Effects of colostrum management practices on the neonatal dairy calf

by

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Abstract

The timely feeding of an adequate volume of high quality colostrum immediately after birth is one of the key factors influencing the health and survival of the neonatal dairy calf. Therefore, the aim of this thesis was to investigate the effect of current colostrum management practices on the passive transfer of IgG, intestinal bacterial colonization, and the presence of bovine colostrum oligosaccharides (bCOs) in the gastrointestinal tract (GIT) of neonatal calves. In order to assess the effect of delaying the first colostrum meal, in chapter 2 calves were fed pooled, pasteurized colostrum at either 0 h, 6 h, or 12 h of life. Results indicated that feeding colostrum at 6 h and 12 h decreased the passive transfer of IgG when compared to calves fed at 0 h. Yet, no differences were observed in passive transfer between 6 h and 12 h calves, indicating that between 1-6 h of life the absorptive capacity of the intestine may decrease, thus leading to similar IgG concentrations in these groups. In addition, at 51 h of life, 12 h calves tended to have a lower prevalence of bacterial groups in the colon, specifically *Bifidobacterium* and *Lactobacillus,* suggesting that delaying the delivery of colostral nutrients may impact early life microbial colonization. In chapter 3, the effect of heat-treatment of colostrum on the concentration of oligosaccharides in bovine colostrum and in the intestine of neonatal calves was assessed. Results revealed that heat-treatment at 60°C for 60 min increased the concentration of free bCOs when compared to unheated colostrum. It is hypothesized that this may be due to the cleavage of bCOs from proteins and lipids in colostrum during heat-treatment. In contrast, calves fed heat-treated colostrum displayed a lower concentration of bCOs in the small and large intestine at 6 h of life compared to calves fed unheated colostrum. This result may be due to the metabolism of bCOs as a carbon source for beneficial intestinal bacteria, however further research is needed. The results of this thesis have a significant impact on the dairy industry as it

demonstrates that delaying the first feeding of colostrum – even by increments of 6 h – can have a large influence on the neonatal calf. Moreover, it emphasizes potential prebiotic compounds that may be used to improve gastrointestinal health during the early life of the dairy calf.

Preface

This thesis encloses the original work of Amanda Judy Fischer with collaborations led by Dr. Michael Steele at the University of Alberta. Animal experiments in chapter 2 and 3 were conducted at the Dairy Research and Technology Centre at the University of Alberta. Co-authors for chapter 2 include Yang Song and Dr. Zhixiong He of the University of Alberta, who contributed to experimental design, experimental sampling, and manuscript preparation. In addition, chapter 2 co-authors include Dr. Deborah Haines of the Saskatoon Colostrum Company Ltd., who provided pooled colostrum and assisted in manuscript preparation, as well as Dr. Le Luo Guan of the University of Alberta who contributed to experimental design and manuscript preparation. Additionally, Dr. Le Luo Guan and Dr. Nilusha Malmuthuge of the University of Saskatchewan conducted the animal experiment described in chapter 3 and contributed to manuscript preparation.

The research projects conducted in this thesis received ethics approval from the Animal Care and Use Committee for Livestock at the University of Alberta and all procedures performed on animals were conducted according to the guidelines of the Canadian Council of Animal Care (Ottawa, ON, Canada, 2009).

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List of Abbreviations

AEA	Apparent Efficiency of Absorption
AcN	Acetonitrile
AUC	Area Under the Curve
BW	Body Weight
BBW	Birth Body Weight
bCO	Bovine Colostrum Oligosaccharide
BGOS	*Bifidobacterium* Galacto-Oligosaccharide
C_{max}	Maximum Concentration
DIM	Days in Milk
EcN	*Escherichia coli* strain Nissle 1917
ETEC	Enterotoxigenic *Escherichia coli*
FA	Fatty Acids
FcRn	Neonatal Fc Receptor
FPT	Failure of Passive Transfer
GIT	Gastrointestinal Tract
HC	Heat-treated Colostrum Calves
HILIC	Hydrophilic Interaction Chromatography
hMO	Human Milk Oligosaccharide
HPLC	High Performance Liquid Chromatography
HT	Heat-treated
LAB	Lactic Acid Bacteria
LA	Lactic Acid
LC-MS	Liquid Chromatography-Mass Spectrometry
MOS	Mannan-Oligsoaccharide
MRM	Multiple Reaction Monitoring
NC	No Colostrum Calves
NCD	Neonatal Calf Diarrhea
OS	Oligosaccharide
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
SCFA	Short Chain Fatty Acid

T_{max}	Time to Maximum Concentration
UH	Unheated
UC	Unheated Colostrum Calves

1.0 Chapter 1: Literature Review

1.1 Introduction

The heifer calf is the future of the dairy herd. Yet, amongst all animals present on the dairy farm, the highest morbidity and mortality rates occur in calves pre-weaning, with rates as high as 46% and 5%, respectively (NAHMS, 2011). This can be costly to producers and also causes concern from an animal welfare standpoint. The three most prevalent ailments resulting in illness and death in the pre-weaning calf include septicemia, pneumonia, and most commonly worldwide, neonatal calf diarrhea (NCD) (Meganck et al., 2014). Economic losses not only occur from mortality, but also the costs involved in managing morbidity, including treatment, diagnostics, labour, veterinary intervention and impaired growth performance (Bazeley, 2003; El-Seedy et al., 2016). Therefore, knowledge and strategies focusing on how to prevent and decrease the prevalence of infectious disease and subsequent illness and mortality in calves are fundamental to ensuring a profitable dairy industry.

The neonatal calf is born agammaglobulinemic, thus rendering it immune deficient and susceptible to disease during the neonatal period. Therefore, the calf relies on immunoglobulin-rich colostrum to provide it with passive immunity to protect it against pathogens it may encounter during early life. Despite the established benefits of the timely feeding of adequate volumes of colostrum containing over 50 g of IgG per L, many producers continue to use poor colostrum management practices that increase the risk of morbidity and mortality of dairy calves (Vasseur et al., 2010). Major risk factors include failing to assess the quality of colostrum fed to calves, relying on the dam to provide colostrum – which may have inadequate IgG concentrations or be contaminated by bacteria – and low night time surveillance, which results in

calves receiving their first colostrum meal at 6 h of life or later (Vasseur et al., 2010). These poor management practices likely contribute to or cause the high prevalence of failed passive transfer (FPT) of IgG in neonatal calves. Specifically, it was estimated that 19% of heifer calves fail passive transfer in the USA and that 41% of dairy operations had at least one calf with FPT (Beam et al., 2009).

Although achieving successful passive transfer of IgG has long been identified as a key factor influencing the overall health and survival of the neonatal calf, the proper succession and establishment of a healthy gastrointestinal microbiota is also critical. Studies using human and mouse models have demonstrated the importance of the gut microbiota to the development of the mucosal immune system; moreover, microbes are involved in the digestion of carbohydrates which supply energy to gastrointestinal epithelial cells and prevent the establishment of pathogenic organisms (Guarner, 2006). In pre-weaned dairy calves, the presence of specific bacterial genera and species has been associated with weight-gain and diarrhea incidences, further emphasizing their role in calf performance and health (Oikonomou et al., 2013). Colostrum has been demonstrated to play a key role in the establishment of gastrointestinal microbiota (Malmuthuge et al., 2015a), yet knowledge regarding these specific colostral compounds and how current colostrum management strategies affect the microbiome of the neonatal calf are limited. Understanding how early life nutrition influences the gut microbiota is essential to developing effective strategies to reduce infection and subsequent disease in the calf and this knowledge may be used to develop prebiotic and probiotic formulas to assist the calf in achieving a balanced microbiome and overall gut health.

1.2 Current Dairy Farm Colostrum Management Practices

The placenta of the bovine dam is cotyledonary, in which 100-140 focal villous aggregations (cotyledons) develop and attach to the maternal caruncles to form placentomes that function as the main exchange for oxygen, carbon dioxide, nutrients and fetal metabolic products (Haeger et al., 2016). However, the bovine placenta is also epitheliochorial, meaning that the uterine epithelium and the maternal blood vessels remain in tact throughout gestation due to the non-invasive nature of the trophoblast (Pereira et al., 2013). This phenomena results in complete separation of the maternal and fetal vascular systems and thus there is no passive transfer of antibodies to the calf in utero (Wooding, 1992). Therefore, the calf is born immune-deficient and relies on colostrum to protect it against any disease challenges it may encounter in early life.

Among all immunoglobulin classes in colostrum, IgG is present at the highest concentration and colostrum containing 50 g of IgG per L or greater is considered good quality (Godden, 2008). However, the IgG content of colostrum can widely vary, with analysis of more than 150 colostrum samples from 7 dairy farms in the U.S. revealing that the concentration of IgG can ranges from 7.1 to 159 g/L, with16% of samples containing less than 50 g/L (Quigley et al. 2013). Given this high variation, accurate measurement of colostral IgG concentrations before feeding it to calves is essential for proper management. Unfortunately, analysis of colostrum to determine IgG concentrations is not easily done and is only evaluated by 13% of producers, with 56% of those estimating the quality solely based on visual inspection (NAHMS, 2007). Typical on-farm tools to determine colostrum quality include the colostrometer, which measures the specific gravity of the colostrum, and the Brix refractometer that approximates the percentage of total solids (Fleenor & Stott, 1980; Quigley et al., 2013). The gold-standard for estimating IgG

concentration is radial immunodiffusion, however this analysis is only used for research purposes and not performed on-farm as it is time-consuming and expensive.

Passive transfer is defined as the transfer of IgG from colostrum to the neonate, and serum IgG concentrations ≥ 10 mg of IgG per ml at 1 to 7 d of age signifies successful passive transfer (BAMN, 2001; Tyler et al., 1996). When serum IgG concentrations fall below this concentration, indicating FPT, this predisposes the neonate to the development of disease (Weaver et al., 2000). High rates of FPT have been associated with increased calf morbidity and mortality (DeNise et al., 1989), and inadequate amounts of good quality colostrum is associated with the future productivity of heifer calves by decreasing first- and second-lactation milk production (Robison et al., 1988; Faber et al., 2005). Although the consequences of FPT are well known, serum samples collected from ~1800 heifer calves across 394 dairy operation in the U.S. indicated that FPT rates are alarmingly high (~19%) and that almost half of the farms had at least one calf that has experienced FPT (Beam et al., 2009).

Ensuring the passive transfer of IgG to the calf is highly dependent on the volume and IgG concentration of the colostrum feeding, as well as the method used (esophageal tube, bottle feeding or suckling from the dam) (Weaver et al., 2000; Johnson et al., 2007; Godden et al., 2008). The rate of FPT is dramatically decreased when calves receive more than 100 g of IgG (Besser et al., 1991) and it has been demonstrated that calves fed colostrum at 10% of their body weight (BW) (e.g. 4 L of colostrum for a 40 kg calf) achieve greater serum IgG concentrations for the first 3 d of life compared to calves fed at 5% BW (Dunn et al., 2017). It is also recommended that colostrum be fed using an esophageal tube feeder, as it is rare that a newborn would consume 3-4 L via nipple bottle immediately after birth in one feeding (Besser et al., 1991; Weaver et al, 2000). Specifically, calves receiving colostrum via suckling the dam, a

nipple bottle, or an esophageal tube had FPT rates of 61%, 19%, and 10%, respectively, further emphasizing the advantages of esophageal tube feeding (Besser et al., 1991).

The timing of the first meal of colostrum is also an essential factor in ensuring successful passive transfer, as the absorption of IgG is optimal during the first hours of life and decreases in a linear trend as the calf ages (Stott et al., 1979a; Bush & Staley, 1980; Matte et al., 1982). Stott et al. (1979) fed neonatal calves 0.5, 1, or 2 L of colostrum at seven different time periods after birth and determined that closure of the small intestine occurs at approximately 21 h for calves fed at 0 h of life. Yet if colostrum feeding is delayed until 24 h then closure occurs at 33 h of life, indicating that the length of time the calf is actually absorbing colostrum is reduced from 21 h to 9 h. This is likely the reason for increased FPT and risk of illness and death in calves that were fed colostrum at 12 h and 24 h of life (Stott et al., 1979b). Although the importance of timely feedings of colostrum has been known for decades, a survey conducted in Canada in 2010 noted that many calves are still receiving their first colostrum meal more than 6 h after birth (Vasseur et al., 2010). Exactly how feeding colostrum at 6 h of life or later will affect the IgG status of the calf using present day colostrum recommendations (~3-4 L of colostrum containing ≥ 50 g/L of IgG) is unknown, however previous work suggests that that calves fed earlier in life will achieve greater passive transfer than those fed later, regardless of the volume or amount of IgG (Stott et al., 1979a; Bush & Staley, 1980; Matte et al., 1982; Dunn et al., 2017).

Although colostrum contains IgG that can protect the calf, it also contains substances that may be harmful, such as pathogenic bacteria. Maternal colostrum may be subjected to contamination directly from the mammary gland or through unhygienic or improper collection and handling (Stewart et al., 2005). In order to reduce the presence of bacteria, on-farm pasteurization of colostrum – the process of heating to a temperature high enough to adequately

reduce the bacterial load to a level that poses little risk of infection – has become a common management practice, with 20% of dairy heifers receiving heat-treated (HT) colostrum in the U.S. (Butler et al., 2000; Keswani & Frank, 1998; NAHMS, 2016). Heat-treatment of colostrum at a temperature of 60°C for 30-60 min results in no differences in IgG concentration when compared to pre-heated colostrum, yet calves fed HT colostrum achieve higher serum IgG concentrations than those fed unheated colostrum (Godden et al., 2006; McMartin et al., 2006; Johnson et al., 2007; Elizondo-Salazar and Heinrichs, 2009; Kent-Dennis, 2014). Recent large-scale studies (Godden et al., 2012; Armengol & Farile, 2016) containing more than 500 animals also demonstrated that calves receiving HT colostrum had higher serum IgG concentrations and were at significantly lower risk for illness and death compared to calves fed unheated colostrum. It has been determined that IgG absorption is negatively associated with colostrum total bacteria counts (Godden et al., 2012; Cummins et al., 2017) and it is speculated that this relationship may be due to: (1) pathogens binding to and neutralizing IgG from colostrum; (2) pathogenic bacteria accelerating the replacement of cells permeable to IgG or damaging these cells; or (3) non-specific pinocytosis of viable bacteria blocking the absorption of IgG (Corley et al., 1977; James et al., 1981). Regardless of the mechanism by which this may occur, it is clear that on-farm heat-treatment of colostrum has both welfare and economic benefits and should be promoted in a similar manner to the timely feeding of adequate volumes of colostrum.

1.3 Immunoglobulin G and Passive Transfer in the Neonatal Dairy Calf

Several classes of immunoglobulins exist, with IgG, IgA, and IgM being the major immunoglobulin classes found in mammary secretions. The half-life of IgG is approximately 1-3 weeks, which is typically much longer than that of IgA or IgM (1-2 d) (Cervenak & Kacskovics,

2009). As mentioned previously, IgG is the primary class found in bovine colostrum, with subclass IgG_1 accounting for approximately 90% of the total IgG in maternal colostrum (Barrington et al., 2001; Larson et al., 1980). The IgG molecule is a monomeric immunoglobulin weighing approximately 160 kDa and is composed of two identical light chains and two identical heavy chains, both with constant and variable regions (Butler, 1983). The heavy and light chains are bound together by a disulfide bond, giving the immunoglobulin its classic Y-shape (Figure 1-1) (Mix et al., 2006). Each IgG molecule has two antigen-binding sites on one end (the antigen binding fragment, Fab), and the constant fragment (Fc) on the other end, which can bind to Fc receptors on various cell types (Figure 1-1) (Hurley & Theil, 2011).

For over 40 years, the absorption of IgG from colostrum into the intestinal epithelial cell of the neonatal calf was thought to be non-specific and receptor independent, mainly because the absorption is confined to such an intense, short period, that the role of any specific receptor would presumably be negligible (Brambell, 1970). The intestinal cells of the neonatal calf have the ability to non-selectively absorb macromolecules within 24-36 h after birth (Staley & Bush, 1985; Stott et al., 1979b) – an ability that is thought to be unique to fetal enterocytes (El-Nageh, 1967). Macroscopically, Smeaton & Simpson-Morgan (1985) demonstrated that immediately after colostrum is ingested, protein-filled vacuoles are present all along the intestinal villi; however after the second day of life only the upper portion of villi contains these vacuoles. By the third day of life, the enterocytes containing vacuoles are no longer present and an entirely new type of cell covers the intestinal surface. Other researchers suggested a similar mechanism in the pig, with antibodies being transported from the lumen through a tubule at the base of the microvilli, followed by the formation of small vesicles that assemble into large vacuoles that are then released at the cell surface and absorbed into the blood (Kraehenbuhl & Campiche, 1969;

Ockleford & Whyte, 1980). Histological analysis of the newborn calf intestine also revealed that ileal enterocytes were the most active in IgG uptake and that the ileal vacuoles were larger compared to those in jejunal enterocytes (Staley et al., 1972). The cessation of the absorption of macromolecules from the gut into the blood of neonates may not only be attributed to the turnover of fetal intestinal cells, but may also be influenced by hormones. In particular, cortisol concentrations are increased in calves deprived of colostrum, which may induce early closure of the intestine and prevent the subsequent absorption of IgG (Kruse & Buus, 1972; Stott et al., 1976; Stott et al., 1980). However, reports are conflicting and the exact mechanism by which this occurs has yet to be determined (Johnston and Oxender, 1979; Stott et al., 1978).

