Effects of Delayed and Extended Colostrum Feeding Strategies on Small Intestinal Growth in Neonatal Holstein Bull Calves

by

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Abstract

Enhancing passive transfer of immunoglobulins (Ig) in calves has been the main focus of colostrum feeding in the dairy industry. Currently, it is not yet clear how the timing and extended duration of colostrum feeding impacts neonatal calf intestinal development. The aim of Study 1 was to determine how the timing of the first colostrum meal after birth (0, 6, or 12h delay) impacts the growth of the neonatal calf's small intestine based on histological and ultrastructure analysis at 51 h of life (Chapter 2). Study 1 demonstrated that at 51 h of life, fetal enterocyte population was still evident. It also showed that calves fed colostrum later in their lives (6 h and 12 h) had larger intestinal surface area and villi height compared to calves receiving colostrum immediately after birth (0 h). However, growth factors such as insulin-like growth factor 1 and glucagon-like peptide 2 did not differ among the treatments. The unexpected growth response in 6 and 12 h treatments from study 1 may be explained in two ways: 1) compensatory growth and/or 2) residual colostrum trophic effect in 6 and 12 h calves. This study was not conclusive and led to more questions about the biological significance of the results so it is still recommended to feed the initial colostrum within the first hours of life to ensure the benefits of passive transfer. Study 2 examined the impact of extending colostrum feeding on intestinal structure and growth factor concentrations in blood (Chapter 3). Calves had the same initial colostrum feeding at 2 h of life, then were randomly assigned to one of three treatment groups: colostrum (COL), diet of 50% colostrum and 50% milk (MIX), or milk (MILK) from 12 h of life to 72 h of life. The results revealed that villi height was larger in COL and MIX calves compared to MILK calves, and surface area in the small intestine was larger in MIX calves than both COL and MILK calves. Proliferation in crypts of the ileum was also greater in MIX calves compared to MILK calves. Glucagon-like peptide 2 levels were higher in COL calves compared to MILK

calves, while levels of insulin-like growth factor 1 tended to be larger in COL calves than in MILK calves. The reasons for the similar trophic response in MIX calves compared to COL calves are hypothesized to be: 1) saturation of the absorptive capacity of the enterocytes, 2) high amount of total solids in colostrum negatively affecting absorption, or 3) abrupt transition from the high energy diet to low energy diet affecting intestinal growth negatively. Based on the results from study 2, supplementing milk with colostrum is beneficial for neonatal calf intestinal growth.

Preface

This thesis contains the original work of Jade Pyo in collaboration with Dr. Michael Steele at the University of Alberta. Unless otherwise specified, the work and data presented herein was completed by Jade Pyo. Both animal experiments in chapter 2 and 3 were conducted at the Dairy Research and Technology Center at the University of Alberta. For chapter 2 co-authors include Amanda Judy Fischer and Yang Song from University of Alberta. Dr. Zhixiong He from Institute of Subtropical Agriculture, Chinese Academy of Sciences, who contributed in the experimental design and sampling. For chapter 3, co-authors include Yudai Inabu and Dr. Toshihisa Sugino from Hiroshima University for sample analysis, and Sarah Pletts of the University of Alberta for assisting with sample collection. For both chapter 2 and 3, co-authors include Dr. Deborah Haines of the Saskatoon Colostrum Company Ltd., who provided pooled colostrum, and Dr. Le Luo Guan of the University of Alberta who contributed to experimental design and aid in sample analyses.

The experiments conducted in this thesis received ethics approval from the Animal Care and Use Committee for Livestock at the University of Alberta (AUP00001595), and all procedures on animals were conducted following the guidelines of the Canadian Council of Animal Care.

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List of Abbreviations

ABS	Avidin-Biotin complex
ACS	Apical canalicular system
ALS	Acid labile subunit
AUC	Area under the curve
BGAR	Biotinylated goat anti-rabbit
BW	Body weight
COL	Colostrum fed calves
DAB	3,3'-Diaminobenzidine
EGF	Epidermal growth factor
Fab	Variable / antigen binding region on antibody
Fc	Constant region on antibody
FPT	Failure of passive transfer
GH	Growth hormone
GHBP	Growth hormone Binding Protein
GHR	Growth hormone Receptor
GLP-2	Glucagon-like peptide 2
Ig	Immunoglobulin
IgG	Immunoglobulin G
IgA	Immunoglobulin A
IgM	Immunoglobulin M
IGF-1	Insulin-like growth factor 1
IGF-2	Insulin-like growth factor 2
IGFBP	Insulin-like growth factor binding protein

LSM	Least square means
MIX	Mixed diet (50% colostrum & 50% milk) fed calves
MILK	Milk fed calves
NGS	Normal goat serum
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy

1 Literature Review

1.1 Introduction

Colostrum is defined as the first milk produced by the dam after parturition that plays a critical role in neonatal calf health and development (Yang et al., 2015). Due to the structure of bovine placenta, calves do not receive immunoglobulins from maternal circulation (Weaver et al., 2000). Therefore, passive transfer of Ig, especially immunoglobulin G (IgG) in neonatal calves is critical to support their immune defense until they can develop a fully functional adaptive immunity (Godden, 2008). Consequently, colostrum management practice and research has been focused on enhancing passive transfer of Ig. However, colostrum contains other components that are also critical in calf health and growth, such as proteins, fatty acids, hormones, vitamins, and minerals that require more investigation (Odle et al., 1996; Hammon and Blum, 2000). These bioactive components of colostrum could be important for modulating intestinal growth, health and development but very little information has been published about this topic. This is surprising because the digestive tract is where digestion and absorption of nutrients occur and also serves an important role in the immune system (Steele et al., 2016). In mature ruminants, the majority of the digestion and absorption occurs in rumen, while in neonatal calves most of the absorption occurs in the lower gut, or more specifically, the small intestine (Gilloteau et al., 2009). Though the small intestine plays a major role in neonatal calf digestion and nutrient absorption it is relatively neglected in dairy research.

The high mortality of pre-weaning calves (11%) in Canada due to the digestive diseases (55% of total mortality) can be attributed to impaired intestinal health and function (OMAFRA, 2018; Gilloteau et al., 2009; Sanglid et al., 2003). It has been suggested that the degree of structural changes and nutritional adaptation that the neonatal digestive tract is subjected to

during the neonatal phase is comparable to the weaning period in dairy calves, which is often considered the most stressful period in a calf's life (Sanglid et al. 2003). In a recent study by Inabu et al., (2018b) it was also suggested that increased small intestinal growth can lead to improved glucose absorption which will aid neonatal calves in overcoming hypoglycemia and negative energy balance. One method of inducing intestinal growth is to increase the number of colostrum meals as shown in a study by Bühler et al. (1996) that compared intestinal growth in calves fed colostrum or milk replacer for all meals. A better understanding of dairy calf colostrum feeding strategies that can improve the growth and adaptations of the intestine may benefit the dairy industry with improved overall health, welfare, and growth of young stock. This may not only affect calf performance in a short-term but it may also lead to long-term economic benefits as calves that do become sick and are treated with antibiotics have been shown to have reduced lifetime milk production (Soberon et al., 2012).

