred due to the higher concentration of RONS, as described aforementioned. It is important to notice that high concentration of RONS in TRT group started in the very first week of sampling even before starting the supplement. Despite TRT cows exhibiting higher values of haptoglobin, they were relatively lower in comparison with values reported for lactating animals (Pohl et al., 2015) while Alb was relatively higher, which was expected because we were looking at pre-calving animals and usually inflammation is exacerbated post-calving (Ceciliani et al., 2012). To the best of our knowledge, no clear relationship was observed between inflammation during late gestation and the risk of lower colostrum volume and quality in dairy cows by analyzing the results as in a whole (Table 3.4 and Figure 3.1 A – D).

Because the approach of pen-level design, animals were found in different levels related to colostrum volume and Ig content. For instance, TRT group already started on the first week of enrollment with higher levels of pro-oxidants compared to CON group and kept constant throughout the entire study period (Table 3.4). This pen-level design was chosen based on the feasibility of completing a dietary supplement study in commercial dairy farm settings where cows cannot be individually fed, otherwise it would not be feasible to investigate this supplementation trial on commercial dairy farms. In addition, we wanted to execute the study on these farms, because the results acquired from it better depict the real challenges of dairy farms. Knowing the limitations of this design, we tried to minimize as much possible the overlapping of the groups related to consumption of the diets and differences that the diet may have had, enrolling cows in the groups, TRT vs CON, 3 weeks apart from each other. Unfortunately, despite all these

efforts we found wide discrepancies in the results of biomarkers of MS that may be attributed to the study design approach rather than the supplementation treatment.

CONCLUSION

This study evaluated, for the first time, the supplementation of DATS to dairy cows during the last 3 wk of gestation as a strategy to improve colostrum volume and Ig content by improving the antioxidant status of the dairy cows. Indeed, the supplementation with DATS increased yields of colostrum in dairy cows with statistical significance. Nevertheless, the distribution of colostrum volume was similar between TRT and CON groups. Despite cows in TRT group produced lower IgG content, when we take in consideration the industry standard of colostrum quality $\geq 50\text{g/L}$, most cows were above this threshold, as similar as it was in CON group. No differences were found on IgA and IgM concentrations between groups. It is important to note that as per the pen-level design approach, animals were found in different levels related to colostrum volume and Ig content. Therefore, we discovered significant disparities in the findings of biomarkers of MS, which may be due to the study design methodology rather than the supplementation procedure. Collectively, the supplementation with DATS produced beneficial results in terms of performance of dairy cattle, which may aid dairy farms to better meet the calves feeding requirements of $\geq 6\text{L}$ of colostrum. Thus, potentially reduces the risk of failure of transfer passive immunity and diseases in calves.

CHAPTER 4: SUMMARY AND CONCLUSIONS.

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This chapter will be submitted for publication in a peer-reviewed journal.

SUMMARY AND CONCLUSIONS

In our studies, we observed that the volume and immunoglobulin content of colostrum were associated with changes in the metabolic status of dairy cows during late gestation. The study described in *Chapter 2* reported, for the first time, that dry period nutritional and different degrees of metabolic adaptation associated with the quality and quantity of colostrum production, assessed by the immunoglobulins G, A, and M. In this project, cows that produced high colostrum volume exhibit higher concentration of antioxidants in blood. This led us to investigate if, by increasing the circulating antioxidant potential in dairy cows with increments of antioxidants to the diet would enhance the production of colostrum volume. In the study reported in *Chapter 3*, additional dietary antioxidant supplement, containing 25-hydroxyvitamin D3, vitamin E and β -carotene to the basal diet of dairy cows during the final three weeks of gestation could improve colostrum production. Nevertheless, despite finding higher volume of colostrum in treatment group with statistical differences compared to control, the distribution of colostrum volume between groups are relatively the same, as well for IgG, IgM, and IgA.

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