1.4 Bioactive Factors and Oligosaccharides in Colostrum

Aside from providing the calf with passive immunity, colostrum is also rich in nutrients that aid the calf in the necessary GIT adaptations required for proper function. Colostrum contains almost two times the amount of gross energy and crude fat, and four times the amount of protein compared to mature milk (Blum & Hammon, 2000; Nissen et al., 2017). In addition to an abundance of energy and macromolecules, colostrum also contains fatty acids (FA), essential and non-essential amino acids, lactose, vitamins, and minerals (Blum & Hammon, 2000). These nutrient factors, along with IgG, have long been recognized, and over the past few decades it has become apparent that there are also compounds with bioactive properties in colostrum that may have an effect on the neonate. For example, aside from immunoglobulins there are 50 proteins found exclusively in colostrum, including complement factors, growth and differentiation proteins, and proteins which exhibit an inhibitory effect on proteolytic activity (Nissen et al.,

2017). Additional bioactive factors include peptide and steroid hormones, such as insulin and growth hormone, cytokines, nucleotides and enzymes (Blum & Hammon, 2000).

Small polymers of simple sugars, known as bovine colostrum oligosaccharides (bCOs), are among the bioactive molecules present in colostrum. Although bCOs are indigestible to the host and resistant to hydrolysis throughout the GIT, they are hypothesized to play an important role in the establishment of commensal microbiota. The primary oligosaccharide (OS) in bovine secretions, 3'sialyllactose (3'SL), is 4 times higher in colostrum compared to mature milk (Martin-Sosa et al., 2003). All OS, whether derived from a human or bovine source, are composed of a lactose core at the reducing end that is elongated by N-acteyllactosamine units (Zivkovic et al., 2011). Structural variation is introduced by extensive fucosylation or sialylation at the terminal ends of the lactose compound. Over 200 OS have been identified in human colostrum and milk that differ in their charge, size, and sequence with approximately 50-70% of human milk oligosaccharides (hMOs) being fucosylated and 5-15% sialylated (Ninonuevo et al., 2006). In contrast, only ~40 different OS in bovine colostrum and milk have been identified, the majority (>70%) of which are sialylated and consist of shorter chains than hMOs (Tao et al., 2008). Sialylated OS possess the ability to inhibit the adhesion of pathogenic bacteria to receptors on the intestinal epithelium (Sohanpal et al., 2004) and act as carbon sources for beneficial bacteria to promote their growth and establishment in the intestine (Yu et al., 2013). Therefore, these acidic compounds may play a key role in modulating a balanced gut microbial community and overall gut health in the neonate.

Once synthesized in the mammary gland, OS are passed via the colostrum or milk to the neonate. From an evolutionary standpoint, it is obvious that the content of colostrum and milk throughout the various stages of lactation is specifically tailored to meet the needs of the

newborn (Martin-Sosa et al., 2003; Zivkovic et al., 2011). For instance, Martin-Sosa et al. (2003) demonstrated that five primary sialylated bCOs are present among the various lactation stages and that there are significant differences between the main compounds present in colostrum when compared to transition milk or mature milk. Aside from their production by the dam, the concentrations of free OS in colostrum may be affected by the heat-treatment of colostrum before it is fed to the calf. Nesser et al. (1991) demonstrated that sialylated OS are mainly bound to milk proteins and lipids and that their availability is higher in HT milk compared to fresh milk. It can be hypothesized that when heat-treated, milk OS normally bound to proteins and lipids are cleaved, which results in an increase in the concentration of free OS. Regardless of whether colostrum is fed fresh or heat-treated, this evidence suggests that specific bCOs may be produced during the colostral period that influence the immature neonatal calf GIT. However, exactly how these OS influence the GIT and microbiota of the dairy calf has not yet been determined.

1.5 Digestion and Beneficial Effects of bCOs

Small amounts of intact hMOs are found in the feces of breast-fed infants, suggesting that the majority of milk OS must either be hydrolyzed in the upper GIT to constituent monomers and absorbed or degraded by intestinal microbes (Sabharwal et al., 1988; Coppa et al., 1993; Engfer et al., 2000). Engfer et al. (2000) used an *in vitro* model to demonstrate that both acidic and neutral hMOs are indeed able to resist the pH of the stomach and hydrolysis by secreted pancreatic and mucosa-bound glycosidases, and thus likely reach the intestine intact. Overall, in the immature oro-gastrointestinal tract the ability of enzymes to degrade milk OS is low and unlikely to occur (Engfer et al., 2000). It was suggested that colostrum and milk OS reach the neonatal colon intact, yet a study conducted by Janschter-Krenn et al. (2013) demonstrated that

these carbohydrate compounds may be hydrolyzed by bacteria in regions as early as the jejunum. In this experiment, neonatal rats were orally administered either a commercial formula (control) or a formula containing 15 mg/ml of a mixture of hMOs, and were euthanized at 2, 4 and 8 h after administration. Interestingly, lacto-N-fucopentose 1 (LNFP1), an OS that was not the dominant compound in the original formula, was one of the main OS present in the intestinal samples and increased over time. As LNFP1 increased, smaller and structurally similar OS decreased, suggesting that smaller-molecular weight OS are incorporated into LNFP1 and may never actually reach the colon. Although this study yields novel information about where OS may be metabolized by micrbiota and how their composition may be altered, the rat model itself poses a major challenge when considering the calf. There are many differences in the intestinal anatomy and physiology of a rat and a neonatal calf, which makes it difficult to predict whether this information translates to dairy calf research.

With regards to colostrum and milk OS hydrolyzation and digestion, one of the most important differences between the rat and the neonatal dairy calf lies in early life intestinal microbial colonization. Colonic bacteria express a variety of enzymes capable of hydrolyzing colostrum and milk carbohydrates, including fucosidases and sialidases (Rhodes et al., 1985; Corfield et al., 1992). However, not all bacterial species possess this ability and those that are able to hydrolyze carbohydrates seem to do so in their own unique way. Species belonging to the genus *Bifidobacteria* are typically associated with the metabolism of milk OS, as they thrive in carbohydrate-rich environments and are among the dominant intestinal colonizers of breast-fed infants (Goh et al., 2015). More specifically, *Bifidobacteria* cluster OS-active genes within conserved regions that consist of upstream regulatory elements, ABC (ATP-binding cassette) transporters and one or more glycoside hydrolases (Barrangou et al., 2009; Lee et al., 2008;

Schell et al., 2002; Sela et al., 2008). Although these regions are conserved, the way in which milk OS are metabolized is species-specific. For instance, certain species import intact OS into the cell for metabolism, while others cleave OS structures extracellularly and import specific OS components for metabolism (Sela et al., 2008; Sela & Mills, 2010; Wada et al., 2008; Suzuki et al., 2008). Hydrolyzation of milk OS by species belonging to the genus *Lactobacillus* is limited to tri- or tetra-saccharides, including hMO building blocks, such as lactose, glucose, and galactose (Schwab & Ganzle, 2011). However, extracellular hydrolysis by other microbes may liberate smaller monosaccharides for *Lactobacilli* use. Species belonging to the *Bacteroides* genus are also predominant residents of the neonatal gut and these members are able to metabolize host mucous glycans as a carbon source, as well as possess the ability to cleave the linkages found in milk OS and uniquely cleave sialic acid (Marcobal et al., 2010; Marcobal et al., 2011). Thus, *Bacteroides* may have a selective advantage in the bovine neonatal GIT, as the majority of bCOs are sialylated, and other dominant gut genera, such as *Bifidobacteria* and *Lactobacillus,* may lack the machinery to catabolize sialic acid and preferentially catabolize neutral oligosaccharides or the underlying sugars.

In addition to promoting the growth of beneficial bacteria, it has been hypothesized that milk OS also possess the ability to inhibit pathogenic bacteria in the neonatal gut. A large amount of pathogens are required to adhere to epithelial surfaces in order to colonize or invade the host and cause disease and generally do so using host glycans on the surface of the cells, which can resemble milk glycans (Smilowitz et al., 2014). Milk OS are thought to inhibit the binding of pathogens to the epithelial cell surface by acting as "receptor decoys" and binding to the pathogen surface, thus inhibiting their ability to bind to host glycans and cause subsequent infection and disease (Zivkovic et al., 2011; Newburg, 2000; LoCasio et al., 2009). The large

repertoire of colostrum and milk OS differing in size, structure, and charge suggest that they possess a vast array of decoy functions (Bode & Jantscher-Krenn, 2012). For example, sialylated OS, such as 6'sialyllactose (6'SL) and 6'sialyllactosamine (6'SLN), can block the adhesion of enterotoxic *E. coli* (ETEC) to erythrocytes (Martin-Sosa et al., 2002). Milk OS can also bind to HIV (Hong et al., 2009), rotavirus (Huang et al., 2012), *Vibrio cholerae* (Coppa et al., 2006), and *Streptococcus pneumoniae* (Andersson et al., 1986), further demonstrating their diverse capability to maintain a balanced and healthy microbial community in the gut.

Through consumption by beneficial bacteria in the GIT, milk OS are able to have indirect effects on the host, specifically supporting intestinal barrier function and modulating immunity. The effects of bacterial species grown on milk OS on intestinal barrier function seems to be predominately mediated by the up-regulation of tight junction proteins and by preventing their redistribution from the intercellular junctions to the cytoplasm (Chiclowski et al., 2012; Ewaschuk et al., 2008). In addition, bacteria that consume OS as a carbon source are able to positively regulate cytokines involved in inflammatory responses, specifically by inducing higher expressions of anti-inflammatory cytokines and decreasing pro-inflammatory cytokines (Ewaschuk et al., 2008; Chiclowski et al., 2012; Ganguli et al., 2013). Gill et al. (1999) demonstrated that the binding of IgG to the epithelium, and its subsequent uptake, can be enhanced by the presence of sialic acid from milk OS on the surface of intestinal microvilli. This phenomenon may explain the high abundance of sialylated OS in bovine colostrum and transition milk, as the passive transfer of IgG is one of the most important factors in promoting the health and survival of the neonatal dairy calf.

1.6 Intestinal Microbial Colonization in the Pre-weaned Calf

The GIT is able to provide a barrier against foreign agents and pathogens, while simultaneously maintaining an ideal environment for growth and colonization of beneficial microbial species. The GIT microbiome plays a fundamental role in shaping key aspects of early life gut maturation, including the utilization of nutrients, the development of the immune system and influencing the physiology of the host (Mazmanian et al., 2005; Peterson et al., 2007). Consequently, dysbiosis of the microbial community can lead to digestive disorders and increase the risk of bacterial infections, which are the main causes of morbidity, mortality, and economic loss in livestock (Collado & Sanz, 2007; Stone, 2004; Anadon et al., 2006). Bacterial colonization of the neonatal GIT begins during birth, with exposure to the microflora of the cervix and vagina (Bezirtzoglou, 1997). Several studies have demonstrated that the infant neonatal GIT is first colonized by aerobes or facultative anaerobes, which utilize oxygen and create the proper environment for the colonization of obligate anaerobes (Bezirtzoglou, 1997; Jost et al., 2012). Smith (1965) highlighted this shift, showing that the first bacteria to colonize all gut regions of the calf at 8 h of life is *E. coli* and *Streptococcus*, followed by *Clostridium perfringens* at 18 h of life. *Lactobacilli* and *Bacteroides* were only observed after 1 d and 2 d post-partum, respectively, with *Lactobacilli* eventually displacing coliforms and predominating all regions of the GIT within the first week of life. However, Smith (1965) only enumerated the plate counts of each bacterial group using a single selective media for all groups, which inidicates a significant limitation due to the considerable differences in efficiencies of this media for the different kinds of bacteria.

More recently, Malmuthuge (2016) utilized next-generation sequencing techniques to confirm the results of Smith (1965). It was found that the intestine of the newborn calf is

immediately dominated by facultative anaerobes. Specifically, the epimural community of the calf was dominated by *Proteobacteria* (53%), followed by *Firmicutes* (18%), *Actinobacteria* (20%) and *Bacteroidetes* (6%) (Malmuthuge, 2016). In infants, *Actinobacteria,* which contains the genus *Bifidobacteria,* dominates the fecal microbiome for three to four months of age (Turroni et al., 2012; Azad et al., 2013). This is similar to pre-ruminants, in which this phylum is present in the gut of 3 to 7 d old calves and continues to predominate until 6 to 9 months of age (Rada et al., 2006; Vlkova et al., 2006). After birth, obligate anaerobes, such as *Firmicutes* and *Bacteroidetes,* establish gradually and eventually become the predominant bacteria as the calf ages (Ley et al., 2008; Mamluthuge, 2016).

In addition to age-related changes in the microbial community, the sample type and location also has a large impact on the prevalence and diversity of bacteria phylotypes reported. Regional differences in microbial composition are detected as early as immediately after birth, with *Firmicutes* dominating the lumen of the ileum, and *Actinobacteria* dominating the lumen of the distal jejunum (Malmuthuge, 2016). At 3 weeks of age, the small intestine continued to be composed primarily of *Firmicutes*, while the colon is co-dominated by both *Bacteroidetes* and *Firmicutes* (Malmuthuge et al., 2014). In the small intestine, even greater regional differences are seen in regards to the mucosa-associated communities. Although *Firmicutes* are present in both the epimural and luminal communities of the small intestine, *Bacteroidetes* and *Proteobacteria* are also found attached to the mucosal surface, with these three phylum containing 17 genera that are not present in any other regions of the GIT (Malmuthuge et al., 2014). This regional specificity suggests that studies using fecal-based samples, or even intestinal digesta, may not reveal the true mucosa-associated intestinal microbiome and its effects on the host (Malmuthuge et al., 2015a).

As mentioned previously, intestinal microbial colonization begins during the birth process, when the neonate is exposed to the maternal microbial environment of the cervix and vagina (Bezirtzoglou, 1997). In dairy calves, the composition and diversity of the microbiome of the neonatal small intestine is different from the birth canal and rectal microbiota of the dam, as well as the birth environment (Malmuthuge, 2016). Following birth and early life, the calf is either kept with the dam, transferred to an individual pen, or placed in a group environment, all of which subject the neonate to an abundance of diverse microbes. To date, the only studies to determine the effect of the rearing environment on the gut microbiota have focused solely on the rumen, demonstrating that allowing pre-ruminants to stay with the dam results in a decrease in aerobic bacteria, a higher concentration of total bacteria, and a more rapid development of protozoa in the rumen (Fonty, 1989; Abecia et al., 2014). These results likely have implications for performance measures and health outcomes during weaning, however, how the rearing environment affects the microbial community of the lower GIT is currently unknown.

Similar to the birth and rearing environment, early life nutrition also has an influence on the succession and composition of the microbiota. Infants that are exclusively breast-fed demonstrate a relatively simple microbiome dominated by beneficial *Bifidobacterium* species within the first weeks of life. This is in contrast to formula-fed neonates, whose microbiome consists of a more diverse population, including *Enterobacteriaceae, Enterococcus* and *Bacteroides* (Yoshioka et al., 1983; Dai & Walker, 1999; Favier et al., 2002). Breast-feeding throughout the first months of life can have beneficial effects on the neonate, including protection against infectious diseases, a low incidence of immune disorders, and reduced morbidity and mortality (Saarinen & Kajosaari, 1995; Schack-Nielsen & Michaelsen, 2007; Le Huerou-Luron et al., 2010; Jost et al., 2012). In contrast to infants, dairy calves are typically

raised without the dam, yet the maternal influence on gut microbiota persists through the feeding of colostrum. The feeding of colostrum accelerates the bacterial colonization of the calf small intestine, as evidenced by calves receiving fresh colostrum reaching a total bacteria density of 10^{10} 16S rRNA gene/g of sample at 12 h of life, whereas calves who do not receive colostrum only achieve 10^{8} 16S rRNA gene/g of sample (Malmuthuge et al., 2015b). As well, the heat-treatment of colostrum can result in a higher prevalence of *Bifidobacteria* in the small intestine throughout the first 12 h of life (Malmuthuge et al., 2015b). This increased prevalence may explain the reduced incidence of enteric infections when calves are fed HT colostrum (Godden et al., 2012), indicating that colostrum plays a key role in the establishment of a healthy GIT.