1.2 Colostrum

Colostrum contains a multitude of nutrients such as proteins, amino acids, fatty acids (both non-essential and essential), vitamins, minerals, as well as non-nutritive components such as Ig, peptide hormones, growth factors, lactoferrin, thyroid hormones (T3 and T4), and different types of enzymes (Campana and Baumrucker, 1995; Foley and Otterby, 1978; Lucey and Home, 2009; McGrath et al., 2016; Odle et al., 1996). Following the first milking which is always defined as colostrum, the subsequent 3 to 5 milkings are considered transition milk, and milkings after that are referred as mature milk (Parrish et al., 1958). Concentrations of bovine colostral components, including bioactive molecules, as well as the number of cells are the highest in colostrum (Bastian et al. 2001; Hammon and Blum, 2000). The level of colostral nutrients drop rapidly during the first days of lactation, though some variance among the species exist (Bastian et al.,

2001). Overall, bovine colostrum contains twice as much gross energy and four times as much protein than mature milk (Blum and Hammon, 2000). Colostrum also contains higher levels of insulin-like growth factor 1 and 2 (IGF-1 and IGF-2), epidermal growth factor (EGF), insulin, and prolactin than milk. (Campana and Baumrucker, 1995; Hammon and Blum, 2000; Odle et al., 1996; Parrish et al., 1950). The importance of IGF-1 and IGF-2 will be discussed in more details later in the section 1.8. These bioactive hormones also decrease in quantity in a time-dependent manner after the first milking.

The colostral trophic effects in the intestine of calves are stimulated by the high nutrient contents and various growth factors in a synergistic manner (Hammon and Blum 1997). The colostral IGF-1, for example, is thought to have a systemic effect in initiating endogenous IGF-1 production, by altering the insulin and prolactin level, which will indirectly lead to growth of the intestine (Hammon and Blum, 2000). Furthermore, it was demonstrated that calves (8 d old) that were fed colostrum for three days had higher binding capacities for IGFs and insulin in the small intestine and colon than calves fed milk replacer, suggesting that colostrum feeding also alters intestinal growth through modification on growth factor functionality (Hammon and Blum, 1997). The high level of nutrients in colostrum also stimulates the endogenous production of the glucagon-like peptide 2 (GLP-2), another potent trophic factor in the intestine (Odle et al., 1996; Dube et al., 2006). Glucagon-like peptide 2, in turn, promotes enterocytes to increase the uptake of nutrients and initiate proliferation and growth (Dube et al., 2007). Colostrum feeding has been shown to impact the secretion of GLP-2 in calves but it is unclear if feeding methods for colostrum have an impact (Desjardins-Morrissette et al, 2018; Inabu et al., 2017). A more detailed review of GLP-2 can be found in the section 1.8.

1.3 Immunoglobulins

Immunoglobulins (Ig) are part of a large protein family with an approximate size of 150 KDa, present in serum, colostrum and milk (Gapper et al., 2007). Unlike other species, such as humans, IgG is the most abundant class in bovine colostrum (Gapper et al., 2007). Other classes, such as immunoglobulin A (IgA) and immunoglobulin M (IgM), are present in colostrum but at much lower concentrations (Barrington et al., 1997). The immunoglobulin IgG_1 comprises the majority of the total IgG population, while IgG_2 exisits in a smaller percentage (Barrington et al., 1997; Gapper et al., 2007). Similar to other bioactive components in colostrum, immunoglobulin concentration is highest in the colostrum then drops rapidly in the first five days of parturition (Parrish et al., 1950).

Two kinds of polypeptide chains, called light and heavy chains, make up an IgG molecule (Janeway et al., 2001). The light and heavy chains have constant (Fc) and variable (Fab) regions, and antigen binding sites are located at the variable regions comprised of both light and heavy chains (Stanfield et al., 1990). IgG antibodies defend the digestive tract by complement activation, opsonisation, and direct binding to the pathogens (Lilius and Marnila, 2001). Furthermore, IgG is the major agent of passive transfer in neonatal calves, providing immune protection until the calves develop their functional immune system approximately 2-4 weeks of life (Butler, 1983; Chase et al., 2008; Godden, 2008).

1.4 Passive Transfer

The bovine placenta is described as cotyledonary synepitheliochorial, referring to the two morphological characteristics of the placenta. The term cotyledonary refers to the presence of cotyledons, or areas of ectodermal proliferation that lines the fetal placenta. Caruncles, which are present on the maternal placenta, form an interconnected structure with the cotyledon, forming

the placentome (Assis Neto et al., 2010). The term synepitheliochorial refers to how the uterine placentome and maternal blood vessels remain intact, leading to the separation of the maternal and fetal blood stream (Wooding, 1992). Consequently, the calf does not get transfer of antibodies *in utero*, and essentially relies on the passive transfer of the colostral Ig for the adaptive immune protection.

The IgG in the colostrum is absorbed in the small intestine of neonatal calves, during the period of nonselective pinocytosis (Fetcher et al. 1983; Bush and Staley, 1980). The rate of IgG absorption increases as it progresses down to the small intestine, with the maximal absorption occurring in the distal small intestine (Fetcher et al., 1983). The absorption of IgG occurs transcellularly, and by forming endosome surrounding the IgG. This phenomenon is termed "transcytosis" (Jochims et al. 1994). The nonselective pinocytosis of luminal macromolecules including Ig only lasts until 1- 3 days of life (Bush and Staley, 1980; Campbell et al., 1977; Hurley and Theil, 2011). It is thought that after this time window, pinocytosis ceases, and this cessation is called gut closure (Bush and Staley 1980; Stott et al., 1979a). The exact mechanism of gut closure is currently unknown and widely debatable (Smeaton et al. 1985; Stott et al., 1979a). Large macromolecules, including Ig, cannot be absorbed via pinocytosis after closure (Stott et al., 1979a). Thus, it is imperative for calf health that calves obtain Ig and macromolecules in adequate amount and within the time frame where absorption is optimal.

1.5 Failure of Passive Transfer

When the serum IgG concentration falls below 10mg/mL, the condition is called failure of passive transfer (FPT), which has been associated with the calf morbidity and mortality (De Nise et al. 1998; Weaver et al., 2000). There are several factors that can lead to FPT: the quality of the colostrum, amount of the colostrum fed, the feeding method (i.e. esophageal tube or

bottle), as well as the timing of the initial colostrum feeding (Godden et al., 2008; Weaver et al., 2000).

In regards to the quality of the colostrum, Besser et al. (1991) demonstrated that the rate of FPT was reduced when greater than 100g of colostral IgG was administered. Feeding method of the colostrum also affects the FPT rate. Vasseur et al. (2009) showed that 22% of dairy calves with 2-6 h of age could not consume 2 L of colostrum using bottle. Due to this reason, several authors have suggested that administration of the colostrum with an esophageal tube ensures consumption of adequate amounts of colostrum compared to suckling from the dam or bottle feeding (Besser et al., 1991; Vassuer et al., 2010; Weaver et al., 2000). However, other than the advantage of ensuring adequate volumes of colostrum are fed to calves, no difference was observed in the efficiency of IgG transfer between bottle feeding and esophageal tube feeding (Desjardins-Morrissette et al., 2018). The timing of the initial colostrum ingestion also plays a critical role in determining the success of passive transfer (Stott et al., 1979b; Weaver et al., 2000). If the feeding is delayed, the closure of the gut is also delayed up to 36 hours in calves (Stott et al, 1979b). However, the IgG absorption is most optimal within the first 4 h after birth, and decreases rapidly after 12 h (Bush and Staley 1980; Stott et al., 1979c; Vasseur et al., 2010). Hence, ensuring colostrum feeding at least 12 h after birth is essential in order to prevent FPT. Interestingly, a recent study by Fischer et al. (2018) showed that calves fed colostrum immediately after birth showed higher peak serum Ig concentrations compared to those fed at 6 h and 12 h after birth. In addition, abundance of research suggests that calves that received high quality colostrum within the optimal window (4 h of life) will most likely have successful passive transfer, leading to the better growth and health (Georgiev, 2008; Stott et al., 1979c; Weaver et al., 2000).