1.7 Functional Roles of the Intestinal Microbiota

The commensal microbiota within the intestine are not opportunistic residents, but often have mutualistic and functional relationships with the host. The metabolic capacity of the GIT microbiota is equal to that of the liver, and thus it can essentially be considered as an additional organ (Gill et al., 2006). Commensal microbes are critical to several diverse aspects of host biology, such as facilitating the metabolism of otherwise indigestible compounds, producing essential vitamins, and providing protection against invasion by pathogenic organisms (Sommer & Backhed, 2015; Smith et al., 2007). In addition, the presence of intestinal microbes is necessary for the development of secondary lymphoid structures, such as mesenteric lymph nodes, Peyer's patches and isolated lymphoid follicles (Sommer & Backhed, 2015). One particular group of bacteria that are potentially health-enhancing are those belonging to the lactic acid bacteria (LAB) group, such as *Bifidobacteria* and *Lactobacilli* (Picard et al., 2005; Salminen et al., 1998*).* Neither of these genera includes any pathogenic species and their high prevalence

in the feces of exclusively breast-fed infants has been shown to provide protection against infection (Yoshioka et al., 1983; Harmsen et al., 2000). The major fermentation products of these bacteria are short chain fatty acids (SCFAs), as well as lactic acid (LA) and gases. In the colon, SCFAs act as the principal anions in the lower gut (Blaut et al., 2002; Marteau et al., 2001) and can be absorbed by enterocytes and stimulate salt and water absorption (Picard et al., 2005). Among the main SCFAs, butyrate has the highest energy value per mole and acts as an important energy source for the colonic epithelium (Maynard, 1979; Boffa et al., 1992). Butyrate has been shown to regulate cell growth and differentiation (Boffa et al., 1992; Bugaut & Bentejac, 1993), as well as promote the expression of differentiation markers *in vitro* (Cummings, 1995). In addition, LAB possess the ability to protect the host through inhibition of pathogens and interactions with the gut associated lymphoid tissue. Beneficial bacteria also prevent infection by out-competing pathogens for binding sites on epithelial cells, (Perdigon et al., 1995; Duffy et al., 1994), stimulating intestinal IgA antibody responses (Fukushima et al., 1998), and increasing the phagocytic activity of peripheral blood leukocytes and macrophages (Gill et al., 2000).

Similar to *Bifidobacteria* and *Lactobacillus, Faecalibacterium* spp. is able to produce butyrate (Duncan et al., 2004). Calves with a higher prevalence of *Faecalibacterium* spp. in feces during the first week of life had a lower incidence of diarrhea within the first four weeks of life, as well as increased body weight gain (Oikonomou et al., 2013). The ability of *Faecalibacterium* to produce butyrate and block the production of pro-inflammatory cytokines is likely the reason behind the decreased incidence of diarrhea in calves displaying a higher prevalence of this microorganism (Oikonomou et al., 2013; Sokol et al., 2008; Ley et al., 2005; Turnbaugh et al., 2006; Tak & Firestein, 2011). The ability of this species to harvest energy has also recently been implicated in weight gain in a mouse model and was shown to play a role in the increased

incidence of obesity of children (Balamurugan et al., 2010; Ley et al., 2005). Therefore, this microorganism provides energy to the host and likely has beneficial effects on the neonatal and pre-weaning calf, however, if *Faecalibacterium* dominance persists into adult life, this may have negative implications during the first lactation or on the transition cow.

While some of the microorganisms residing in the gut may be beneficial to the host, others may be harmful (Picard et al., 2005). For example, neonatal calf diarrhea is caused by a variety of microorganisms, including rotavirus, *Cryptosporidium parvum*, ETEC and *Salmonella* (Acha et al., 2004). El-Seedy et al. (2016) reported a prevalence of *E. coli* and *Salmonella* in neonatal calf diarrhea of 18% and 76%, respectively, and these two bacteria have also been reported as the most common identified pathogens in scouring calves less than two months of age (Acha et al., 2004; Cho & Yoon, 2014). Pathogenic *E. coli* typically adheres to the ileum and colon (Moxley & Francis, 1986; Orskov et al., 1975) and the production of bacterial enterotoxin causes damage to epithelial cells, resulting in fluid secretion and diarrhea (Acres, 1985).

1.8 Knowledge Gap

It is well known that the timely feeding of adequate volumes of colostrum is a key factor in ensuring early passive transfer in the neonatal calf. However, farms continue to struggle with colostrum management and it has been reported that many calves may not receive the first colostrum meal until 6 h of life or later (Vasseur et al., 2010). The majority of studies conducted in regards to delaying colostrum feeding and its effects on the passive transfer of IgG were conducted more than 30 years ago, and whether these results hold true using current day colostrum recommendations (3-4 L of colostrum containing ≥ 50 g of IgG per L), standardized colostrum quality and volume among treatments warrants further research. Moreover, how

feeding colostrum in a delayed manner affects the prevalence of intestinal bacteria, which are essential for development of the mucosal immune system and providing energy for intestinal cells during early life, requires further research.

As mentioned previously, the initiation of a balanced microbial community in early life is a key factor influencing the future health and performance of the dairy calf. Unfortunately, little is known about the compounds from colostrum that may have a prebiotic effect on the GIT microbiota, as well as how the heat-treatment of colostrum may affect these prebiotic structures. More specifically, heat-treatment may affect the structure and concentrations of free bCOs present in colostrum, as evidenced by a recent study which determined that calves fed colostrum heated at 60°C for 60 min displayed a higher prevalence of *Bifidobacteria* in the small intestine within the first 12 h of life compared to calves fed fresh colostrum (Malmuthuge et al., 2015b). This is likely due to the increased availability of free prebiotic bCOs in HT colostrum, however, whether or not this occurrs and the concentrations of free bCOs in the small and large intestine of the neonatal calf are currently unknown.

1.9 Hypothesis and Objectives

The overall hypothesis of this thesis is that the concentration of prebiotic bCOs in the calf intestine and colostrum, the passive transfer of immunity, and the establishment of region-specific intestinal microbiota, varies with colostrum management practices, specifically the timely feeding and heat-treatment of colostrum. Therefore, the specific objectives of the this thesis were: 1) to elucidate the effects of delaying the first colostrum feeding after birth on the passive transfer of IgG and the prevalence of bacterial groups in the distal small intestine and colon; and 2) to determine the effects of heat-treatment of colostrum on prebiotic bCOs and to

demonstrate the regions in which bCOs are present in the intestine of the neonatal calf. The present thesis is the first to identify how delaying colostrum feeding affects the prevalence of bacteria in the lower intestine, and further, to identify the intestinal regions in which prebiotic bCOs are present in the neonatal calf. This knowledge may be used to optimize colostrum feeding management protocols, as well as to formulate prebiotic and probiotic supplements for neonatal calves to improve GIT health.

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1.11 Tables and Figures

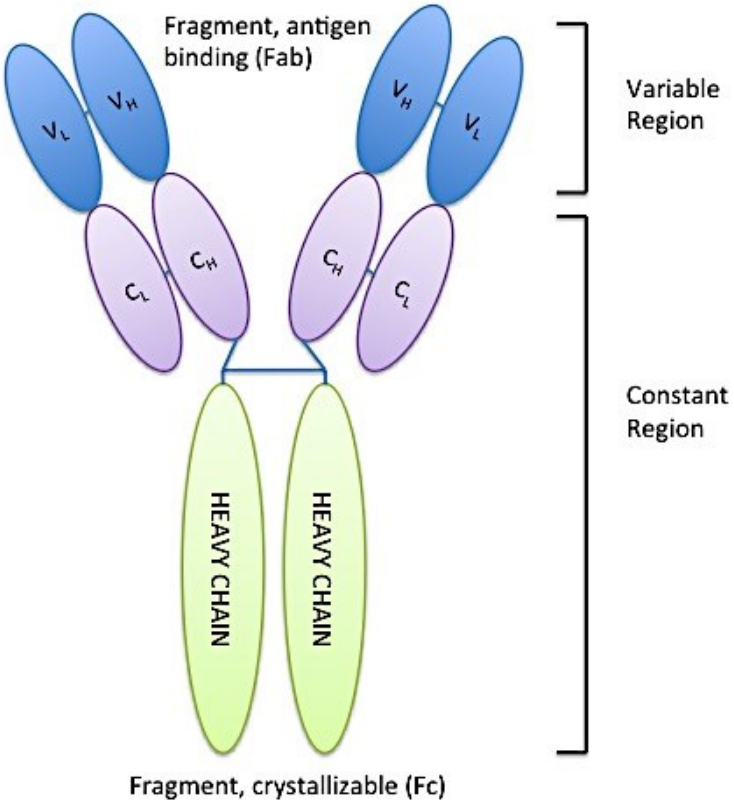

Figure 1-1. The immunoglobulin G molecule. C = constant region, V = variable region, subscript H = heavy chain, subscript L = light chain.

2.0 Chapter 2: Effect of delaying colostrum feeding on passive transfer and intestinal bacterial colonization in neonatal male Holstein calves

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2.1 Abstract

The objective of this study was to investigate the effect of time of first colostrum feeding on the passive transfer of IgG, as well as bacterial colonization in the intestine of neonatal dairy calves. Twenty-seven male Holstein calves were randomly assigned to 1 of 3 treatments at birth: calves fed colostrum at 45 min (0 h, n = 9), 6 h (6 h, n = 9), or 12 h after birth (12 h, n = 9). Calves were fed pooled, heat-treated colostrum (62 g of IgG per liter) at their respective feeding times at 7.5% of birth body weight (BBW), and fed milk replacer at 2.5% of BBW at 12 h after the colostrum meal, followed by every 6 h thereafter. Blood samples were taken every 3 h using a jugular catheter and were analyzed for determination of serum IgG by radial immunodiffusion. At 51 h after birth, calves were euthanized for collection of tissue and digesta of the distal jejunum, ileum, and colon. Quantitative real-time PCR was used to estimate the proportion of *Bifidobacterium* sp*., Lactobacillus* sp*., Faecalibacterium prausnitzii, Clostridium Cluster XIVa,* and total *Escherichia coli*. Delaying colostrum feeding by 6 h (35.6 ± 1.88%) and 12 h (35.1 ± 3.15%) decreased the maximum apparent efficiency of absorption of IgG compared to calves fed colostrum immediately after birth (51.8 ± 4.18%) and delayed the time to maximum serum IgG concentration (24 h vs. 15 h, respectively). Moreover, 12 h calves tended to have a lower

proportion of *Bifidobacterium* sp. (0.12 ± 0.017%) and *Lactobacillus* sp. (0.07 ± 0.019%) associated with the colon mucosa compared to 0 h calves (1.24 ± 0.648% and 0.26 ± 0.075%, respectively). In addition, 6 h (0.26 ± 0.124%) and 12 h (0.49 ± 0.233%) calves had a lower proportion of total *E. coli* associated with ileum mucosa when compared to 0 h calves (1.20 ± 0.458%). These findings suggest that delaying colostrum feeding within 12 h of life decreases the passive transfer of IgG and may delay the colonization of bacteria in the intestine, possibly leaving the calf vulnerable to infections during the pre-weaning period.

2.2 Introduction

Pre-weaning dairy calves are at high risk of morbidity and mortality, with recorded rates as high as 46% and 5%, respectively (NAHMS, 2011). Calves are born without passive immunity as the placenta of the cow separates the maternal and fetal blood supply, thus preventing the transfer of immunoglobulins during gestation (Godden, 2008). To protect the calf, the dam produces colostrum, which contains high levels of immunoglobulin G (IgG) in addition to nutrients and other bioactive factors. Ensuring the passive transfer of IgG by early and adequate intake of good-quality colostrum is recognized as one of the most important aspects in determining health and survival of the neonatal dairy calf (Godden, 2008). Unfortunately, poor colostrum management continues to be a problem, with 31% of calf mortality in the first 21 d of life being due to the failure of passive transfer (FPT) of IgG from colostrum (Wells et al., 1996). In addition, it has also been demonstrated that calves fed 2 L of colostrum produce 9% and 14% less milk in the first and second lactation, respectively, compared to calves fed 4 L of colostrum (Faber et al., 2005), emphasizing the importance of proper colostrum management on future productivity.

Multiple factors are responsible for insufficient circulating IgG leading to the failure of passive transfer, including feeding contaminated or low-quality colostrum and of particular interest in the current study, the first ingestion of the first colostrum meal occurring more than 6 h after birth (Vasseur et al., 2010). The termination of the absorption of macromolecules, including IgG, across the gut epithelium is termed "gut closure" and is thought to occur by approximately 24 h postpartum in calves (Stott et al., 1979a). The passive transfer of IgG across the small intestinal epithelium has been shown to be optimal within the first 4 h of life and rapidly declines after 12 h (Stott et al., 1979b; Weaver et al., 2000). It is therefore generally accepted that calves fed colostrum immediately after birth will achieve higher maximum serum IgG concentrations than those fed colostrum more than 4 h after birth. However, the majority of the studies concerning the effect of the timely feeding of colostrum only feed ~2 L of colostrum of an unknown IgG concentration within the first hours of life, while current day recommendations are to feed colostrum at 10% of body weight (~3-4 L) (Dunn et al., 2017). Therefore, how delaying colostrum feeding may affect passive transfer in the neonatal dairy calf using standardized colostrum among treatment groups and present day quantity recommendations is unknown.

The successful passive transfer of IgG is generally associated with a decreased risk of disease in the young calf. Similarly, the early establishment of gut microbiota has been reported to be directly associated with the health of calves (Oikonomou et al., 2013). For example, enterotoxigenic *E. coli* (ETEC) is typically associated with an increased incidence of diarrhea, while beneficial species, such as *Bifidobacterium* sp., are associated with a healthy gut microbiome and immunity (Apgar et al., 1993; Picard et al., 2005; Uhde et al., 2008). The establishment of gut microbiota in the neonate is also associated with the development of the

mucosal immune system and secondary lymphoid structures, and certain bacterial genera are able to produce energy substrates for the intestinal epithelia (Guarner, 2006; Sommer & Backhed, 2015). Current knowledge of the commensal bacteria present in the calf microbiome and their effects on the host are limited. Variation of microbial composition in the rumen has been shown to be higher in neonates or young animals than in adults (Jami et al., 2013). This knowledge may translate to the intestinal microbial composition, and if so, implies that the gut microbiome may be more easily influenced during this time. This may provide the opportunity to establish beneficial bacteria during early life to decrease the possibility of pathogenic bacterial colonization and subsequent disease (Malmuthuge et al., 2015b). It has been demonstrated that feeding colostrum within the first 12 h of life can result in a higher proportion of small intestinal mucosa associated *Bifidobacterium* and lower total *E.coli* when compared to calves not fed colostrum (Malmuthuge et al., 2015a). However, how delaying the first colostrum feeding after birth may affect the bacteria colonizing the small and large intestine is currently unknown.

The objective of the present study was to investigate the effect of time of first colostrum feeding on the passive transfer of IgG as well as bacterial colonization in the intestine of neonatal dairy calves. It was hypothesized that delaying colostrum feeding in the first 12 h of life would progressively decrease the passive transfer of IgG, as well as the proportion of beneficial bacteria in the small intestine (distal jejunum and ileum) and colon.

2.3 Materials and Methods

2.3.1 Calving and Early Calf Life

The animal experiment was conducted following the guidelines of the Canadian Council of Animal Care (CCAC, 1993) at the Dairy Research and Technology Centre of the University of

Alberta. The animal use protocol was approved by The Livestock Care Committee of the University of Alberta (AUP00001595). Approximately 3-10 d before parturition, Holstein heifers and cows were moved to maternity pens. After the area was cleaned using 1% iodine, an iVET® birth-monitoring device (manufactured by iVET®, Papenburg, Germany; devices and system provided by the Livestock and Research Branch of Alberta Agriculture and Forestry, Government of Alberta) was inserted into the vagina. The iVET® system is composed of a transmitter (an electronic sensor embedded within iVET® device) and a cellular receiver (Dutra et al., 2015). The iVET® was thoroughly cleaned with and disinfected prior to insertion. After insertion, the iVET® remained in the vagina until calving (~3-10 d). When parturition began, the iVET® was pushed out, which activated the sensor due to changes in the intensity of temperature and light, at which time the iVET® receiver sent out a text message and voice alert to researchers (Dutra et al., 2015).

Only bull calves from a singleton birth with a body weight between 35-55 kg were included in the current study. Calves were weighed in a calibrated electronic scale (Digi-Star SW300, Digi-Star L.L.C., Fort Atkinson, WI) and transferred to individual pens bedded with shavings and fresh straw. Calves were dried using two clean towels for 10 min, following which calves' navels were dipped with 7% iodine.

2.3.2 Animal Experiment and Feeding

Male Holstein calves (n = 27) born from February to September of 2016 were randomly allocated into 3 treatment groups: calves fed colostrum within 1 h (0 h, n = 9), 6 h (6 h, n = 9), or 12 h after birth (12 h, n = 9). Pooled, heat-treated colostrum containing 62 g of IgG per litre was provided by the Saskatoon Colostrum Company Ltd. (SCCL, Saskatoon, SK, Canada) and fed to

calves at their respective feeding times at 7.5% of birth body weight (BBW) (need to provide reference for why we chose this percentage). Colostrum was thawed and heated to 39 °C prior to feeding in a water bath kept at a consistent temperature of 50°C. Once heated, colostrum was poured into two 2 L esophageal tubing bottles and transferred to the calf pen in a bucket of warm (~39°C) water, where it was then tube fed to the calf in less than 5 min. Twelve hours after their colostrum feeding, calves were hand-fed milk replacer with a 26:18 crude protein:fat ratio (Excel Pro-Gro Calf Milk Replacer, Grober Nutrition, Cambridge, Ontario, Canada) by nipple bottle at a volume of 2.5% of BBW per meal every 6 h until euthanasia at 51 h after birth. Milk replacer was prepared by mixing 150 g of milk replacer powder per 1 L of water in a clean bucket, poured into a clean bottle with a unique nipple, and heated in the water bath to 39°C. If calves did not consume the milk replacer meal within 30 min by nipple bottle, the remainder of the meal was fed using an esophageal tube. If calves were tubed more than 50% of their milk replacer intake within a 24 h period, they were excluded from the study.