1.6 Current Neonatal Calf Diet Management

Considering the significance of colostrum in neonatal calf health and growth, and the risk of failure of passive transfer (FPT), an effective colostrum feeding regimen is required. Such feeding regimen should include employing the proper method, volume, quality, and timing to avoid FPT, in addition to allowing absorption of colostral bioactive molecules. Minimum feeding of 100g of IgG, or 10% - 12% of the birth body weight (BW) in colostrum is recommended in practice (Besser et al., 1991; Dunn et al., 2017; Khan et al., 2007; Weaver et al., 2000). Weaver et al. (2000) further recommended that feeding 4 L of high quality colostrum (regardless of BW) by esophageal tube is required to ensure the adequate absorption of minimal IgG to prevent FPT. However, in Canada, the average dairy producer feeds only 2.5 L within the first 12 h post-natal, which may lead to health and mortality risks for calves (Vasseur et al., 2010). Based on this research the guidelines for colostrum feeding has changed to feeding over 150g of IgG to the calf in the first meal of life (Haines, D., personal communication, 2018).

Regarding the common practice to ensure the quality of the colostrum, the same Canadian survey indicated that none of the dairy producers in Canada evaluated the quality of the colostrum (typically evaluated using a Brix refractometer on farm), and some producers (15.6 %) relied solely on the dam as the source of colostrum (Vasseur et al., 2010). In another survey in the USA it was determined that 31% of producer feed one colostrum feeding, 39.8% feed two feedings, and 23.9% feed more than three meals of colostrum (Fulwider et al., 2008), though the quality of each meal was not verified (therefore transition milk may be included). Considering the wide variance of immunoglobulin concentration in colostrum (Gapper et al., 2007), feeding colostrum from a dam without testing its quality may lead to FPT in neonatal calves.

Delivery of the initial colostrum feeding within the optimal period of absorption (4 h postnatal) is also critical for calf health (Weaver et al., 2000; Stott et al., 1979a). According to the same survey of 119 farms in Quebec, 94.8% of the farms gave the first meal of colostrum within the first 6 h of birth (Vasseur et al., 2010). However, given the low average visiting frequency along with low surveillance camera availability (7.8%), the percentage of producers feeding within the first 6 h of birth may actually reflect the time when the calves were found, not born (Vasseur et al., 2010).

Another important part of the pre-weaning calf diet is the transition from colostrum to milk. In nature, calves experience a gradual transition from colostrum to mature milk which is not the case on most dairy farms. Transition milk in Canada is however often discarded as it is deemed unsalable due to the high somatic cell count (Bauer, J.P. personal communication, 2018). Instead, of transition milk, calves are fed milk or milk replacer immediately after the colostrum meals. There is limited information available evaluating the effect of transition milk feeding, and to our knowledge there are no studies comparing the effect of abrupt transition with gradual transition on calf health and growth.

1.7 Digestive Tract

1.7.1 Overview of Ruminant Digestive Tract and Small Intestine Structure and Function

Digestion and absorption of nutrients are the primary functions of the digestive tract, however it also has a barrier function to prevent pathogens and toxins from entering the circulation of the animal (Gäbel et al., 2002). Therefore, efforts to further understand and maintain digestive tract functions are imperative to maintain neonatal calf growth and health. In

mature cattle, the rumen is the primary organ of digestion and absorption, hosting a large diversity and quantity of commensal microbiota (Steele et al., 2016). While volatile fatty acids are mostly absorbed in rumen, other important processes occur in the small intestine, such as the absorption of the majority of fat and protein (Zhao et al., 1998). The small intestine starts with a short segment of the duodenum followed by the jejunum and ileum. All compartments of the small intestine contain absorptive villi, which are joined at the crypto-villi junction with the embedded crypts of Lieberkuhn (Hofmann, 1988). Two types of mucous layers exist to protect the small intestine from the pathogens: the loosely adherent outer layer (approximately 100μm) and the tightly adherent and more viscous inner layer (approximately 30μm) (Steele et al., 2016).

Brunner's gland is a characteristic feature of the duodenum. The Brunner's glands produce alkaline secretion to protect the duodenum from the acidic digesta entering from the abomasum (Ohwada and Suzuki, 1992). Jejunum and ileum are major sites of lipid and peptide absorption compared to duodenum (Chen et al. 1999; Noble 1978). Both segments have Peyer's patches, which are major sites of lymphocyte production (Landsverk et al., 1991). Jejunal and ileal Peyer's patches have two distinct population; as such the ileal Peyer's patches have higher proportion of younger lymphocyte and lack CD4+ cells (Hein et al., 1991).

The small intestine is made up of simple columnar epithelium. Villi project into the lumen while crypts are embedded under the crypto-villi junctions (Korinek et al., 1998; Rowland et al. 2008; Figure 1-1). The crypts host a pool of undifferentiated and actively dividing cells that over the span of 2-7 days migrate and replace the older cell layer in villi (Crosnier et al., 2006; Korinek et al., 1998; Rowland et al., 2008). In murine models, approximately 8-11 crypts supply the new cells for one villi (Rizvi et al., 2005). In addition to the enterocytes, goblet and

enteroendocrine cells also differentiate and migrate from the pool of stem cells at the base of the crypts (Crosnier et al., 2006; Figure 1-1). Paneth cells, which secrete anti-microbial substances, stay at the bottom of the crypts in the vicinity of stem cells (Figure 1-1). Villi (and microvilli) increase the absorptive surface area by several folds (Mandara and Trier, 1994), allowing the small intestine to absorb nutrients efficiently. Thus, growth of the small intestine via increasing overall length or villi length and width, and a consequential increase in surface area will equate to increased efficiency of and capacity for nutrient absorption.

1.7.2 Neonatal Calf Digestive Tract

Unlike mature cattle, neonatal calves have underdeveloped digestive tracts and are considered pseudomonogastrics (Orskov et al., 1970). When neonatal calves are fed, the meals bypass rumen due to the reflexive reticular groove closure and enter the abomasum (Baldwin et al., 2004; Orskov et al. 1970). During weaning, rumen maturation is initiated with different stimuli. Absorption of butyrate and propionate, as well as the direct action of butyrate, stimulates ruminal papillae development, while roughages increase the muscularity of the rumen (Tamate et al., 1962). Until rumen maturation is complete, the small intestine is the main area of lactose digestion with high number of glucose transporters, which declines as the rumen develops (Wood et al., 2000). Therefore, understanding the function of the small intestine in neonatal calves may be essential for improving neonatal calf health and growth management.

At the cellular level, neonatal calves still possess fetal enterocytes, which differ from the adult-type enterocytes in that they lack functional brush boarder enzymes and permeability regulation (Guilloteau et al., 2009). Instead, these fetal enterocytes employ vacuolar pinocytosis and are completely replaced by adult-type enterocytes within 5-7 days of life (Guilloteau et al., 2009). The vacuolar pinocytosis allows rapid and non-selective absorption of colostral

macromolecules including Ig in the 1d – 3 d of life, as mentioned previously. The immature digestive system of neonatal calves also allows calves to absorb and utilize the colostral bioactives without digesting them. For example, hydrochloric acid and proteolytic enzyme activity in neonatal calves are not adequate to digest the growth factors and peptide hormones (Xu et al., 1996). Additionally, colostrum contains proteolysis inhibitor, also allowing growth factors to reach the small intestine undigested (Gerogiev, 2008; Xu et al., 1996).