2.3.3 Blood Sampling

At approximately 20 min of life, a 3 mL serum sample was collected from the jugular vein using a vacutainer for the purpose of generating baseline values. At 2 h after birth, a 2.5" 16-gauge I.V. catheter (Terumo Medical Corporation, Somerset, New Jersey, USA) was inserted into a jugular vein of each calf for the duration of its life. To insert the catheter, the calf was gently restrained by a handler and its neck was shaved, followed by disinfection using chlorhexidine and 70% ethanol and catheter insertion. Blood samples were collected every 3 h, left at room temperature for 3 h to clot and the serum collected following centrifugation at 3,000 x g at 4 °C for 20 min. The serum was transferred into three 1.5 mL microcentrifuge tubes in

equal volume aliquots and frozen at -20 °C. Immediately following blood collection, the catheter was flushed with saline, followed by infusion of 1.5 ml of heparinized saline in order to keep the catheter patent.

2.3.4 Intestinal Tissue and Digesta Sampling

Calves were euthanized at 51 h (3 h after the final meal) of life using a pentobarbital sodium injection (Euthanyl, Vetoquinol, Lavaltrie, Quebec, Canada) at 12.5% of euthanization body weight administered through the jugular catheter. Once the calf reached a surgical plane of anesthesia, exsanguination was performed and the entire gut contents were removed after ligation of the rectum and esophagus. Following this, 10 cm intestinal segments of pre-defined intestinal regions were collected. The distal jejunum segment was defined as 30 cm proximal to the collateral branch of the cranial mesenteric artery; the ileum segment was defined as 30 cm proximal to the ileo-cecal junction; and the colon segment was defined as 30 cm distal to the ileo-cecal junction (Malmuthuge et al., 2015b). Intestinal content was removed from the sample using tweezers and placed in a 50 mL Falcon tube. Then, the tissue was washed in phosphate buffered saline (PBS) until clean (~3-4 washes) and placed in a sterile bag. Both intestinal content and tissue samples were immediately snap-frozen in liquid nitrogen and transferred to an -80 °C freezer until further use.

2.3.5 Analysis of Serum Immunoglobulin G

Serum samples were thawed and centrifuged at 3,000 g for 20 min at 4 °C, after which supernatant was transferred to a new tube. Serum samples were re-frozen in -20 °C for 24 h and then shipped overnight on ice to the Saskatoon Colostrum Company Quality Assurance

Laboratory for determination of serum IgG concentrations by radial immunodiffusion analysis (Chelack et al., 1993) with modifications as described in Chamorro et al. (2014). In brief, samples obtained before colostrum feeding were undiluted and samples obtained after colostrum feeding were diluted 1:4 using PBS (Chamorro et al., 2014). Antiserum against bovine IgG (Jackson Laboratories, West Grove, Pennsylvania, USA) (2.5% in PBS with a pH of 7.25) was distributed evenly throughout immunodiffusion plates prepared from 2% agarose. Standard curves from 1.06 to 8.5 mg/ml were produced using triplicate samples of bovine serum IgG (Midland BioProducts Corportation, Boone, Iowa, USA). All samples were run in triplicate and any replicates deviating more than 1.5 mg from the mean were repeated. After application to the gel, samples were incubated at 25 °C for 18-24 h (Chamorro et al., 2014). The ring diameters were then measured with a computer-assisted plate reader (The Binding Site Group, Birmingham, England) and the concentrations were calculated using the linear standard curve.

The maximum apparent efficiency of absorption (AEA, %) of IgG for each treatment group was calculated using calf BBW, calf serum IgG concentration, and colostrum IgG mass. The formula used is previously described by Quigley et al. (2002), with the assumption of a plasma volume of 9.9% of birth weight. The formula is as follows:

$$AEA\ (\%) = \left[\frac{(IgG\ conc. - IgG\ baseline)\ \times\ plasma\ volume}{IgG\ consumed}\right] \times\ 100$$

Parameters relative to colostrum feeding were calculated from the raw data, including time to reach maximum concentration (T_{max}), the maximum concentration reached (C_{max}), ratio of C_{max}/T_{max}, the change in concentration (delta change, baseline IgG concentration substracted from the maximum IgG concentration) and the IgG concentration at 12 h, 24 h, and 36 h after the

colostrum meal (IgG_{12}, IgG_{24}, IgG_{36}). The positive incremental area under the curve (AUC) for IgG was determined using the trapezoid rule over the first 12 h (AUC_{12}), 24 h (AUC_{24}), 36 h (AUC_{36}) after birth. The formula for AUC for IgG during a 3 h period is described below:

$$AUC = \left[\frac{(a+b) \times c}{2}\right]$$

Where: a = the height of one side (IgG conc., mg/ml), b = the height of the other side (IgG conc., mg/ml), c = base (amount of time, h).

The AUC_{12} was determined by the summation of the previous 3 h intervals ($AUC_{0-3} + AUC_{3-6} + AUC_{6-9} + AUC_{9-12}$). The same method was used for determination of AUC_{24} and AUC_{36}

2.3.6 DNA Extraction from Tissue and Digesta Samples

The total DNA from intestinal digesta was extracted using the repeated bead beating plus column method (Yu and Morrison, 2004). Briefly, the digesta sample (~0.3 g) was washed twice with TE buffer. After the addition of cell lysis buffer containing 4% SDS, they were subjected to physical disruption at 4,800 rpm for 3 min using Biospec Mini Beads Beater 8 (BioSpec, Bartlesville, OK), followed by incubation at 70°C for 15 min and centrifugation for 5 min at 16,000 x g. The bead-beating, incubation and centrifugation were repeated once and impurities were removed from the supernatant using 10 M ammonium acetate, followed by DNA precipitation using isopropanol. After precipitation, DNA was further purified using QIAmp fast DNA stool mini kit (Qiagen Inc., Germantown, MD). DNA quantity and purity were evaluated using NanoDrop 1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE) and stored

at -20 °C until further use. For processing of tissue samples, the tissue was ground in liquid nitrogen prior to DNA extraction. Approximately 0.1 g of the ground tissue was subjected to DNA extraction using the bead-beating method as described by Li et al. (2009). The DNA quantity and purity were evaluated as described above.

2.3.7 Quantification of Bacterial Groups in the Calf Distal Jejunum, Ileum and Colon using Quantitative Real Time PCR

For intestinal digesta and tissue samples, the densities of total bacteria, *Bifidobacterium* sp.*, Lactobacillus* sp.*, Clostridium Cluster XIVa, Faecalibacterium prausnitzii,* and total *E. coli* were estimated by measuring their respective 16S rRNA gene copy numbers using quantitative real time PCR (qRT-PCR). Bacterial populations from digesta samples were estimated using the StepOnePlus real time PCR system (Applied Biosystems, Thermo-Fisher Scientific, Waltham, MA) and bacterial populations associated with the tissue were estimated using the high throughput Viia 7 Real-Time PCR System (Thermo-Fisher Scientific). For both tissue and digesta, qRT-PCR was performed using SYBR green chemistry (Fast SYBR Green Master Mix, Applied Biosystems, Foster City, CA) with specific primers targeting each bacterial group (Table 2-2). Standard curves were generated for each bacterial group using purified 16S rRNA genes of *Butyrivibrio hungatei, Bifidobacterium adolescentis, Lactobacillus acidophilus* ATCC4356, *Roseburio hominis* A2-183*, Faecalibacterium prausnitzii* A2-165 and *Escherichia coli* K12, respectively. The copy number of 16S rRNA genes per g of digesta or tissue was calculated using the equation described by Li et al. (2009). The proportion (% of total bacteria) of each bacterial group was obtained and is defined as copy number per gram of each bacterial group

divided by the copy number per gram of total bacteria multiplied by 100 (Malmuthuge et al., 2015b). The formulas used are described below:

$$total\ DNA\ (ng) = DNA\ conc.\ (ng/\mu l\ )\ \times\ elution\ volume\ (\mu l)$$

$$copy\ number\ per\ gram\ of\ sample = \frac{\left[\left(\frac{total\ DNA\ (ng)}{qRTPCR\ conc.\ (ng)}\right)\ \times\ quantity\ mean\right]}{weight\ of\ sample\ (g)}$$

$$proportion\ (\%\ of\ total\ bacteria) = \left(\frac{copy\ \#/g\ \ of\ bacterial\ group}{copy\ \#/g\ \ of\ total\ bacteria}\right)\ \times\ 100$$

2.3.8 Statistical Analysis

To determine the effect of colostrum treatment, all data were analyzed using the MIXED procedure of the Statistical Analysis System (SAS Institute, Cary, NC). For serum IgG concentrations and AEA, repeated measures were used with the model including the fixed effects of treatment, age, and treatment by age interaction. For IgG parameters calculated relative to the meal, as well as the AEA_{max}, only the colostrum treatment was included as a fixed effect. As well, for the proportion and copy number of 16S rRNA gene per g of sample for the bacterial groups, data were analyzed by colostrum treatment by bacterial target (*Bifidobacterium, Lactobacillus,* total *E. coli, Clostridium Cluster XIVa, F. prausnitzii,* and total bacteria), by type (tissue, digesta) within each region of the intestine (distal jejunum, ileum, colon). All values reported are least squares means (LSM) with significance declared at $P \leq 0.05$ and tendencies at $0.05 < P < 0.10$.

2.4 Results

2.4.1 Effect of Delaying Colostrum Feeding on the Passive Transfer of IgG

Feeding colostrum within the first hour of life (0 h) increased the passive transfer of IgG compared to calves fed colostrum at 6 h and 12 h of life (Figure 2-1; Table 2-1). Calves fed at 0 h had a higher AUC for the first 12 h after the colostrum meal (AUC_{12}) compared to 6 h and 12 h calves (Table 2-1). Similarly, 0 h calves had higher AUC_{24} and AUC_{36} compared to 6 h and 12 h calves. There was no difference in passive transfer of 6 h and 12 h calves. In general, calves fed at 0 h had significantly higher mean concentrations of IgG (mg/mL) for the first 27 h of life when compared to 6 h and 12 h calves (Figure 2-1).

Calves fed at 0 h of life displayed a 28.0% increase in the maximum concentration of IgG (C_{max}) when compared to 6 h and 12 h calves, while no difference was detected between 6 h and 12 h groups (Table 2-1). In addition, a 32.4% increase in the maximum AEA (AEA_{max}, %) was observed for 0 h calves when compared to 6 h and 12 h calves. No differences were observed among treatments with regards to the time to maximum concentration (T_{max}) relative to the colostrum meal (Table 2-1).

2.4.2 Effect of Delaying Colostrum Feeding on Tissue Mucosa Associated Bacteria in the Small Intestine and Colon

In general, a high variation between calves was observed for the copy number of 16S rRNA genes per g of fresh sample for both mucosa and digesta associated bacterial groups. Calves fed at 6 h tended to have a lower ($P = 0.08$) total bacteria density associated with the

distal jejunum mucosa compared to 0 h calves, while no differences were detected between 0 h and 12 h calves (Figure 2-2). The prevalence of *F. prausnitzii* associated with the distal jejunum mucosa was higher in 12 h calves ($P = 0.06$) and 6 h calves ($P = 0.04$) compared to calves fed immediately after birth (Table 2-3). No further differences were detected in the bacterial groups associated with the mucosa of the distal jejunum among treatment groups.

In the ileum, a 33.3% decrease ($P = 0.08$) in *F. prausnitzii* associated with the mucosa was observed in 6 h calves compared to 0 h calves, while no differences were detected between 12 h and 0 h calves. Moreover, a lower proportion of total *E. coli* was found associated with the ileum mucosa when calves were fed colostrum at 6 h ($P = 0.05$) and 12 h ($P= 0.09$) of life compared to calves fed colostrum at 0 h (Table 2-3).

In regards to bacterial groups associated with the mucosa of the colon, 12 h calves tended to have a lower proportion of *Bifidobacterium* sp. ($P = 0.08$) and *Lactobacillus* sp. ($P = 0.05$) when compared to 0 h calves, while no differences were observed between 0 h and 6 h calves for these genera (Figure 2-3). No differences were detected among treatment groups with regards to the proportion of *Clostridium Cluster XIVa, F. prausnitzii* and total *E. coli* in the colon mucosa associated microbiota.

2.4.3 Effect of Delaying Colostrum Feeding on Digesta Associated Bacteria in the Small Intestine and Colon

In comparison to mucosa associated bacteria, fewer differences among treatments were detected in the bacterial groups associated with intestinal digesta. In the distal jejunum digesta, 12 h calves displayed a lower ($P = 0.04$) proportion of total *E. coli* compared to 0 h calves (Table 2-4). In the ileum digesta, there was a tendency ($P = 0.09$) for higher *Clostridium Cluster XIVa*

in 6 h calves in comparison to 0 h calves, while no differences were observed between 12 h and 0 h calves. Similarly, 6 h calves also tended ($P = 0.09$) to have a higher proportion of *Clostridium Cluster XIVa* in the colon digesta when compared to 0 h calves. No differences were observed for total bacteria (Figure 2-2), *Bifidobacterium* sp.*,* *Lactobacillus* sp.*,* and *F. prausnitzii* proportion in the intestinal digesta among treatment groups.

2.5 Discussion

To our knowledge, the present study is the first to systematically determine how a delay in colostrum feeding using current colostrum feeding recommendations, highly standardized colostrum and frequent blood-sampling affects the passive transfer of IgG in the neonatal calf. It was hypothesized that delaying the first colostrum meal would progressively decrease the passive transfer of IgG in neonatal calves. In accordance with our hypothesis, feeding colostrum immediately after birth increased the maximum concentration of serum IgG reached, as well as the apparent efficiency of absorption of IgG compared to calves fed colostrum at 6 h or 12 h after birth. The increased absorption of IgG demonstrated by calves fed within the first hour of life was consistent with previous reports, which stated that IgG transfer across the enterocyte is optimal within the first 4 h after birth (Stott et al., 1979). Moreover, 0 h calves displayed a peak in IgG absorption at approximately 15 h of life (Figure 2-1), which is in contrast to earlier studies that report maximal levels at 24 h (Stott et al., 1979). The current study found peak serum levels of 24 h only for 6h and 12 h fed calves. This is an important finding that deserves further consideration and study as many sampling protocols for passive transfer experiments have relied upon the notion that peak serum IgG levels occur at 24 h after birth, irrespective of the time of the first feeding. After reaching maximum IgG concentration at 15 h of life, 0 h calves displayed

a progressive decrease and plateau in serum IgG concentrations. Staley et al. (1972) demonstrated that when transported across the intestinal cell, IgG first enters the lymphatics by exocytosis, followed by its entry to the circulatory system via the thoracic duct (Weaver et al., 2000). Similarly, colostral IgG infused into the duodenum of neonatal piglets can be transported into the lymph (Kiriyama, 1992), and this phenomema may explain the decrease and plateau of IgG in 0 h calves. If IgG is indeed being absorbed into the lymphatics, this likely leaves calves fed colostrum immediately after birth better protected against pathogenic challenges they may encounter in early life compared to calves fed colostrum later.

Contrary to our hypothesis, no differences were found in IgG parameters relative to the colostrum feeding for calves fed at 6 h and 12 h of life, demonstrating that these delays do not progressively decrease the absorption of IgG. These findings are in contrast to an earlier study that reports a linear decline in absorption over this same time period (White, 1993). The result of our study suggests that there may be a critical time point between 1 h and 6 h of life when the closure of the small intestine, defined as the cessation of absorption of macromolecules from the gut into the blood of neonates, progresses to a finite degree (Leece & Morgan, 1962). Previous studies demonstrated the presence of a tubular vesicle-vacuolar mechanism in neonatal enterocytes, with the amount of vacuoles that transport IgG from the intestinal lumen to the blood becoming reduced over time as the gastrointestinal tract matures (Kraehenbuhl and Campiche, 1969; Ockleford and Whyte, 1980; Smeaton and Simpson-Morgan, 1985; Louis and Lin, 2009). More specifically, as the calf ages the vacuoles migrate to the upper portion of the villi and by the third day of life an entirely new type of cell covers the intestinal surface (Smeaton & Simpson-Morgan, 1985). The reduced ability of 6 h and 12 h calves to absorb IgG as efficiently as 0 h calves may be due to the turnover of fetal intestinal cells into mature

enterocytes with a decreased ability to absorb IgG from colostrum (Smeaton and Simpson-Morgan, 1985). Yet, 6 h and 12 h calves did not have failure of passive transfer, as they achieved serum IgG concentrations above 10 mg/mL, suggesting that the small intestine remains permeable to colostral IgG at this time. Early studies also suggest that there may be a hormonal influence involved in the closure of the small intestine, as it has been reported that when the first colostrum meal is delayed calves experience a "cortisol shock" which may induce changes in the absorptive capacity of the intestine (Kruse and Buus, 1972; Nightengale, 1979).

The exact mechanism by which passive transfer and closure occurs has not been demonstrated and findings are conflicting (Johnston & Oxender, 1979; Stott et al., 1978). The majority of studies concerning delayed colostrum feeding, the absorption of IgG and its effect on calf endocrinology and intestinal cell development were conducted more than 30 years ago, when recommendations for the volume and quality of colostrum were not as elevated as in present day. As well, in older studies the colostrum fed was often not well standardized and blood was taken less frequently than in the present experiment, which may have affected the reported hormone concentrations, specifically cortisol, due to large diurnal variations (Gardy-Godillot et al., 1989). Thus, the causative factors of reduced IgG absorption in calves not fed colostrum until 6 h or 12 h of life, or later, are still to be elucidated and further research using present day recommendations of colostrum quality and volumes, as well as frequent blood sampling, is needed.