1.8 Methodology to assess intestinal growth and structure

1.8.1 Use of Brighfield Microscopy as an Indicator of Intestinal Growth

The major small intestinal structure, villi, contains rapidly proliferating cells with relatively short cell cycles of 5-7 days (Gerbe et al., 2012). The growth of the intestine can be determined using the brightfield microscopy by measuring the villi height and crypt depth (Wongdee et al., 2016). In several studies including bovine and poultry models, increased amounts and nutritional levels in the diet led to increased villi height and crypt depth, and surface area as indicators of intestinal growth (Buhler et al., 1998; Laudadio et al., 2012; Moghaddam and Alizadeh-Ghamsari, 2013). In addition to the villi height and crypt depth measurements, mucosal surface area measurements can also provide further evidence of intestinal growth and development (Kisielinski et al., 2002). In the equation derived by Kisielinski et al., (2002), the mucosal surface area can be derived from either a cross section or a longitudinal section using three parameters: villi width, height, and crypt width. Older methods of surface area quantification, such as the Harris method, require 14 parameters from both longitudinal and cross section samples (Harris et al., 1988), which are often unobtainable depending on how the samples are cut and processed into slides. When the measurements taken using the new method, introduced by Kisielinski et al. (2002), were compared to the older

methods, the results showed no significant difference from the Harris method. For the studies in this thesis, villi height and crypt depth were measured from the crypto-villus junction (base) to the tip of the villus and crypt, as described by Wongdee et al. (2016). For consistency, the widths for the surface area were measured at the middle of the crypts for all samples.

1.8.2 Use of Transmission Electron Microscope

Ultrastructure, the basic architecture of cells, can be found using a transmission electron microscope (TEM), which transmits a beam of electrons through a thin slice of sample, then refracts to the electron sensor to form an image (Williams and Carter, 1996). Unlike the brightfield microscope, TEM can show microvilli, the smaller projecting structure on villi which can increase the surface area in several folds (Mandara and Trier, 1994). Furthermore, visual presentation of cellular organelles and cellular processes, such as pinocytosis, can be identified using this method, as shown in previous studies (Moretti et al., 2013; Bush and Staley, 1980). Labeling macromolecules, typically with gold, can also be performed to identify the location of macromolecules within the cell, thus providing valuable information regarding the cell mechanics and functions (Bessi et al., 2002). Transmission electron microscopy (TEM) is commonly used as a qualitative assessments of the samples. However, some studies have shown methods to use TEM for quantifying microvilli height and width (Ferrer et al., 1995; Weis et al., 2015)

1.8.2 Use of Proliferating Cell Nuclear Antigen to Detect Proliferation

Proliferating cell nuclear antigen (PCNA) is a 36 kD DNA clamp protein that plays a critical role in the cell cycle. It is highly conserved among species such as plants, yeast, and mammals, as it provides a scaffold for the proteins required for DNA replication and repair

(Olaisen et al., 2018; Strzalka and Ziemienowicz, 2011). It was also demonstrated recently that PCNA plays an additional role in rapidly detecting DNA damage and the consequential cellular signalling in the DNA damage response in both yeast and human cells (Olaisen et al., 2018). Since this methodology does not require radioactive molecules or labelling *in vivo* or *in vitro*, histological methods of determining proliferation, such as PCNA, are preferred and widely used (Hall et al., 1990). As scaffold proteins only existing during the DNA replication and repair, PCNA responses are only identified in the proliferating cells (Hall et al., 1990). Therefore, by quantifying PCNA response, it is possible to determine the proliferative state of the particular tissue. Researchers have presented PCNA data in various ways. In a study examinig murine crypts, PCNA index was calculated by quantifying the number of PCNA+ cells in a half crypt (Tomita, 2012). In another study conducted with the murine kidney tissue, PCNA+ cells were quantified over high power field (Hpf), which is the area viewed under the maximal magnification of a microscope (Herrera et al., 2014). Another method, which we chose to use in this thesis, is to count PCNA+ cells over the total number of crypt cells to account for the different sizes of the crypts and villi (Kubben et al., 1994).

1.9 Factors Affecting Small Intestine Growth

1.9.1 Growth Hormone and Somatotrophic Axis in the Digestive Tract

Growth hormone (GH) is a polypeptide hormone released from somatotrophic cells in the pituitary (Gahete et al., 2016). Though GH is mostly known for trophic effect, GH also regulates a wide range of physiological functions such as feeding, reproduction, osmoregulation, and immune function both directly and indirectly (Bergan-Roller and Sheridan, 2018; Björnsson, 1997). Most of the GH activities are mediated by insulin-like growth factor 1 (IGF-1), and the GH-IGF-1 axis is highly conserved across the species (Reinecke et al., 2005). The GH from the

pituitary stimulates the secretion of hepatic IGF-1, which is the major source of IGF-1 in the body (Wood et al., 2005). The GH action and bioavailability is modulated by GH binding proteins (GHBPs), and downstream IGF-binding proteins (IGFBPs) (Duan and Xu, 2005).

Transcripts of GH receptor (GHR) were detected throughout the digestive tract of many species, including the murine, bovine, and human small intestine (Delehaye-Zervas et al., 1994; Komatsu et al., 2012; Nagano et al., 1995). This prevalence suggests that there is a potential direct GH effect on these tissues, though the trophic role of GH is most likely mediated via local IGF-1 (Shulman, 2000). There are other roles that GH plays in the digestive tract, such as increasing water and sodium chloride absorption, which was observed in rodent intestine (Mainoya, 1982). Together with IGF-1, GH was also shown to increase glutamine transport in small intestine (Shulman, 2000). This finding is especially notable as glutamine is the preferred source of energy in small intestine metabolism and nucleic acid synthesis (Windmueller and Spaeth, 1974).

1.9.2 Insulin-Like Growth Factor 1

Insulin-like growth factors 1 and 2 are the most abundant growth factors in colostrum (Marnila and Krohnen, 2002). Both IGF-1 and 2 receptors were identified in the duodenum, jejunum, ileum and colon of the neonatal calves (Baumrucker et al., 1994; Hammon and Blum, 2002), suggesting that the enterocytes are capable of detecting IGFs. In the intestine, IGFs work in autocrine, paracrine, and endocrine manners (Kuemmerle, 2012; Plath-Gabler et al., 2001).

Both IGF-1 and IGF-2 are single chained polypeptides with a large range of physiological capacities due to their similarity with the insulin in structure (Kuemmerle, 2012). The binding of IGF-1 to the IGF-1 receptor (IGF-1R) causes the phosphorylation of the tyrosine residue of IGF-1R, which in turn causes phosphorylation of insulin receptor substrate 1, a

signalling protein (Izumi et al. 1987). The phosphorylation of the signalling protein then leads to a downstream signalling cascade, mainly the Ras-Erk1/2 pathways (Kuemmerle et al. 2012). One of the main effects of IGFs is the modulation of transport and metabolism of glucose and amino acids (Georgiev et al., 2008), thereby affecting energy supply for cell growth and development. Additionally, IGFs regulate cell proliferation and differentiation, as well as apoptosis inhibition (Bühler et al., 1998; Jehle et al. 1999).

It is also known that IGFs are involved in complex modulatory systems with other growth factors. A study conducted by Roffler et al. (2003) demonstrated that calves fed colostrum showed a greater villi circumference and increased villi length when compared to calves administered any single growth factor alone. Additionally, there are other modulators that are not in colostrum that can further affect the physiological functions of IGFs. An example of this is Glucagon-like peptide 2 (GLP-2), which has not been identified in the colostrum. However, GLP-2 level can affect both insulin-like growth factors 1 and 2 levels (Anini et al., 2002; Inabu et al., 2018a). The GLP-2 and IGF-1 axes have been attributed as key promoters of villi and crypt growth in the small intestine (Dube et al, 2007; Rowland et al., 2008).

Despite the evidence of IGF-1's trophic effect, the oral ingestion of colostral IGF-1 and its absorption has been a topic of debate in neonatal calves. A study by Vacher et al., (1995) has shown that orally administered IGF-1 did not get absorbed in the small intestine, nor did it appear in the serum of the neonatal calves. Another study by Hammon and Blum (1997), using radioactive IGF-1, also showed similar results. However, orally administered IGF-1 was shown to incur systemic effect leading to the production of endogenous IGF-1, as well as to increase plasma levels of prolactin and insulin, which are also part of the pathways associated to intestinal growth (Baumrucker and Blum, 1994). Further studies on the exact mechanisms of IGF-1 and its

interactions with other hormones in neonatal calves will elucidate the exact role of colostral IGF-1.

1.9.3 Insulin-Like Growth Factor 2

Despite the structural similarity to IGF-1, the mode of action of IGF-2 is distinct from IGF-1. While IGF-1 plasma concentration shows a strong correlation with colostrum intake, as well as increased weight gain and body weight thereafter, the plasma level of IGF-2 is not dependent on the colostrum intake (Hammon and Blum, 1997). Furthermore, studies suggested that IGF-2 has distinctive trophic roles. Using the murine model, Jehle et al. (1999) demonstrated that IGF-2 and insulin regulate enterocyte differentiation, while IGF-1 is responsible for proliferation of intestinal crypts. Bennet et al. (2002) also demonstrated that the IGF-2 plays a role in increasing the rate of crypt fission in the murine model.

1.9.4 Insulin-like Growth Factor Binding Proteins (IGFBPs)

In addition to IGFs, bovine colostrum contains high concentration of IGF-binding proteins (IGFBPs) (Campbell et al., 1991). By forming complexes, IGFBPs modulate the bioavailability of the IGFs (Odle et al., 1996). In fact, IGF-1 and IGF-2 are almost always (95 – 99%) found as a complex with IGFBPs (Odle et al., 1996). Thus, the aforementioned low absorption of oral IGF-1 in neonatal calf digestive tract may be related to the IGFBPs. There are many variants of IGFBPs, and six IGFBPs are known to directly modulate IGF-1(Jones and Clemmons, 1995; Vacher et al., 1995). Growth hormone, insulin, and protein level in lumen were shown to affect level of IGFBPs in older ruminants, but the same was not demonstrated in neonatal calves (Breier et al., 1988; Skaar et al., 1994).

The main carrier of IGF-1 in blood is IGFBP-3 (Hammon and Blum, 1997; Odle et al., 1996). Because of its bulky acid-labile subunit (ALS), IGFBP-3 protein forms a large complex (150 kDa) (Baxter et al., 1989). The large size of IGFBP-3 complex prevents the complex from crossing the epithelium, thereby reducing the bioavailability of the bound IGF-1. On the other hand, IGFBP-2 – IGF-1 complex can cross the epithelium, though the half-life of IGFBP-2 is shorter than IGFBP-3 (Hammon and Blum, 1997; Zapf et al., 1990).

1.9.5 Glucagon-Like Peptide 2

Glucagon-like peptide 2 (GLP-2) is a member of the glucagon superfamily and is produced in the digestive tract, as well as in the central and peripheral nervous system (Kieffer and Habener, 1999). Both GLP-2 and glucagon-like peptide 1 (GLP-1) are secreted by the enteroendocrine L cell upon the ingestion of nutrients (Burrin et al. 2003; Elsabagh et al., 2017; Figure 1- 2). The ingestion of nutrients in duodenum activates the vagus nerve, resulting in indirect stimulation of the ileal L cells via acetylcholine (Burrin et al., 2000; Figure 1- 2), as well as directly stimulating L cells in the intestine (Anini et al., 2002; Figure 1- 2). There was no notable level of GLP-2 in both colostrum and amniotic fluid (Burrin et al., 2003), suggesting that the majority of the GLP-2 functions are carried on by endogenous GLP-2.

The trophic effect of GLP-2 has been well defined, including an increase in villi length and crypt depth, and crypt cell proliferation, as well as modulating intestinal functions such as permeability, digestion, motility, and blood flow (Dube et al., 2006). Unlike other growth factors, GLP-2 does not have specific cells with receptors but rather has receptors found in enteroendocrine cells, enteric neurons, and subepithelial myofibroblasts (Dube et al 2006; Figure 1- 2). Yusta et al. (2000) demonstrated that the administration of GLP-2 in mice increased IGF-1 mRNA expression in the small intestine. Together with the lack of GLP-2 receptor presence in

villi and crypt, these results suggest that GLP-2 most likely exerts its trophic effect on villi and crypt via mediators such as IGF-1 (Dube et al., 2006; Yusta et al., 2000; Figure 1-2). Additionally, Burrin et al. (2003) demonstrated that the infusion of GLP-2 in piglets led to the almost immediate increase of glucose uptake via the portal blood vessel, as well as protein synthesis and induction of jejunal nitric oxide synthase (NOS). This result suggests yet another GLP-2 mode of action without nutrient presence in the lumen. Furthermore, this result also suggests that GLP-2 may be stimulated by nitric oxide, which in turn stimulates NOS to increase the nutrient flux necessary for GLP-2 trophic effects (Burrin et al., 2003) in the intestine.

1.10 Knowledge Gap

Studies have shown that early life nutrition in dairy calves can have long-term implications in their health and production (Faber et al. 2005; Soberon et al., 2012). The dairy calf mortality on dairy farms is alarmingly high (11%), of which most is due to digestive diseases (55% of the total mortality) (OMAFRA, 2018). Setting up the calf for success is dependent on assuring passive transfer of IgG, while enhancing gut growth and health. Unfortunately, most of the research focuses on the passive transfer, rather than the gut growth and health. To our knowledge, no study has examined whether the delayed initial colostrum feeding can impact growth of the small intestine, where most of the carbohydrate, protein and lipid absorption occurs in pre-weaning calves (Zhao et al., 1998). Additionally, no study has observed the impact of the delayed colostrum feeding on the structure of the small intestine and ultrastructure of the neonatal enterocytes. In fact, there is limited knowledge available regarding the ultrastructure of enterocytes in neonatal calves, with the few available studies, all of which were conducted decades ago (Hadad and Gyles, 1982; Jochims et al., 1994; Staley et al., 1972).

Moreover, to our knowledge, no study has demonstrated the effects of extended colostrum feeding duration on digestive tract growth. Considering how critical that optimal intestinal function is to growth and health of a calf, more studies have to be designed to investigate the intestinal physiology in greater detail in response to modern feeding programs.

1.11 Objectives and Hypothesis

The overall hypothesis for this thesis is that colostrum feeding strategies can impact small intestinal structure and growth. In particular, we hypothesize that delaying colostrum feeding after birth would negatively impact small intestinal growth. In addition, we hypothesize that extending colostrum feeding in the first days of life will result in increased small intestinal growth.

The objectives are thus: 1) to determine whether the delayed feeding of initial colostrum affects the growth of the neonatal calf small intestine and ultrastructure, 2) to determine how extended colostrum feeding protocols affect the growth of the neonatal calf small intestine.