The gastrointestinal tract of the neonatal calf is a complex organ that must absorb IgG and digest nutrients, provide a barrier against potential pathogens, all while maintaining a hospitable environment for beneficial microbes. The gut microbial community plays a key role in developing the immune system, utilizing nutrients, and influencing the overall physiology of the

host (Mazmanian et al., 2005; Peterson et al., 2007). In the current study, calves fed colostrum at 12 h after birth tended to have a lower proportion of *Bifidobacterium* sp. and *Lactobacillus* sp. associated with the colon mucosa compared to calves fed colostrum immediately after birth. The microbiota associated with the mucosa of the neonatal intestine are in close contact with intestinal cells and thus have the ability to interact with the host. Species belonging to the *Bifidobacteria* and *Lactobacillus* genera are considered to act as beneficial bacteria, as they produce lactic acid (LA) and short chain fatty acids (SCFAs), which have trophic and regulatory effects on colonocytes (Boffa et al., 1992; Cummings, 1995). In addition, these microbes may alter the gut mucosal barrier through the stabilization of the intestinal mucosa, normalizing intestinal permeability and improving the complex immune system of the gut, which can prevent the overgrowth of pathogenic bacteria (Brandtzaeg et al., 1989; Yasui et al., 1995; Picard et al., 2005). A study conducted by Malmuthuge et al. (2015a) determined that feeding colostrum to neonatal calves increased the proportion of *Bifidobacteria* and the density of total bacteria in the small intestine within the first 12 h of life, compared to calves not fed colostrum. The delay in the delivery of colostral nutrients for 12 h after birth in the present study likely affects the microbial dynamics during early life by shifting the establishment of certain bacterial groups to correspond with the timing of the delivery of nutrients. Preventing the immediate initiation of the growth and establishment of beneficial genera may have lasting effects on the dynamic bacterial community in the large intestine and hinder the ability of the gut to face challenges later in life, such as neonatal calf diarrhea (Oikonomou et al., 2013). However, a limitation of the present study is the standardized euthanasia time for all calves at 51 h of life. It may be suggested that if calves were allowed identical time for bacterial colonization after colostrum feeding (e.g. calves fed at 6 h euthanized at 57 h, and calves fed at 12 h euthanized at 63 h of life), no differences

would be observed in regards to the proportions of bacterial groups among treatments. Yet, it has previously been demonstrated that when grown on bovine or human milk, bacterial groups, such as *Bifidobacterium,* achieve a stationary phase of growth at approximately 8-24 h after exposure to a milk medium (Turroni et al., 2011). Therefore, although the time of euthanasia may pose a limitation to the present study, it is assumed that the proportions of bacterial groups measured achieved a stationary phase of growth from the colostrum treatment prior to the collection of intestinal segments at 51 h of life and that similar differences would have been observed if calves were euthanized according to colostrum feeding time.

In addition, while some microorganisms may be beneficial to health, others may be harmful (Picard et al., 2005). For example, enterotoxigenic *E. coli* (ETEC) K99, *Crytosporidium parvum* and rotavirus account for 5.5%, 55.0% and 58.7% of diarrhea incidences in pre-weaning calves, respectively (Uhde et al., 2008). Pathogenic *E. coli,* including ETEC K99, typically binds to the ileum and colon (Orskov et al., 1975; Corley et al. 1977; Moxley & Francis, 1986). Therefore, the lower proportion of total *E. coli* associated with the ileum mucosa in 6 h and 12 h calves when compared to 0 h calves was not expected in the present study. Malmuthuge et al. (2015a) demonstrated that calves not fed colostrum within 12 h after birth displayed a significantly lower density (copy of 16S rRNA genes/g of sample) of total bacteria compared to calves fed fresh or heat-treated colostrum soon after birth, suggesting that bacterial colonization occurs at a slower rate in the absence of colostrum feeding. On the other hand, in mammals, generic *E. coli* can also benefit the host by preventing the colonization of pathogenic bacteria (Reid et al., 2001), producing vitamin K2 (Sharma et al., 1992), and by creating an anoxic environment in the intestine in order for beneficial obligate anaerobes, such as *Bifidobacterium*, to become established (Madigan et al., 2015). For example, the beneficial effects of microcin-

producing *E. coli* strain Nissle 1917 (EcN) include limiting the growth of competitive bacteria in the inflamed intestine and reducing subsequent colonization of the pathogen (Sassone-Corsi et al., 2016). Moreover, a high proportion of *E. coli* is not uncommon in young calves, with *E. coli* previously reported as the dominant bacterial group present in the feces of calves 1-7 d old (Mayer et al., 2012). Therefore, although in the current study only the proportion of total *E. coli* was measured and no conclusions can be drawn in regards to the specific beneficial or pathogenic strains present, this high proportion in calves fed colostrum early in life is not uncommon and may have beneficial effects in the newborn calf intestine.

Many of the bacterial groups associated with the mucosa and digesta of the distal jejunum, ileum, and colon were not statistically different among treatment groups. This may be due to the large individual variation in the detected copy number of the 16S rRNA genes per gram of sample. High genetic, microbial and transcriptomic variation in the neonatal calf has been previously reported, with the exact reasoning for the variation not yet determined (Liang et al., 2014; Malmuthuge et al., 2015a). The specific microbial environment of the birth canal of the dam and the site of parturition may have an effect on the phenotype, as well as the nutrition provided to the animal. Regardless of the exact causative factors for this high individual variation, at this time the neonatal calf intestine, which before birth is devoid of microorganisms, is undergoing unfamiliar, dynamic microbial changes that have previously never occurred. The newborn microbiome is only just beginning to establish itself, and thus it is expected that the microbes within each individual interact with the host in a unique way, leading to the existing high individual variation.

2.6 Conclusion

In conclusion, delaying colostrum feeding by 6 h or 12 h after birth decreases the passive transfer of IgG and the time to maximum serum IgG concentration compared to calves fed colostrum immediately after birth. It is speculated that the gut decreases in permeability when colostrum is not fed immediately to newborn calves, however the exact mechanism by which this occurs is unknown and warrants further investigation. To our knowledge, this is the first study to demonstrate the effects of delaying colostrum feeding on intestinal microbial colonization. It was determined that delaying colostrum feeding tended to decrease the proportion of bacteria associated with the intestinal mucosa, specifically total *Escherichia coli, Bifidobacterium* sp. and *Lactobacillus* sp., which play important roles in gut health. How the presence of these specific bacterial groups during the first days of life may affect future growth and productivity needs to be explored further.

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2.8 Tables and Figures

Table 2-1. Effect of delaying colostrum feeding on IgG parameters relative to ingestion of first colostrum meal.[1] Different letters represent significance at $P < 0.05$. Values represent mean ± SEM.

Parameter	Treatment (Mean ± SEM)			*P*-value
	0 h	6 h	12 h	
AEA_{max} (%)	51.8 ± 4.18[a]	35.6 ± 1.88[b]	35.1 ± 3.15[b]	0.007
AUC_{12}	130.4 ± 14.20[a]	98.4 ± 5.90[b]	96.0 ± 7.60[b]	0.038
AUC_{24}	408.3 ± 35.00[a]	297.8 ± 16.00[b]	293.1 ± 22.30[b]	0.006
AUC_{36}	657.2 ± 50.60[a]	483.7 ± 26.30[b]	485.0 ± 38.40[b]	0.006
IgG_{12} (mg/ml)	23.2 ± 2.00[a]	15.2 ± 0.80[b]	15.3 ± 1.30[b]	0.001
IgG_{24} (mg/ml)	22.3 ± 1.40[a]	17.0 ± 1.00[b]	16.9 ± 1.40[b]	0.008
IgG_{36} (mg/ml)	19.3 ± 1.50[a]	14.9 ± 0.90[b]	15.6 ± 1.40[b]	0.058
T_{max} (h)	17.0 ± 0.71	21.0 ± 1.22	20.7 ± 2.03	0.111
C_{max} (mg/ml)	25.5 ± 2.00[a]	18.2 ± 1.10[b]	18.5 ± 1.40[b]	0.003
C_{max}/T_{max} (mg/ml/h)	1.5 ± 0.13[a]	1.2 ± 0.90[b]	1.0 ± 0.14[b]	0.003
Delta change (mg/ml)	25.0 ± 2.03[a]	17.8 ± 1.08[b]	18.1 ± 1.34[b]	0.004

[1] AEA_{max} = maximum apparent efficiency of absorption; AUC_{12} = area under the curve during the first 12h after colostrum feeding; AUC_{24} = area under the curve during the first 24h after colostrum feeding; AUC_{36} = area under the curve during the first 36 h after colostrum feeding; IgG_{12} = immunoglobulin G concentration at 12 h after the colostrum feeding; T_{max} = time to maximum concentration; C_{max} = maximum concentration, Delta change = baseline IgG concentration subtracted from maximum IgG concentration

Table 2-2. Primers used to determine the copy number of bacterial 16S rRNA genes in the calf intestine.

Bacterial Group	Primer	Product Size (bp)	Annealing Temperature (°C)	Reference
Total Bacteria	Forward (F): 5'-actcctacgggaggcag-3' Reverse (R): 5'-gactaccagggtatctaatcc-3'	467	62	Stevenson & Weimer, 2007
Lactobacillus	F: 5'-gaggcagcagtagggaatcttc-3' R: 5'-ggccagttactacctctatccttcttc-3'	120	62	Delroisse et al., 2008
Bifidobacterium	F: 5'- atcttcggaccbgaygagac-3' R: 5'- cgatvacgtgvacgaaggac-3'	196	66	Cleusix et al., 2010
Total *Escherichia coli*	F:5'- ggaagaagcttgcttctttgctgac-3' R: 5'- agcccggggatttcacatctgactta-3'	544	62	Sabat et al., 2014
Faecalibacterium prausnitzii	F: 5'- ggaggaagaaggtcttcgg-3' R: 5'- aattccgcctacctctgcact-3'	248	60	Vital et al., 2013
Clostridium Cluster XIVa	F: 5'- cggtacctgactaagaagc-3' R: 5'- agtttyattcttgcgaacg-3'	415	60	Rintilla et al., 2004

Table 2-3. Effect of delaying colostrum feeding on the proportion (% of total bacteria) of bacteria associated with the intestinal mucosa in neonatal calves (mean ± SEM).

Region	distal jejunum			ileum			colon		
Treatment	0 h	6 h	12 h	0 h	6 h	12 h	0 h	6 h	12 h
Bifidobacteria	0.03 ± 0.003	0.02 ± 0.009	0.03 ± 0.006	0.03 ± 0.007	0.02 ± 0.005	0.02 ± 0.003	1.24 ± 0.648[a]	0.50 ± 0.400[ab]	0.12 ± 0.017[b]
P-value		0.8471			0.2959			0.2083	
Lactobacillus	0.03 ± 0.007	0.08 ± 0.029	0.073 ± 0.021	0.05 ± 0.021	0.01 ± 0.009	0.02 ± 0.011	0.26 ± 0.075[a]	0.20 ± 0.087[ab]	0.07 ± 0.019[b]
P-value		0.2448			0.3114			0.1424	
Clostridium	0.02 ± 0.005	0.02 ± 0.004	0.033 ± 0.012	0.01 ± 0.002	0.01 ± 0.001	0.01 ± 0.002	2.59 ± 1.618	2.00 ± 0.435	2.11 ± 0.566
P-value		0.3419			0.4059			0.8934	
F. prausnitzii	0.04 ± 0.005[a]	0.05 ± 0.008[b*]	0.05 ± 0.005[b]	0.03 ± 0.003[a]	0.02 ± 0.001[b]	0.03 ± 0.004[ab]	0.16 ± 0.093	1.91 ± 0.979	1.61 ± 0.822
P-value		0.0818			0.1867			0.2414	
Total *E. coli*	0.09 ± 0.025	0.11 ± 0.047	0.07 ± 0.011	1.20 ± 0.458[a]	0.26 ± 0.124[b*]	0.49 ± 0.233[b]	1.70 ± 0.406	2.59 ± 0.875	2.55 ± 0.910
P-value		0.6593			0.1108			0.5617	

[a*,b*]Means with a * and different superscript are significant within intestinal region and bacterial target among treatment groups at $P < 0.05$.

[a,b]Means with a different superscript are significant within intestinal region and bacterial target among treatment groups at $0.05 < P < 0.10$.

Table 2-4. Effect of delaying colostrum feeding on the proportion (% of total bacteria) of intestinal bacteria associated with the digesta in neonatal calves (mean ± SEM).

Region	distal jejunum			ileum			colon		
Treatment	0 h	6 h	12 h	0 h	6 h	12 h	0 h	6 h	12 h
Bifidobacteria	0.05 ± 0.018	0.08 ± 0.030	0.07 ± 0.028	0.01 ± 0.005^{b}	0.05 ± 0.021^{a}	0.01 ± 0.003^{b*}	8.06 ± 6.678	1.04 ± 0.878	0.39 ± 0.262
P-value		0.6462			0.072			0.2224	
Lactobacillus	0.66 ± 0.254	0.41 ± 0.166	0.63 ± 0.280	0.30 ± 0.110	0.89 ± 0.398	0.31 ± 0.078	0.77 ± 0.284	0.71 ± 0.281	0.28 ± 0.094
P-value		0.7432			0.2282			0.2696	
Clostridium	0.01 ± 0.003	0.02 ± 0.019	0.01 ± 0.001	0.01 ± 0.001^{a}	0.04 ± 0.018^{b}	0.01 ± 0.006^{ab}	5.40 ± 2.225^{a}	10.23 ± 2.079^{b}	7.72 ± 1.299^{ab}
P-value		0.4777			0.1603			0.2244	
F. prausnitzii	0.05 ± 0.008	0.05 ± 0.019	0.05 ± 0.016	0.02 ± 0.014	0.03 ± 0.013	0.01 ± 0.002	0.13 ± 0.049	0.60 ± 0.377	0.36 ± 0.137
P-value		0.9915			0.3028			0.3794	
Total *E. coli*	13.29 ± 3.554^{a*}	9.48 ± 5.744^{a*b*}	1.21 ± 0.243^{b*}	13.96 ± 4.613	17.36 ± 6.324	17.23 ± 4.788	5.20 ± 2.413	8.83 ± 3.607	10.18 ± 3.511
P-value		0.1053			0.8941			0.5442	

^{a*,b*}Means with a * and different superscript are significant within intestinal region and bacterial target among treatment groups at $P < 0.05$.

^{a,b}Means with a different superscript are significant within intestinal region and bacterial target among treatment groups at $0.05 < P < 0.10$.

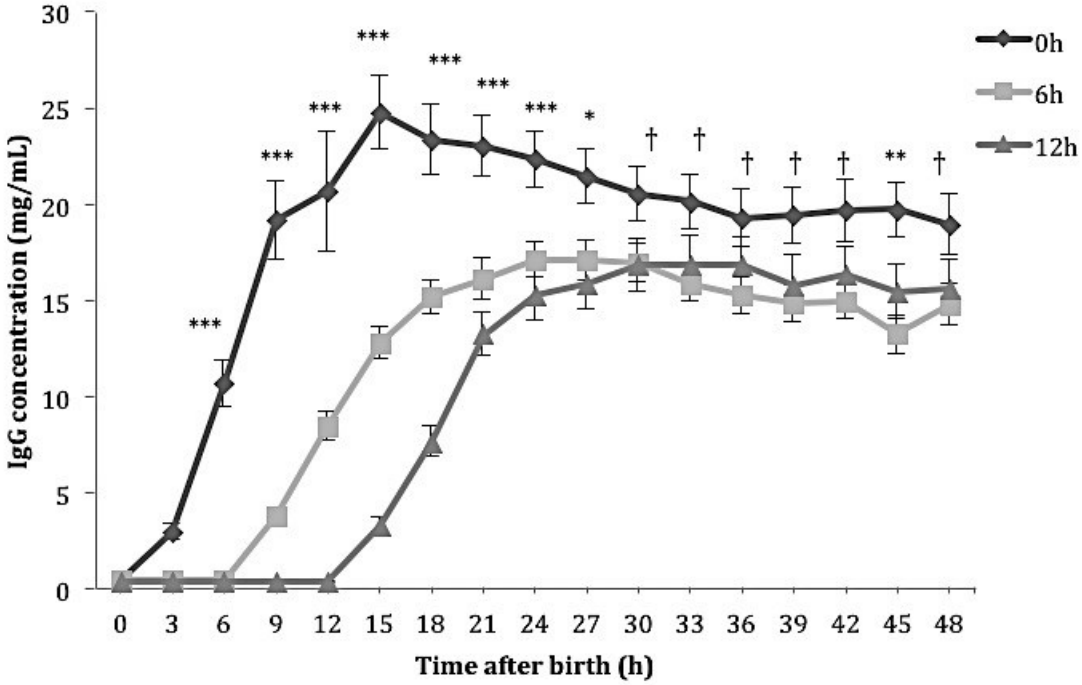

Figure 2-1. The effect of delaying colostrum feeding on serum concentrations of IgG (mg/mL) relative to the time of birth. ***$P < 0.001$, **$0.001 < P < 0.01$, *<$0.01 < P < 0.05$, †$0.05 < P < 0.10$. Points represent mean ± SEM.

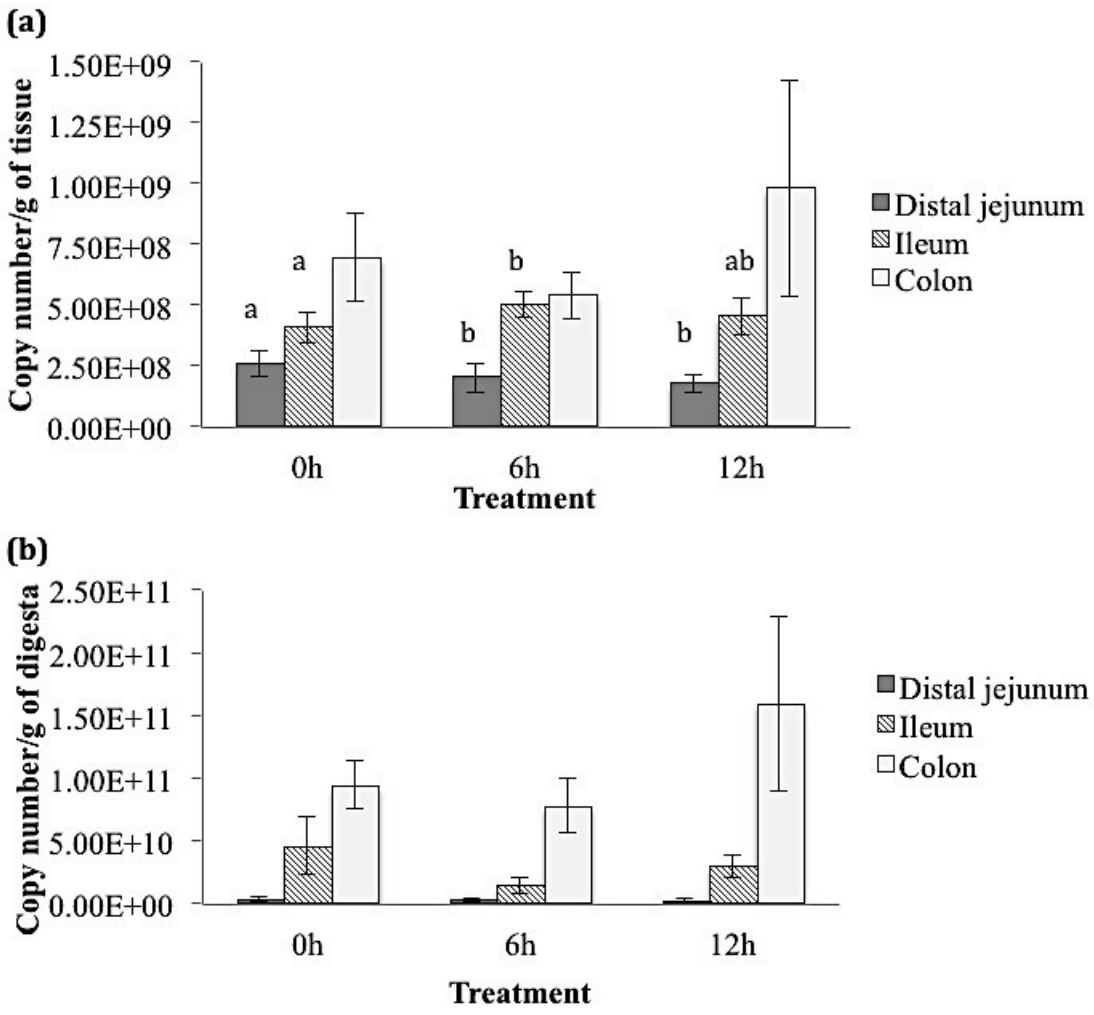

Figure 2-2. Effect of delaying colostrum feeding among treatment groups on the copies of 16S rRNA genes of total bacteria associated with the (a) mucosa and (b) digesta of the distal jejunum, ileum and colon of neonatal calves at 51 h of life. Different letters represent the average copies of 16S rRNA genes of total bacteria have a tendency to be different within intestinal region among treatments at $0.05 < P < 0.10$. Bars represent mean ± SEM.

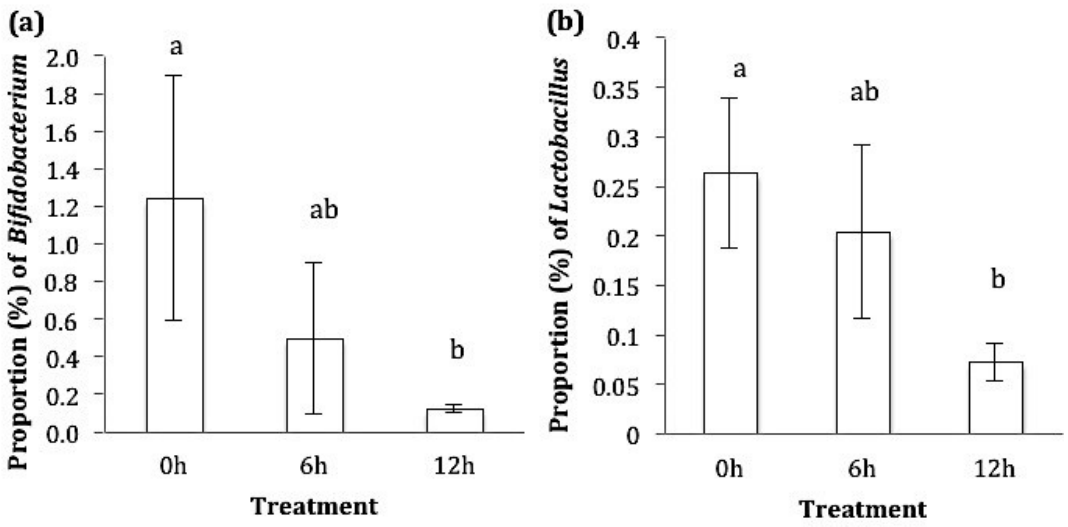

Figure 2-3. Effect of delaying colostrum feeding on the proportion (% of total bacteria) of (a) *Bifidobacterium* sp. and (b) *Lactobacillus* sp. associated with the colon mucosa of neonatal calves at 51 h of life. Different letters represent a tendency to be different among treatments at $0.05 < P < 0.10$. Bars represent mean ± SEM.

3.0 Chapter 3: Effect of heat-treatment of bovine colostrum on the concentration of bovine colostrum oligosaccharides in colostrum and in the intestine of neonatal male Holstein calves

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3.1 Abstract

The objective of this study was to determine the effect of the heat-treatment (60°C for 60 min) on the concentration of bovine colostrum oligosaccharides (bCOs) in pooled bovine colostrum and the intestine of neonatal male Holstein calves after feeding. First-milking colostrum was pooled from both primi- and multi-parous cows, and half of the pooled colostrum was heat-treated at 60°C for 60 min while the other half remained unheated. At birth, thirty-two male Holstein calves were randomly assigned to one of three treatment groups: 1) control calves that did not receive colostrum for the duration of the experiment and were euthanized at 6 h (NC, n = 4) or 12 h (NC, n = 4); 2) calves fed unheated colostrum (UC) and were euthanized at 6 h (UC, n = 6) or 12 h (UC, n = 6); or 3) calves fed heat-treated colostrum (HC) and euthanized at 6 h (HC, n = 6) or 12 h (HC, n = 6). All calves were fed 2 L of colostrum within 1 h after birth. At dissection, digesta of the distal jejunum, ileum and colon were collected and analyzed by liquid chromatography-mass spectrometry to determine the concentration of bCOs within each

intestinal region. The heat-treated (HT) colostrum had a higher concentration of total free bCOs (3511.6 μg/g) when compared to unheated (UH) colostrum (1329.9 μg/g), with 3'sialyllactose being the most abundant bCO in both UH and HT colostrum. In contrast, calves fed HT colostrum had a lower amount of free total bCOs in the distal jejunum (221.91 ± 105.3 vs. 611.26 ± 265.1 μg/g), ileum (64.97 ± 48.39 vs. 344.04 ± 216.87 μg/g) and colon (25.60 ± 13.1 vs. 267.04 ± 125.81 μg/g) at 6 h of life when compared to calves fed unheated colostrum. No differences were observed in regards to the concentrations of total bCOs in the intestine of UC and HC calves at 12 h of life. Lower concentrations of bCOs in the gastrointestinal tract of HC calves at 6 h of life could be due to the early establishment of beneficial bacterial, such as *Bifidobacterium,* in HC calves and their subsequent metabolism of bCOs as a carbon source. These findings suggest that the heat-treatment of colostrum increases the concentration of free bCOs, which may serve as prebiotics available to microbiota within the intestine of the neonatal calf.

3.2 Introduction

The neonatal dairy calf is at high risk of morbidity and mortality (NAHMS, 2011), which causes concern not only from an economic standpoint, but also in regards to welfare. The timely feeding of high-quality, adequate volumes of uncontaminated colostrum is a key factor in determining the survival of the neonatal dairy calf (Weaver et al., 2000). However, although the consequences of poor colostrum management are well known, many farms do not asses the quality of colostrum, which may lead to feeding contaminated colostrum or colostrum with a low concentration of IgG. Moreover, common management practices often fail to feed the first colostrum meal in a timely

manner (Vasseur et al., 2010). Unfortunately, this type of colostrum management plays a pivotal role in decreased calf health and welfare, which contributes to the high rates of morbidity reported in neonatal calves. More specifically, there is an alarmingly high prevalence of enteric infections in neonatal calves, with neonatal calf diarrhea (NCD) being the most common ailment resulting in illness and death (Meganck, 2014) and 25.3% of pre-weaned calves being affected by digestive problems (NAHMS, 2011). Therefore, knowledge regarding how to decrease the prevalence of digestive disorders in pre-weaning calves is necessary to ensure a profitable dairy industry.

In an effort to improve neonatal calf gut health, there has been increasing interest in supplementing bovine colostrum or colostrum replacers with gut active carbohydrates derived from yeast (mannan-oligosaccharides, MOS) and bacteria (*Bifidobacterium* galacto-oligosaccharides, BGOS) (Brady et al., 2015). However, the majority of studies using large sample sizes have found negative or no effect on calf performance and passive transfer of immunity when MOS or BGOS are supplemented (Robichaud et al., 2014; Brady et al., 2015). During early life, the gastrointestinal tract (GIT) of the calf is evolutionarily tailored to respond to compounds secreted by the dam into colostrum and milk, and the structure of an oligosaccharide (OS) is a major determinant of biological function (Short et al., 2016). For instance, MOS are particularly effective at adhering to *Escherichia coli* when present in an α1-3 and α1-6 configuration (Firon et al., 1987), while sialylated OS are most effective as α2-6 isomers (Martin et al., 2002). Therefore, these differences in structure and configuration may provide reasoning as to why the supplementation of MOS may not have a beneficial effect on the calf GIT during early life, as it may better respond to bovine colostrum oligosaccharides (bCO) structures

during this period. Martin-Sosa et al. (2003) determined five primary OS compounds present in bovine colostrum and milk, with significantly higher amounts of specific OS present in colostrum compared to mature milk. More than 70% of the identified OS in bovine colostrum and milk are sialylated (Tao et al., 2008), with 3'sialyllactose being the most abundant isoform in colostrum, followed by 6'sialyllactosamine (Martin-Sosa et al., 2003). It has also been demonstrated that heat-treated milk has a higher concentration of free sialylated OS compared to fresh milk (Nesser et al., 1991), suggesting that the processing of colostrum or milk may have an effect on the types and concentrations of OS present. Using *in vitro* experiments, it has been demonstrated that OS are able to resist enzymatic hydrolysis throughout the upper GIT and it was previously thought that the majority of OS reach the colon intact for fermentation by commensal microbiota (Engfer et al., 2000). However, a recent study using a rat model showed that the intestinal bacteria might metabolize human milk-derived OS as early as the jejunum and that smaller molecular weight OS may actually never reach the colon (Janschter-Krenn et al., 2013).

Currently, there is a lack of knowledge regarding methods to increase the availability of oligosaccharides in bovine colostrum for supplementation in dairy calves, as well as the characterization of bCO concentrations in the neonatal calf intestine. Therefore, the objectives of the present study were to: 1) determine the effect of the heat-treatment of colostrum on the concentration of bCOs, and 2) to determine the concentrations of bCOs in the small and large intestinal regions of neonatal calves fed heat-treated (HT) colostrum compared to calves fed unheated (UH) colostrum. It was hypothesized that HT colostrum would have higher concentrations of free bCOs when

compared to UH colostrum and as a consequence, calves fed HT colostrum would have higher concentrations of bCOs within the intestine compared to calves fed UH colostrum.

3.3 Materials and Methods

The experimental procedures reported were conducted at the Dairy Research and Technology Centre, University of Alberta in accordance with the Canadian Council of Animal Care (CCAC, 1993) and all protocols were approved by the University of Alberta Animal Care and Use Committee for Livestock (AUP00001012). Colostrum (first milking after calving) containing ≥ 50 mg/mL of IgG was collected from 16 multi-parous cows and immediately frozen at -20 ^{o}C after collection. Once the required volume was collected, colostrum was thawed and pooled. Half of the pooled colostrum (24 L) was heat-treated for 60 min at 60°C using a pasteurizer (DT 10G, Dairy Tech Inc., Greely, CO). Both UH and HT colostrum were frozen at -20°C until needed. At birth, male Holstein calves were randomly assigned to one of three treatment groups: 1) control calves that did not receive colostrum for the duration of the experiment and euthanized at 6 h (NC, n = 4) or 12 h (NC, n = 4); 2) calves fed unheated colostrum and euthanized at 6 h (UC, n = 6) or 12 h (UC, n = 6); or 3) calves fed heat-treated colostrum and euthanized at 6 h (HC, n = 6) or 12 h (HC, n = 6). The average birth body weight of UC calves euthanized at 6 h and 12 h were 40.9 ± 3.4 and 39.1 ± 1.5 kg, respectively and for HC calves euthanized at 6 h and 12 h were 47.7 ± 3.3 and 41.4 ± 2.1 kg, respectively. Using a water bath, colostrum was thawed to 38°C and 2 L were fed to each calf using an esophageal tube feeder within an hour after birth. Calves were euthanized by penetrative captive bolt followed by exsanguination. The digesta samples were collected following

the procedures previously reported by Malmuthuge et al. (2015). Briefly, closed intestinal segments (10 cm) of the distal jejunum, ileum and colon were collected with the distal jejunum defined as 30 cm proximal to the collateral branch of the mesenteric artery, the ileum defined as 30 cm proximal to the ileo-cecal junction, and the colon defined as 30 cm distal to the colon-cecal junction. After collection, digesta samples were snap-frozen in liquid nitrogen and transferred to -80°C until further analysis.

After thawing digesta samples for 5 min on ice, approximately 0.10 g of each sample was obtained and placed in a 2 mL microcentrifuge tube. This was followed by the addition of 150 µl of HPLC-grade water and defatting by centrifugation at 10,000 rpm for 15 min and the supernatant was removed and placed in a new tube. After repeating the above step twice, 1 ml of 2:1 chloroform:methanol was added to the supernatant and centrifugation was performed at 2,000 rpm for 60 min to remove any proteins and impurities. The lower phase was then re-extracted using 500 µl of 50% methanol and the resulting supernatant was cooled at 4°C for 30 min. The sample was then centrifuged at 14,800 rpm for 15 min to remove any residual contaminants and diluted 5-fold using 95% acetonitrile (AcN). Colostrum was processed using the same procedures, except that it was only defatted by centrifugation once and diluted 5-fold prior to chloroform:methanol extraction. All samples were stored at 4°C until liquid chromatography-mass spectrometry (LC-MS) analysis. The recovery rate of the OS extraction method was assessed by spiking a known amount of internal standard (β1-3-Gal-N-acetyl-galactosaminyl-β1-4-Gal-β1-4-Glc, GalNAc) prior to OS extraction and was measured by LC-MS and estimated to be 97%.

Oligosaccharide standards, including disialyllactose (DSL), 3'-sialyllactose (3'SL), 6'-sialyllactose (6'SL), 3'-sialyllactosamine (3'SLN), and 6'-sialyllactosamine (6'SLN), and GalNAc (internal standard), were purchased from Dextra Laboratories Ltd. (Reading, U.K.) and diluted using 95% AcN to give a 9-point calibration curve. An LC system with a binary pump and autosampler (Agilent Technologies, Palo Alto, CA, USA) coupled to a 4000 QTRAP mass spectrometer (AB SCIEX, Concord, ON, Canada) was used to determine the concentration of bCOs in colostrum and intestinal digesta. Liquid chromatography separation was performed using an Ascentis Express hydrophilic interaction liquid chromatography (HILIC) column (10 cm×2.1 mm, 2.7 μm in particle size) (Sigma, St. Louis, MO), with the mobile phase being composed of (A) water with 50mM ammonium acetate, and (B) AcN. The total run time was 35 min (including re-equilibration) with a constant flow rate of 200 μL/min. The gradient used was as follows: 0-18 min, 5% to 30 % A; 18-20 min, 30% A; 20.1 min increased to 50% A; 20-24 min held at 50% A and decreased to 5% A at 24.1 min for column re-equilibrium over 11 min prior to the next injection. The auto-sampler temperature was set to 15˚C with 5 μL of injection volume. A turbo spray ion source (electrospray ionization) was used under negative ion mode and multiple reaction-monitoring (MRM) scan mode was developed for the quantification of the analytes of interest. Nitrogen was used as curtain gas, nebulizing gas and drying gas. The instrument was operated using the following settings: curtain gas, gas 1 and gas 2 at 20, 50 and 50 arbitrary units, respectively, and ionspray voltage at -4.5 kV. The ion source temperature was 400 ˚C and quadrupoles Q1 and Q3 were operating at unit mass resolution. The MRM transitions and optimized mass spectrometer parameters for each analyte and their reference internal standard (GalNAc)

are summarized in Table 3-1. The data was processed using Analyst 1.6 software (AB SCIEX, Concord, ON, Canada).