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1.13 Figures

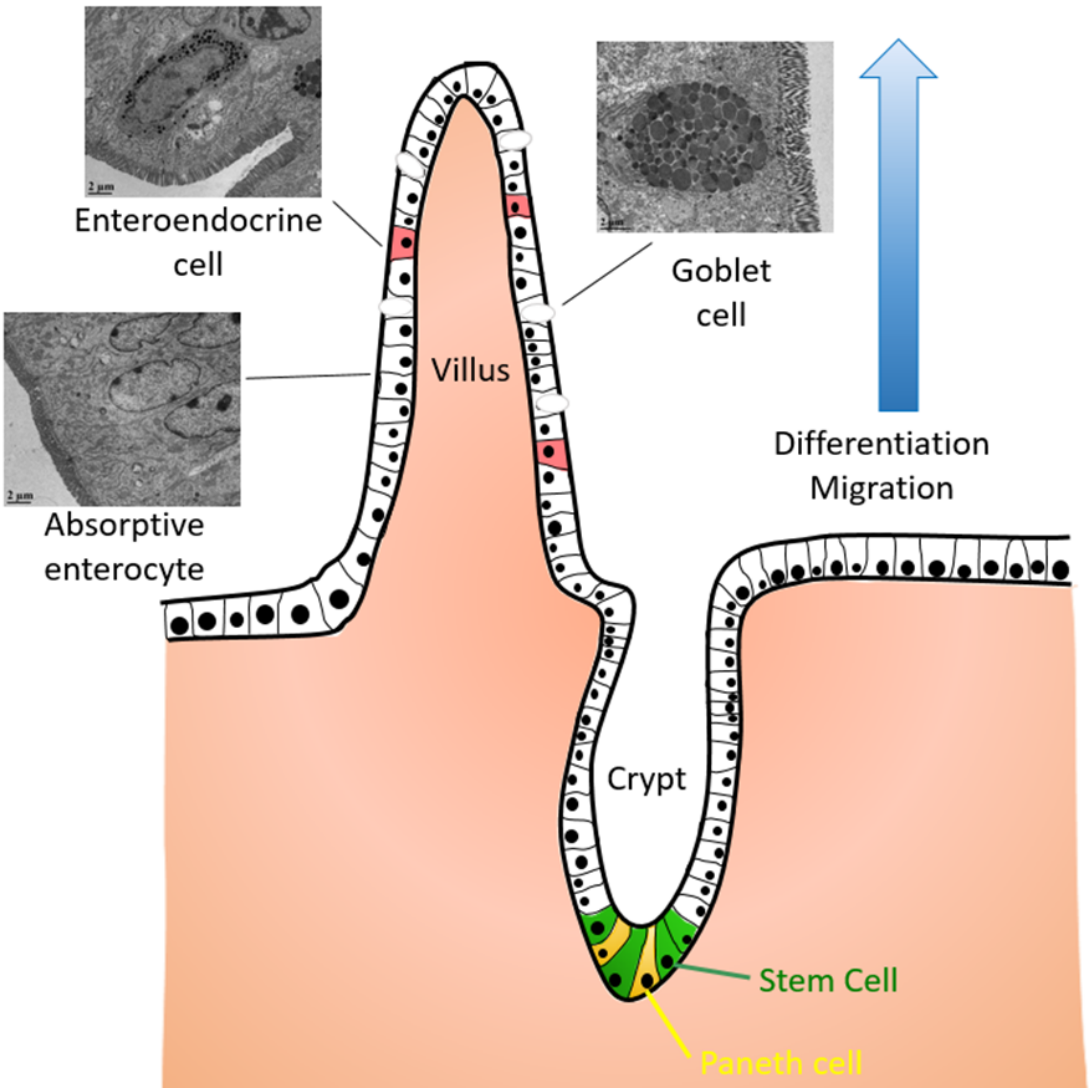

Figure 1-1. Crypt-Villus axis of the small intestine.

All TEM images were obtained from the ileum samples. Green cells = stem cells, Yellow cells = Paneth cells, White cells = absorptive enterocytes (TEM image magnification ×5600), white cells with grey outline = goblet cells (TEM image magnification ×7100), Pink cells = enteroendocrine cells (TEM image magnification ×5600), Blue arrow = direction of differentiation and migration of the enterocytes, goblet cells, and enteroendocrine cells.

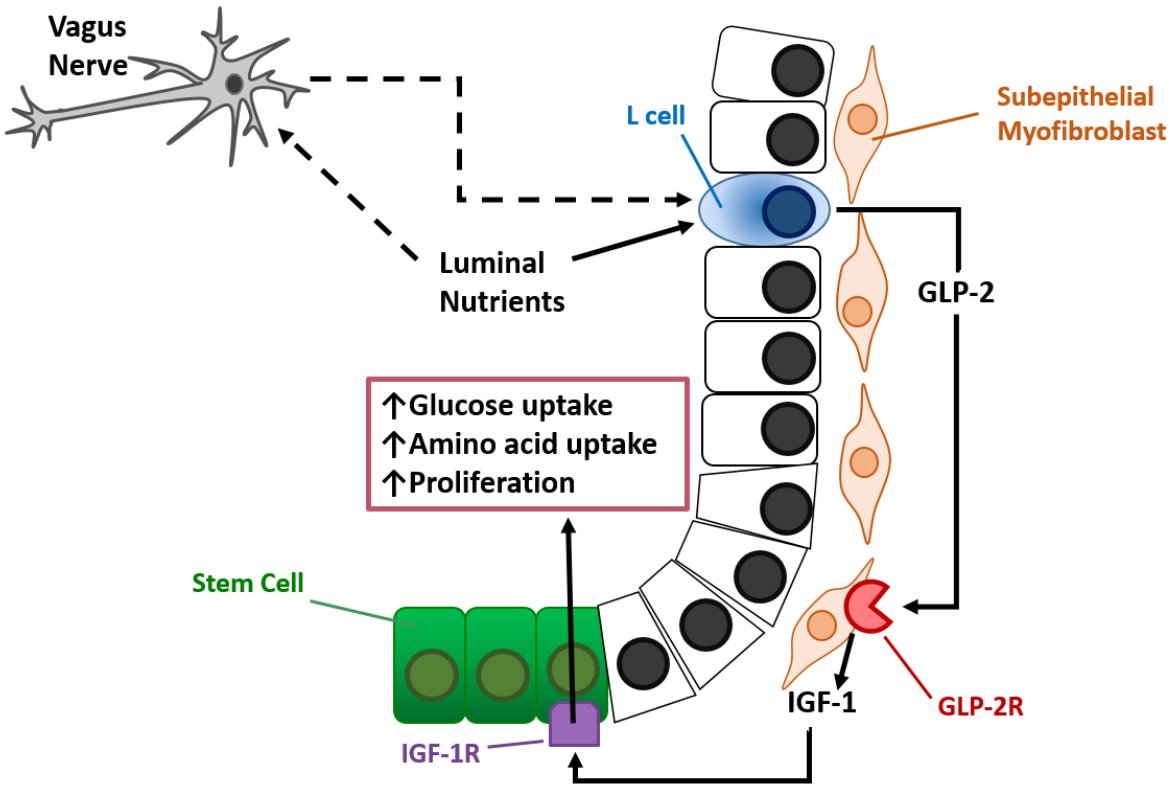

Figure 1-2. Overview of glucagon- like peptide 2 and Insulin-like peptide 1 axis in the small intestine.

Solid arrows stand for direct effects. Dashed arrows stand for indirect effects. White cells represent absorptive enterocytes, GLP-2R stands for GLP-2 receptor, IGF-1R stands for IGF-1 receptor.