To determine the effect of colostrum treatment on the concentration of bCOs within intestinal region, all data were analyzed using the MIXED procedure of the Statistical Analysis system (SAS Institute, Cary, NC). Data were analyzed using the animal as a random effect, and the treatment (HC, UC), sample time (6 h, 12 h), and sample type (distal jejunum, ileum, colon) and their interactions as fixed effects, and body weight was included as a covariate. For the effect of treatment on the concentration of each bCO (3'SL, 6'SL, 3'SLN, 6'SLN, DSL) and total bCOs, data were analyzed among treatment group by sampling time and sample type and the interaction (treat*type, treat*time, time*type and treat*type*time). All values reported are least square means (LSM) with significance declared at $P \leq 0.05$ and tendencies at $0.05 < P < 0.10$.

3.4 Results and Discussion

Using LC-MS, four main types of bCOs were detected in bovine colostrum, regardless of treatment (Fig 3-1(a)). Our study revealed that 3'SL was the dominant bCO in both heat-treated (2390.0 μg/g) and unheated (840.0 μg/g) colostrum samples (Figure 3-1(a),(b),(c)), which is consistent with previous reports (Martin-Sosa et al., 2003; Namakura et al., 2003; Fong et al., 2011). However, the value of 3'SL in UC in our study differed from previous studies that have reported values of 354 μg/g and 1245 μg/g of 3'SL in unheated colostrum (Martin-Sosa et al., 2003; Fong et al., 2011). The differences observed between the present study and previous reports may be attributed to differences in the sampling time after parturition, genetics and sample size of the various

experiments. Specifically, Fong et al. (2011) collected 'second milking' colostrum from a single Freisan cow, and Martin-Sosa et al. (2003) obtained samples from six multiparous Spanish-Brown cows on day 2 of lactation. In contrast to these two experiments, the present study collected pooled, 'first-milking' colostrum from 16 multi-parous Holstein cattle. Further research using large sample sizes and frequent sampling throughout the various lactation stages of both primi-parous and multi-parous cows is needed to determine the factors which may greatly influence the concentrations of bCOs in colostrum.

Although statistical analysis was not performed for pooled colostrum samples, as there was only a single pooled heat-treated sample and a single pooled unheated sample, heat-treatment numerically increased 3'SL by 2.8 times, 6'SLN by 2.3 times, 6'SL by 1.5 times, and DSL by 3.6 times when compared to UC, while 3'SLN was low (1.1-3.8 μg/g) and not different between the two types of colostrum samples (Figure 3-1(a)). In contrast to human milk, bovine milk contains less free oligosaccharides, as they are generally found attached to lipid or protein as glycoconjugate structures (Kobata, 1977; Nesser et al., 1991). The concentration of free oligosaccharides in bovine milk can be increased through heat-treatment (Nesser et al., 1991), likely from their cleavage from these structures. Therefore, it is suggested that the cleavage of bCOs from colostral lipids or proteins may provide reasoning for the increase in the concentration of free bCOs in HT colostrum when compared to UH colostrum.

In contrast to our hypothesis, although higher concentrations of bCOs were detected in heat-treated colostrum, calves' fed heat-treated colostrum had a lower concentration of bCOs within intestinal regions at 6 h when compared to that of calves'

fed unheated colostrum. As expected, no bCOs were detected in the intestine of calves not fed colostrum at either 6 h or 12 h of life. More specifically, at 6 h of life UC calves tended to have a higher concentration of DSL ($P = 0.05$) and a significantly higher concentration of 6'SLN ($P = 0.02$), 6'SL ($P = 0.03$), and 3'SL ($P = 0.01$) in the distal jejunum when compared to HC calves (Figure 3-2). In the ileum, UC calves tended to have a higher concentration of 6'SLN ($P = 0.09$) and 6'SL ($P = 0.06$), and a significantly higher concentration of 3'SL ($P = 0.03$) and DSL ($P = 0.04$) compared to HC calves. Additionally, the colon of UC calves tended to have a higher concentration of 6'SLN ($P = 0.09$), 6'SL ($P = 0.06$), and DSL ($P = 0.06$), and a significantly higher concentration of 3'SL ($P = 0.02$) compared to HC calves. Yet, at 12 h of life no differences were detected in bCOs within the distal jejunum or ileum among treatments, however UC calves had a higher concentration of DSL ($P = 0.04$) in the colon when compared to HC calves. Species belonging to the genera *Bifidobacteria* have been shown to exhibit robust sialidase activity, as well as produce large amounts of acidic fermentation products (e.g. lactate and SCFAs) when grown on 3'SL and 6'SL (Yu et al., 2013). A previous study which focuses on the differences of *Bifidobacterium* sp. in the small intestine of the same calves from the present study reported that calves fed heat-treated colostrum displayed a higher proportion of small intestinal *Bifidobacterium* sp. at 6 h of life compared to calves fed unheated colostrum, while no differences were detected at 12 h among treatment groups (Malmuthuge et al., 2015). Therefore, we speculate that the low concentration of bCOs in the intestine of HC calves may be due to their utilization as a substrate for the growth of *Bifidobacteria*. To support our speculation, further correlation analysis was performed to explore the relationship between bCOs and *Bifidobacterium* in the intestine

of UC and HC calves. A negative correlation (r = -0.47, $P = 0.04$) between *Bifidobacterium* associated with the distal jejunum mucosa and bCOs was observed, suggesting that the decreased concentrations of bCOs may be due to their metabolism by *Bifidobacterium* in the small intestine of HC calves. However, additional bacterial genera in the intestine may also metabolize bCOs, such as *Bacteroides,* and certain species belonging to this genus have been shown to cleave and catabolize sialic acid (Marcobal et al., 2011). Future studies involving more in-depth analysis of the gut microbiota composition and population changes between HC and FC would help us determine the exact causative factors for the observed lower concentrations of bCOs in the gut of HC calves. In addition, studies have reported biological effects of sialylated OS, including their ability to inhibit pathogenic *E. coli* K99 (Martin et al., 2002) and their capability to enhance the absorption of IgG (Gill et al., 1999). Therefore, the high content of sialylated OS (e.g. 3'SL (2390.0 μg/g)) observed in heat-treated colostrum in the present study suggests it may have a potential beneficial effect as a prebiotic to enhance the population of beneficial microorganisms. Future studies are needed to understand the role of bCOs in the intestine of neonatal calves and their interactions with the immune system and intestinal microbiota.

3.5 Conclusion

To our knowledge, the present study is the first to characterize the concentration of bCOs from unheated and heat-treated colostrum throughout the intestine of neonatal dairy calves, as well as to investigate the effect of heat-treatment on the concentration of bCOs in colostrum. In conclusion, the heat-treatment of colostrum increased the

concentration of free bCOs in colostrum compared to unheated colostrum. Heat-treatment at 60°C for 60 min may release sialylated bCOs from colostrum proteins and lipids, thus increasing the amount of free bCOs. It was also determined that UC calves displayed a higher concentration of bCOs at 6 h of life in the intestine compared to HC calves, which may suggest a variation in their metabolism due to the lower proportion of *Bifidobacterium*. Our study suggests that heat-treatment may also have a potential prebiotic benefit in regards to providing more substrate to beneficial microorganisms, such as *Bifidobacterium*. Future research should focus on characterizing the microbial community of the intestine when calves are fed unheated and heat-treated colostrum, as well as the potential beneficial effects of bCOs on the microbiome and immune system of neonatal dairy calves.

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3.7 Tables and Figures

Table 3-1. MRM transitions and optimized parameters for each oligosaccharide compound.

Compound(s)	MRM transitions (amu)	DP[a] (eV)	EP[a] (eV)	CE[a] (eV)	CXP[a] (eV)
3'-SLN/6'-SLN	673→290	-105	-10	-44	-13
3'-SLN/6'-SLN	673→572	50	4	-38	-13
3'-SL/6'-SL	632→290	40	4	35	3
3'-SL/6'-SL	632→572	45	4.5	40	3
DSL	932→632	50	4	40	3
DSL	932→581	45	4.5	40	3
glucose	706→628	50	6	25	6
glucose	706→201	50	6	32	6

[a] DP, EP, CEP, CE and CXP are declustering potential, entrance potential, collision energy and collision cell exit potential.

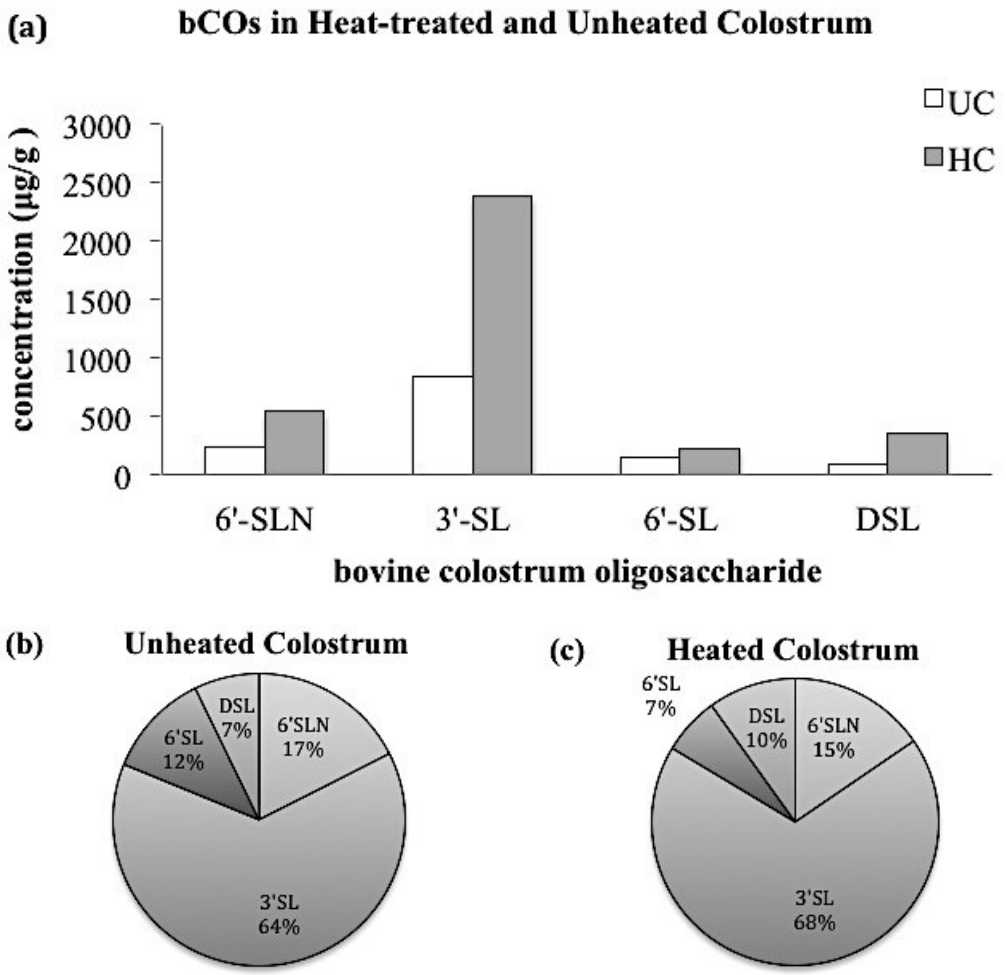

Figure 3-1. (a) Numerical differences between the concentrations of bovine colostrum oligosaccharides in a single pooled unheated colostrum (UC) sample and a single pooled colostrum sample heat-treated at 60°C for 60 min (HC). Bars represent the concentration of each bCO (µg/g) of within each pooled colostrum sample (b) The proportion of bovine colostrum oligosaccharides in unheated colostrum as a percentage of total oligosaccharides (c) The proportion of bovine colostrum oligosaccharides in heat-treated colostrum as a percentage of total oligosaccharides. HC = heat-treated colostrum; UC = unheated colostrum; 3'SL = 3'sialyllactose; 6'SL = 6'sialyllactose; 6'SLN = 6'sialyllactosamine; DSL = disialyllactose.

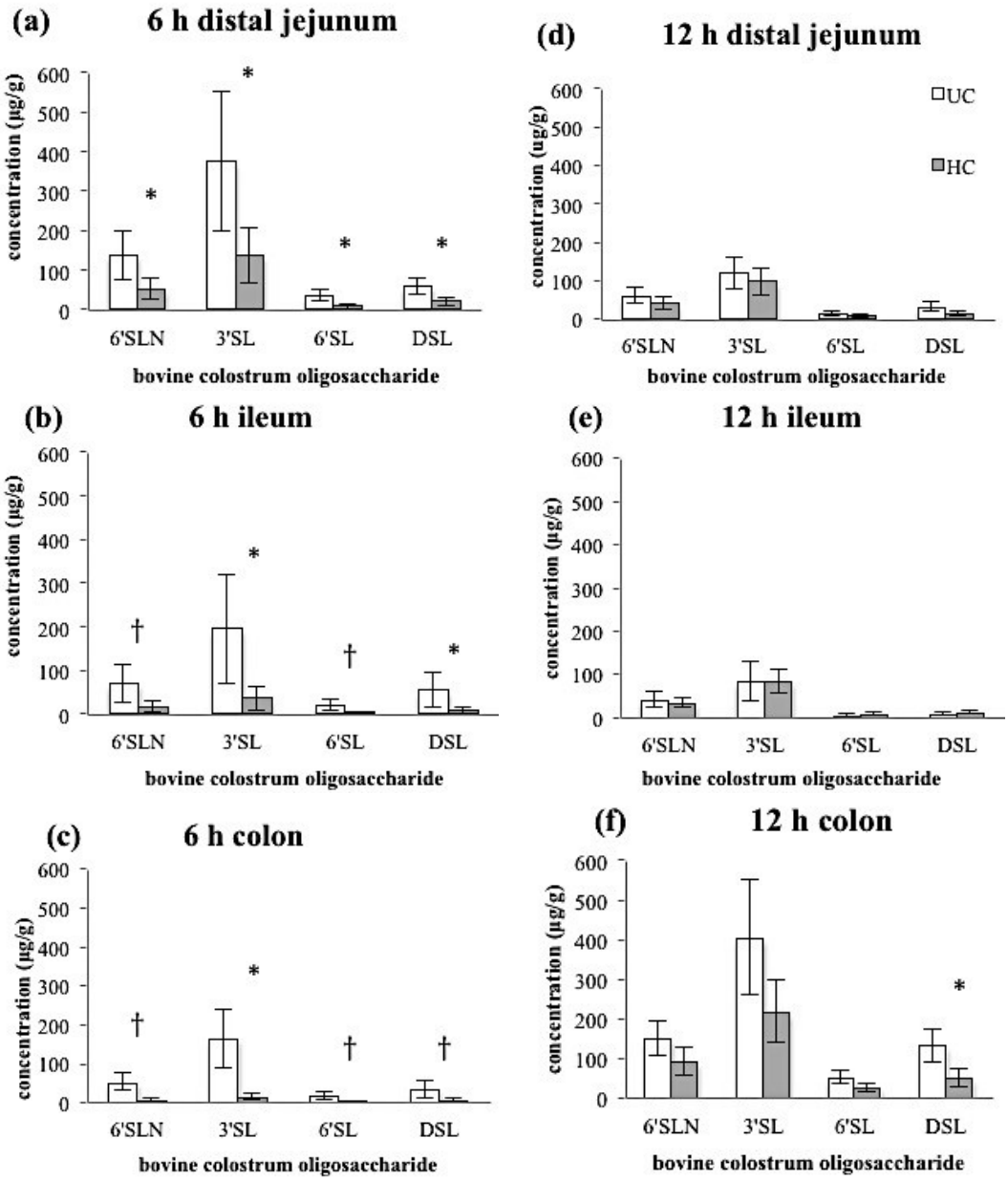

Figure 3-2. The concentration (µg/g) of bovine colostrum oligosaccharides in the intestine of calves fed unheated (UC) and heat-treated (HC) colostrum. Bars represent mean ± SEM. 3'SL = 3'sialyllactose; 6'SL = 6'sialyllactose; 6'SLN = 6'sialyllactosamine; DSL = disialyllactose. A * above bars represent significance between treatment groups at $P < 0.05$ and a † represents a tendency at $0.05 < P < 0.10$.

4.0 Chapter 4: General Discussion

4.1 Significance of Research

Poor colostrum management continues to be a problem on dairy farms, which often leads to increased susceptibility to pathogenic infection and disease in the neonatal dairy calf (Vasseur et al., 2010). However, little is known about how poor colostrum management may affect the health of the neonatal calf, specifically the passive transfer of IgG and intestinal bacterial colonization. As well, current strategies to reduce the incidence of health events during early life, such as neonatal calf diarrhea, are limited and recent studies suggest that there are prebiotic compounds in bovine colostrum that may promote a healthy gastrointestinal microbial community (Yu et al., 2013). Therefore, the experiments presented in this thesis were conducted to provide fundamental knowledge to industry professionals and the scientific community regarding the consequences of delaying colostrum feeding on calf health, as well as the characterization of potential prebiotic compounds from colostrum throughout the intestine (GIT) of the neonatal calf.