2 The Effects of Delaying Colostrum Feeding on Small Intestine Growth of Neonatal Bull Dairy Calves

2.1 Introduction

The absorption of colostral macromolecules such as immunoglobulins (Ig) is critical in calf health and growth (Wooding, 1992; Weaver et al., 2000). Unlike in mature cattle, in neonatal calves, the small intestine is the major organ of digestion and absorption of nutrients. In addition to passive transfer of Ig, colostrum is thought to be important for the growth of the small intestine, which improves absorption and digestion capacity, thereby benefiting calf health and growth (Hof, 1989). One important functional feature of the neonatal intestine is the presence of fetal enterocytes. Calf fetal enterocytes are vacuolar and can rapidly uptake colostral macromolecules via non-selective pinocytosis (Fetcher et al. 1983; Gilloteau et al., 2009). This is a transient macromolecular uptake and stops around 1 - 3 d of life, a phenomenon known as gut closure (Bush and Staley 1980; Campbell et al., 1977; Sangild, 2002; Stott et al., 1979). It was suggested that gut closure occurs due to the replacement of the fetal enterocytes by adult-type enterocytes, which are not capable of macromolecule internalization (Campbell et al., 1977), however more research is warranted to confirm this theory. Even though the internalization of macromolecules continues up to 3 d of life, the absorption of IgG and other macromolecular is optimal in the first 4 h of life (Campbell et al., 1977; Stott et al., 1979). Furthermore, a recent study demonstrated that delaying colostrum feeding by 6 and 12 h tended to decrease overall serum immunoglobulin G (IgG) levels (Fischer et al., 2018). Therefore, along with the quality and quantity of colostrum, the timing of the colostrum feeding is also critical for ensuring successful passive transfer, and consequentially neonatal calf health (Vasseur et al., 2010; Weaver et al., 2008).

Colostrum has benefits beyond supplying IgG for passive transfer. In addition to IgG, colostrum contains high levels of nutrients and growth factors such as insulin-like growth factor 1 (IGF-1) and growth hormones that have trophic effects on the gastrointestinal tract (McGrath et al., 2016; Odle et al., 1996). Another growth factor related to gut health, glugaon-like peptide 2 (GLP-2) has been shown to be responsive to colostrum feeding (Burrin et al., 2000). For surface area measurements, villi length and villi width are often measured as key determinants of surface area (Kvidera et al., 2017). An increase in surface area is in turn associated with an increased capacity for absorption of nutrients (Artursson et al., 2007; Wang et al., 2009), which may improve the overall growth of newborn calves. Colostral trophic effects were demonstrated when calves fed colostrum 6 times showed increased villi height and crypt depth compared to calves that were fed milk replacer only (Bühler et al., 1998). A similar study also demonstrated that feeding colostrum for 3 d of life increased villi length and width, when compared to a milk-based diet with less nutrients and growth factors (Blättler et al., 2001). Considering the optimal time window of macromolecule uptake, the timing of colostrum feeding may have an impact on the absorption of colostral components and consequently on the growth of the small intestine. Despite this, few studies have investigated the effects of delaying initial colostrum feeding on neonatal calf intestinal growth. Moreover, information on neonatal calf intestinal structure and enterocyte ultrastructure is limited, though the unique characteristics of the young enterocyte population may be the key to understanding the process of early nutrient absorption. Because colostrum components are known to elicit trophic effects in the small intestine, we hypothesized that calves fed colostrum at birth have increased small intestine growth compared to calves fed colostrum later in life. The objectives of this current study were: 1) characterize the structure and

ultrastructure of the small intestine of neonatal calves, and 2) to determine the effect of delayed colostrum feeding on small intestinal growth.

2.2 Material and Methods

2.2.1 Animals

This experiment was conducted at the Dairy Research and Technology Center (DRTC) of the University of Alberta, under the approval of the University of Alberta Animal Care and Use Committee (AUP00001595). Bull calves with a birth weight range of 35-55kg (n=27) were removed from the dam immediately after birth transferred to individual pens. Calves were dried with clean towels for 10 minutes immediately after birth, tagged and naval-dipped using 7% iodine following the DRTC protocol. The calves were then randomly placed into one of 3 treatment groups: calves fed colostrum within 1h postpartum (0 h, n=9), 6 h postpartum (6 h, n=9), or 12 h postpartum (12 h, n=9). Within the first hour of life, blood was collected using a vacutainer for a baseline sample. A 16-gauge jugular catheter (Terumo Medical Corporation, Somerset, New Jersey, USA) was inserted at 2 h of life. Using the jugular catheter, blood samples were taken every 3 h for serum IGF-1 analysis. Calves were fed with pooled and pasteurized colostrum with 62 g/L IgG (SCCL, Saskatoon, SK, Canada) at 7.5% of their birth BW at assigned time points (0, 6 or 12 h). Calves were subsequently fed milk replacer (Excel Pro-Gro Calf Milk Replacer, Grober Nutrition, Cambridge, ON, Canada; 26:18 crude protein: fat ratio) every 6 h thereafter at 2.5% of birth BW.

2.2.2 Euthanasia and Tissue Sample Collection

Calves were euthanized at 51 h of life (3 h after 7th meal of life) using Euthanyl injection (Vetoquinol, Lavaltrie, QB, Canada) at 1.25 mL/10kg of BW via jugular catheter. After the calf reached the adequate surgical plane of anesthesia, exsanguination was performed. The digestive

tract was removed then placed on a tray disinfected with 70 % ethanol. Intestinal samples were collected (10 cm segment) from the pre-determined intestinal regions (Malmuthuge et al., 2015): distal jejunum samples were collected from 30 cm proximal to the cranial mesenteric artery; ileum samples from 30 cm proximal to the ileo-cecal junction; colon samples from 30 cm distal to the ileo-cecal junction; proximal jejunum samples were collected from 100 cm distal from abomasal pyloric sphincter; and duodenum samples were collected immediately after the abomasal pyloric sphincter. After the sample collection, small intestine weight and length were measured with a scale and presented as the ratio to BW (Kg). Intestinal samples were immersed in 15 mL of formaldehyde for brightfield microscopy. Small pieces (≤ 2mm in length) of tissue samples were removed and placed in the fixative (2.5% glutaraldehyde & 2% paraformaldehyde diluted in 0.1M Phosphate buffer) filled glass tubes for electron microscopy. Samples were then transferred to Histocore facility for further processing within a maximum of 48 h after collection (University of Alberta, AB, Canada).

2.2.3 Histological Analyses

After being transferred to Histocore facility (University of Alberta, AB, Canada), samples were dehydrated overnight in a series of ethanol solutions, then submerged in paraffin wax and fitted into cassettes, followed by sectioning using microtome (Leica, Wetzlar, Germany) into 5µm-thick sections. Sections were then stained using hematoxylin and eosin, followed by the histological analyses using a Zeiss Brighfield Axio Scope at Biological Sciences Microscope Unit, with Picture Frame software (University of Alberta, AB, Canada). Villi height and crypt depth of the duodenum, ileum, proximal and distal jejunum were measured using the Image J program (National Institutes of Health, Bethesda, MD, USA), by measuring each villus from tip to the villous-crypt junction and from the villous-crypt junction to the bottom of the crypt

(Wongdee et al., 2016). For each calf, 5 measurements from 10 different images were taken per small intestinal compartment, under 100x magnification. Villi to crypt ratios were calculated by dividing the villi height values over the crypt depth values. Surface area index was calculated using the equation described by Kvidera et al. (2017):

$$M = \frac{\left[(v.\ width \times v.\ length) + \left(\frac{v.\ width}{2} + \frac{c.\ width}{2}\right)^2 - \left(\frac{v.\ width^2}{2}\right)\right]}{\left(\frac{v.\ width}{2} + \frac{c.\ width}{2}\right)^2}$$