The findings presented in the second chapter of this thesis demonstrate the importance of the timely delivery of colostrum on passive immunity. To our knowledge, this is the first study to demonstrate the effect of delaying the first colostrum meal using frequent blood sampling at 3 h intervals and highly standardized colostrum of a known IgG concentration. The majority of studies concerning passive immunity assess IgG status at 6 h or 12 h intervals, or even by a single blood sample taken anywhere between 1-7 d of age. Furthermore, many authors fail to report colostrum characteristics and standardize colostrum fed to calves. Therefore, the methods used in the present study

allow for the proper estimation of absorption parameters of IgG at frequent intervals, as well as determining how maximum IgG concentrations are achieved during early life.

This thesis also marks the first study to demonstrate how delaying colostrum feeding may affect intestinal microbial colonization at the end of the second day of life. A recent study conducted by Malmuthuge et al. (2014) indicated the presence of region-specific microbiota throughout the small intestine, which cannot be demonstrated through profiling of the fecal microbiota (Romano-Keeler et al., 2014; Malmuthuge, 2016). Thus, the dissection of specific intestinal compartments - as found in this study - provides a comprehensive analysis of region-specific mucosa and digesta associated bacterial groups when colostrum feeding is delayed, which has previously never been shown before.

In addition to determining the effects of delaying the first feeding of colostrum, the present thesis also demonstrates how heat-treatment at 60°C for 60 min, which is a common management practice on farm, may affect the availability of prebiotic compounds within colostrum and characterizes their presence in the intestine of neonatal calves. Previous research has indicated the effect of heat-treatment of bovine milk on sialylated oligosaccharides (OS) (Nesser et al., 1991), however the present study is the first to demonstrate this effect on the five most abundant OS in bovine colostrum. As well, previous research focusing on the supplementation of yeast- and bacteria-derived OS in colostrum to improve passive transfer and performance measures have found no beneficial effects (Robichaud et al., 2014; Brady et al., 2015). The reasoning for this may be that during the neonatal period, the calf GIT is evolutionarily tailored to respond to compounds secreted by the dam in colostrum. Therefore, with an increased interest in improving calf gut health and decreasing the incidence of GIT disorders, the

characterization of bCOs throughout the calf intestine may have implications for potential prebiotic compounds that may be available and supplemented in colostrum or milk in the future.

4.2 Understanding the Effects of Delaying Colostrum Feeding on Passive Transfer

A primary objective of the first study presented in this thesis was to determine the effect of delaying the first colostrum meal on the passive transfer of IgG in the neonatal dairy calf. In regards to passive transfer, it was demonstrated that calves fed colostrum immediately after birth had higher serum IgG concentrations, as well as maximum apparent efficiency of absorption of IgG, when compared to calves fed colostrum at 6 h or 12 h after birth. Interestingly, no calves experienced failure of passive transfer (serum IgG < 10 mg/ml), even if feeding was delayed to 12 h after birth. It is speculated that the reasoning for no calves failing passive transfer in the current study may be due to the adequate volume of high quality colostrum fed to calves (~197 g of IgG delivered to each calf). However, on farm, the concentration of IgG in colostrum can widely vary, with a reported range of 7.1 to 159 mg/mL, with 16% of samples containing concentrations less than 50 mg/mL (Quigley et al., 2013). Therefore, the present study may not be representative of on-farm scenarios, where the average volume of colostrum fed to calves in Canada is only 2.5 L (Vasseur et al., 2010), which translates to anywhere from 17.5 mg to 397 mg of IgG being fed to the calf (Quigley et al., 2013). The rate of failure of passive transfer of IgG is markedly decreased when calves receive more than 100 g of IgG within the first hours of life (Besser et al., 1991). This study also demonstrated that if only 2 L of colostrum was fed to each calf, then only 36% of colostrum samples would

provide an adequate amount of IgG to ensure successful passive transfer (Besser et al., 1991; Weaver et al., 2000). Moreover, when calves were fed 2 L of colostrum at 12 h of life, serum IgG concentrations only reached a maximum of 7.9 mg/ml and calves were at high risk for illness and death (Stott et al., 1979). Therefore, in industry, it is likely that with the lower volume of colostrum fed to calves and the variable mass of IgG, that calves fed at 12 h of life would fail passive transfer. In addition, irrespective of parameters indicative of the successful passive transfer, the earlier calves that receive colostral antibodies translaters to earlier protection from potential pathogens. For example, not feeding a calf until 12 h of life results in the calf being unprotected in a scenario of pathogenic challenge, such as *E. coli* or *Cryptosporidium* present in the birthing environment, or even the calf pen during early life. Additionally, it has been demonstrated that a large proportion of IgG can be recycled from the circulation into the intestinal lumen (Butler, 1999). Therefore, if the early feeding of colostrum results in increased absorption of IgG into the circulation, then this may contribute to better protection of the GIT against infection during early life (Besser et al., 1988).

In addition to no calves in the present study failing passive transfer, no differences were found between 6 h and 12 h calves in regards to passive transfer parameters. It is suggested that there may be a critical time point between 1 h and 6 h after birth in which the efficiency of the absorption of IgG decreases and the closure of the small intestine increases to a degree when colostrum is not provided. However, little is known about whether or not IgG absorption occurs by a receptor-mediated or non-specific mechanism, and how closure of the intestine is mediated is also unknown. Therefore, the exact mechanism explaining this decreased absorption of IgG in 6 h and 12 h calves cannot be

determined from this study. The intensity by which IgG is absorbed in a short duration of time has led the scientific community to assume the absorption of IgG is non-specific. Early studies further demonstrate the presence of a vesicular-vacuolar mechanism by which IgG is non-selectively absorbed by transcytosis through enterocytes (Smeaton & Simpson-Morgan, 1985). To our knowledge, in neonatal calves, no studies have confirmed the presence of the neonatal Fc receptor (FcRn), which transports IgG from the intestine into the circulation in rodents (Rodewald & Kraehebuhl, 1984). However, histological analysis of the newborn lamb intestine revealed the expression of FcRn on the apical side of duodenal crypt cells (Mayer et al., 2002), but not in enterocytes, which are responsible for the initial absorption of IgG. Thus, although it is likely that IgG absorption occurs in a non-specific manner, it may be assumed that there is a fine-tuned control during the absorptive and closure processes of the intestine, which may be initiated by specific biological factors. Specifically, the docking and fusion of carrier vesicles, as well as the sorting of specific proteins into these vesicles, are mediated through a complex system of signals which are encoded within the apical and basolateral membranes of the enterocyte (Hunziker & Kraehenbuhl, 1998). It may be possible that when colostrum is not fed during the first 6 h or 12 h of life there may be a down-regulation in a crucial protein in the membrane of the enterocyte that mediates the fusion of vesicles to the membrane and uptake of IgG through the enterocyte and into circulation. Research using labeled IgG and serial dissections at frequent intervals after colostrum feeding should be conducted in order to collect tissue samples for histological and transcriptomic analysis to determine the change in cell morphology during the absorptive period, as well as potential proteins which may influence this uptake.

Moreover, *ex-vivo* studies using isolated newborn calf intestinal tissue subjected to an IgG medium in Ussing chambers may be useful to determine the exact mechanism by which this absorption occurs, as well as the factors that influence the decrease in absorptive capacity when calves are not fed during the first 6 and 12 h of life.

4.3 Understanding the Effects of Delaying Colostrum Feeding on Intestinal Bacterial Colonization

Although the successful passive transfer of IgG is essential in ensuring a healthy dairy calf, the composition and establishment of GIT microbiota has also been associated with health and disease outcomes (Oikonomou et al., 2013). The delivery of colostrum is essential in establishing the early life gut microbiota as it has been shown that withholding colostrum feeding can decrease the abundance of total bacteria within the small intestine during the first 12 h of life (Malmuthuge et al., 2015). Therefore, a primary objective of this thesis was to determine how delaying the first colostrum meal would affect intestinal bacterial colonization at 51 h after birth. The results suggest that delaying the first colostrum meal may delay the initial establishment of intestinal microbes, such as *Bifidobacterium, Lactobacillus* and total *Escherichia coli.* However, mainly tendencies were observed in regards to differences in bacterial groups among treatments, which is likely due to the high individual variation in proportion of bacterial genera and species reported. Previous research has demonstrated that the composition and diversity of the neonatal calf small intestinal microbiome is not similar to that of the birth environment, or the dam birth canal (Malmuthuge, 2016). This is in contrast to human studies, in which infants born by Caesarian section have a fecal microbial community

similar to that of the hospital and skin environment (Fanaro et al., 2003; Dominguez-Bello et al., 2010), while infants born by vaginal delivery have fecal microbiota that closely resembles the mother's birth canal (Dominguez-Bello et al., 2010). Furthermore, similar studies also suggest the vertical transmission of bacterial groups, such as *Bifidobacterium,* from the mother to the infant (Makino et al., 2013). Malmuthuge (2016) did not detect the dominant *Bifidobacterium* species in the calf small intestine, *Bifidobacterium longum* subsp. *infantis,* in the dam birth canal, yet did detect other *Bifidobacterium* species, specifically *Bifidobacterium longum* and *Bifidobacterium pseudolongum*. It was suggested that the abundance of *B. longum* subsp. *infantis* may be below the detection limit of PCR in the dam birth canal and that even though it may be transmitted in low amounts, it may become one of the predominant species in the calf small intestine due to the environment of the newborn gut, which contains and abundance of glycans and milk oligosaccharides that are primary substrates for the growth of this organism (Malmuthuge, 2016). Future research should focus on the effects of the dam birth canal on calf gastrointestinal microbiota, and studies using calves born via C-section or delivered vaginally may provide conclusive evidence on this subject. Moreover, it has also been shown that genetics may play a factor in microbial colonization, with twin calves having a more similar fecal microbial composition compared to calves that are unrelated (Mayer et al., 2012) and calves with different microbial communities displaying differential levels of immune genes in the intestine (Liang et al., 2014). It may be of interest to perform RNA-sequencing of the host intestinal tissues in the calves used in the present study in order to determine any correlations between the expression of specific genes and the microbial groups present. Additional factors that may have

influenced the high individual variation observed among calves may include difference in the time of birth, as calves were born from February to September of 2016, as well as variations in the animal handling and the specific research personnel interacting with each calf. In conclusion, it is clear that the differences observed between calves may be due to a multitude of environmental, maternal, and genetic factors, and future research should be conducted to determine the potential influence of each aspect on the newborn calf gastrointestinal tract microbial community.

A major limitation of the present study is the collection of intestinal segments at only 51 h after birth. Repeated sampling, for example using newborn calves not fed colostrum, and calves euthanized at 24 h after feeding, may have provided a better understanding of the environmental (e.g. nutritional) that influence early life bacterial colonization. Regardless, it is not unexpected that newborn calves would exhibit high individual variation in their intestinal microbes. At this time, the newborn microbiome is attempting to find a balance and an abundance of microbes are competing and utilizing a variety of substrates to establish themselves. A metagenomic approach can be used to estimate microbial gene composition and functional abundance of the microbiota, while metatranscriptomics measure the levels of gene expression, allowing for the determination of the active microbial community (Malmuthuge & Guan, 2017). The use of one of these techniques in the present study would have allowed for a more comprehensive analysis of the microbial community in its entirety, in contrast to only five bacterial groups being measured, and would have provided information regarding the functions of these bacterial groups and the factors that may influence their abundance. Moreover, if preventing the immediate establishment of essential early life bacterial

groups occurs as a result of delaying the first colostrum feeding, this may have lasting effects on the intestinal microbiome. Whether this affects the ability of the gut to respond to pathogenic challenges later in life is unknown and further research regarding the effect of delayed colostrum feeding using repeated intestinal sampling throughout the pre-weaning period, along with high-throughput microbial identification on calf health events and future production, should be conducted.

4.4 Understanding the Effects of Feeding Heat-treated Colostrum on Bovine Colostrum Oligosaccharides

The objective of the second experiment presented in this thesis was to determine the effect of the heat-treatment of colostrum on the presence of bovine colostrum oligosaccharides (bCOs) within colostrum and the intestine of neonatal calves. Similar to previous studies, 3'sialyllactose (3'SL) was the primary bCO identified in both fresh and heat-treated (HT) colostrum samples, yet there were variations in the reported concentrations of this oligosaccharide (OS) in the present study compared to other research (Martin-Sosa et al., 2003; Fong et al., 2011). Overall OS abundance, including structures known to have a prebiotic effect, are 2- to 4-fold greater in Angus and Angus hybrid cows compared to Holstein cows (Sischo et al., 2016). The authors reported that dairy cows received a corn-silage and alfalfa hay-based TMR, while beef cows received alfalfa and grass hay ration (Sischo et al., 2016). In addition, there are marked differences in milk production between these production types, with beef cattle 30 DIM producing only 10.6 kg/d and 5.4% lactose (Radunz et al., 2010) and Holsteins 50 DIM producing 31.3 kg/d and 4.8% of lactose (Daniel et al., 2016). Thus, the differences observed in

regards to production and genetics between breeds, in combination with the potential influence of differing diets, may contribute to differing concentrations and types of OS in beef cattle. Research regarding the synthesis of OS in the bovine mammary gland is largely uncharacterized, yet in humans the exact mechanism has been elucidated and begins with the formation of a lactose core by β-galactotransferse in the presence of α-lactalbumin, followed by elongation with N-acetyllactosamine units, fucose, and sialic acid (Smilowitz et al., 2014). Gene expression analysis of colostrum and milk samples from Holstein and Jersey cows demonstrated that there are 121 glycosylation-related genes involved in OS metabolism and synthesis pathways in the mammary gland (Wickramasinghe et al., 2011). This study provides basic knowledge on the enzymes that may catalyze and synthesize OS in the mammary gland, and thus future studies may use these candidate genes as potential targets for analysis when exploring the effect of nutrition and genetics on OS synthesis pathways in order to optimize the content of beneficial OS in bovine colostrum and milk.

In contrast to the higher concentration of bCOs present in HT colostrum, calves fed HT colostrum displayed a lower concentration of bCOs within the distal jejunum, ileum and colon at 6 h of life when compared to calves fed unheated (UH) colostrum. Milk and colostrum OS can act as a prebiotic source for beneficial bacterial groups, such as *Bifidobacterium,* and the growth of bacteria in the presence of milk OS can increase their binding to enterocytes, which positively support the intestinal barrier and modulate the immune system (Ewaschuk et al., 2008; Marcobal et al., 2011; Chiclowski et al., 2012; Yu et al., 2013). Our results suggest that *Bifidobacterium* may metabolize bCOs in the neonatal calf intestine, however, it has also been shown that other bacterial groups

can metabolize sialylated OS. For example, species belonging to the genera *Bacteroides* can utilize larger molecular weight OS than *Bifidobacterium* and can uniquely catabolize and use sialic acid as a carbon source (Marcobal et al., 2011). As well, although *Lactobacillus* cannot utilize in tact OS due to a lack of transport mechanisms, they can metabolize OS constituents, such as lactose, galactose, and glucose, and liberation of these mono- and di-saccharides by other bacteria may promote their growth (Schwab & Ganzle, 2011). A major limitation of this study is the lack of bacterial analysis within the intestine of neonatal calves. A comprehensive analysis of the gut microbiome, using metagenomics, may have provided us with in-depth information regarding the microbial population changes when differing amounts of bCOs are provided and future research should focus on the multitude of bacterial groups in the calf intestine that can utilize bCOs. Moreover, increased sampling frequency, such as every 2 h after feeding, may have provided a better illustration of the intestinal region in which the metabolism of bCOs may have occurred. A study conducted by Janschter-Krenn et al. (2013) characterized the concentrations of different OS, including 3'SL, throughout the intestine of the rat at 2 h intervals after feeding and demonstrated that OS may be hydrolyzed by bacteria in regions as early as the proximal jejunum. This information may translate to the dairy calf, as at 6h of life, HC calves had only small amounts of bCOs present from the distal jejunum onward. Unfortunately, this cannot be determined from the samples obtained and further research is required in regards to the characterization of bCOs in the intestine and their possible metabolism by microbial species.

4.5 Conclusion

This thesis demonstrates the impact of colostrum management practices on the passive transfer of IgG, intestinal bacterial colonization, and potential gut active bCOs. In particular, it was determined that delaying colostrum feeding does not progressively decrease the passive transfer of IgG, yet when calves are fed after 6 h of life their ability to absorb IgG as efficiently as calves fed immediately after birth declines. This may be due to changes in the closure of the intestine within the first 6 h of life, however further research on the mechanism by which IgG is absorbed and the factors facilitating gut closure are necessary. In addition, delaying colostrum feeding may delay the colonization of early life bacterial groups in the intestine and the consequences of this delay on the developing calf microbiome and calf health should be a focus of future research. Lastly, it was determined that the heat-treatment of colostrum increases the concentration of free bCOs, which may be supplemented in the neonatal calf to improve gut health. However, specific sialylated bCOs need to be studied further to elucidate their potential beneficial effects in the neonatal calf intestine. In conclusion, this thesis demonstrates the importance of the timely feeding of the first colostrum meal on passive transfer and intestinal bacterial colonization, as well as pinpoints a promising area of future research in regards to the use of colostrum oligosaccharides as prebiotics to improve neonatal calf GIT health.

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