In formula: M = mucosal-to-serosal amplification ratio, v. width = villi width v. length = villi length, and c. width: crypt width

2.2.4 Electron Microscopy

Scanning electron microscopy (SEM) samples from the duodenum, proximal and distal jejunum, and ileum along with transmission electron microscopy (TEM) samples from ileum and proximal jejunum were processed using the protocol developed by Biological Sciences Microscopy Unit based on Bozzola and Russell (1992). Both SEM and TEM samples were removed from the fixative, but only the TEM samples were post-fixed with 1% osmium tetroxide in 0.1M phosphate buffer. Both samples were dehydrated using a series of ethanol solutions. Dehydrated SEM samples were placed on an adhering carbon tape on an aluminum specimen stub using forceps. The samples were coated with nickel alloy particles using the Hummer 6.2 sputter coater (Anatech USA, Hayward, California, USA). Coated samples were viewed under a Philips / FEI (XL30) Scanning Electron Microscope (Phillips-FEI, Hillsboro, Oregon, USA). Dehydrated TEM samples were immersed in Spurr's Low Viscosity resin. Once the resin hardened, the sample blocks were sectioned using an Ultracut E Ultratome (Reichert-Jung, Germany). Silver-gold sections (80-100 nm) were placed on 300px copper grids (Electron

Microscopy Sciences, Hatfield, Pennsylvania, USA), using a hand-held loop followed by uranyl acetate and lead citrate staining. The grids were viewed under Morgagni 268 TEM (Phillips-FEI, Hillsboro, Oregon, USA) to assess a sub-set of samples from the ileum and proximal jejunum (n=3 per treatment).

2.2.5 Insulin-like Growth Factor 1

Serum samples for IGF-1 analysis were frozen in -20 °C and shipped overnight on ice to Western College of Veterinary Medicine (WCVM, Saskatoon, Saskatchewan, Canada). Serum IGF-1 level analysis were performed using an automated solid-phase chemiluminescent immunoassay kit in conjunction with Immulite/Immulite 1000 systems machine (Siemens AG, Munich, Germany), calibrated following the instructions provided by the manufacturer.

2.2.6 Statistical Analysis

Data was analyzed using Statistical Analysis System version 9.4 (SAS Institute, Cary, North Carolina, USA) using the MIXED procedure. Treatment groups were compared by measurement type (villi height, crypt depth, small intestine length, weight and surface area) and intestinal segment (duodenum, ileum, proximal and distal jejunum). The colostrum treatment was the fixed effect. Tukey-Kramer test was used to correct the values for the multiple comparisons of treatments for the histological measurements. The IGF-1 data were analyzed as repeated measurements over time, including the fixed effects of treatment, time, and treatment by time interaction and considering calf as the repeated measure subject. The covariance structure (compound symmetry, unstructured, autoregressive, or Topelitz) with the lowest Bayesian information criterion (BIC) was chosen. For the repeated measures data, post-hoc tests were performed using Bonferroni adjustment. Least squares means (LSM) by week was assessed using the SLICE command. Normality and homoscedasticity of the residuals were assessed

graphically by the standardized residuals. Significance was defined as $P \leq 0.05$, and tendency as $0.05 < P \leq 0.1$. Results were presented as least squares mean (LSM) ± standard error (SE).

2.3 Results and Discussion

During the characterization of the ultrastructure of the enterocytes, all the tissue samples observed under TEM showed fetal enterocyte populations. Fetal enterocytes can be identified based on several unique features not seen in mature enterocytes, such as large vacuolar formation, apical canalicular system (ACS), and apical mitochondria (Bessi et al., 2002; Moretti et al., 2013). We observed large vacuoles with electron dense materials in all segments of the small intestine (Figure 2-1), which was similar to what Moretti et al. (2013) observed in neonatal goats. Additionally, we observed the presence of apical and basal mitochondria as well as ACS at 51 h of life (Figure 2-1). In a previous study, in which neonatal goats were fed a uniform diet of colostrum and milk replacer (Moretti et al. 2013), ACS and apical mitochondria were uncovered in the jejunal enterocytes at 36 h, but not at 96 h of life. Furthermore, Bessi et al. (2002) demonstrated that the appearance of the apical canalicular system was reduced in 3 d old calves compared to newborn calves. These results suggest that the fetal enterocyte population is replaced by the adult-type enterocytes between 51 h and 96 h of life, which falls within the duration suggested by other studies (Campbell et al., 1977; Hurley and Theil, 2011)

The presence of enterocyte ultrastructural features however might vary in a species-specific manner between goats and calves. For instance, Moretti et al. (2013) observed ACS only in jejunum, while Bessi et al. (2002) showed the presence of ACS in both jejunum and ileum, similarly to the present study. These differences agree with the literature, as both the jejunum and ileum showed higher absorption capacity than the duodenum in calves (Jochims et al., 1994), while the jejunum is considered the major segment for macromolecular absorption in neonatal

goats (Nordi et al., 2012). A future study, evaluating more frequent time points of electron micrograph sample collection up to 72 h of life will be necessary to discern the exact time frame of fetal enterocyte replacement by adult-type enterocytes.

In our study, majority of the histomorphometric results did not support our hypothesis that calves fed at 0 h of life would have increased small intestinal growth. Firstly, small intestine weights and lengths were not different among calves fed their initial colostrum meal at either 0, 6, or 12 h of life (Figure 2-2). Moreover, villi height result did not agree with our hypothesis either, as in some compartments of the intestine 6 and 12 h calves had increased small intestinal growth compared to that of 0 h calves. With the exception of the duodenum, 0 h calves had shorter villi in the proximal jejunum (330.6 ± 17.6 μm; $P < 0.001$), and distal jejunum (368.8 ± 9.9 μm), in comparison to 6 h calves in proximal jejunum (438.1 ± 18.3 μm), and 12 h calves in the distal jejunum (515.1 ± 8.3 μm; $P < 0.0001$; Figure 2-2). There was no difference among treatment groups in the ileum. Differences in crypt depth among treatments were only observed in the distal jejunum, where 0 h calves had deeper crypts (197.3 ± 11.2 μm) than 6 h (157.1 ± 11.2 μm; $P = 0.05$) and tended to have deeper crypts than 12 h calves (163.7 ± 11.8 μm; $P = 0.10$; Figure 2-2). Crypt depth results agreed in the distal jejunum with our hypothesis, as calves fed meals earlier had larger growth in crypts than calves fed meals later in life.

Similar to the villi height results, the villi:crypt ratio was different only when comparing 6 and 12 h groups in the distal jejunum (1.7 ± 0.2 and 2.5 ± 0.3 respectively; $P = 0.05$). The larger villi:crypt ratio in 12 h calves is likely derived from the longer villi, as there was no difference in crypt depth in the distal jejunum. Larger villi:crypt ratios are often interpreted as physiological intestinal development, and an indicator of increased digestive and absorptive capacity (Wang et al., 2009).

Treatment effects were observed in intestinal surface area of the proximal jejunum and ileum. In the proximal jejunum, 6 h calves had larger surface area index (18.4 ± 1.5; $P < 0.0001$) compared to either 0 h (13.0 ± 1.4) or 12 h (13.2 ± 1.4) calves. In the ileum, 12 h calves had larger surface area index (21.6 ± 1.8) than 0 h calves (14.3 ± 2.0; $P = 0.03$) and tended to have a larger surface area index than 6 h calves (15.5 ± 1.8; $P = 0.07$; Figure 2-2). As the surface area index calculation is determined by villi height, crypt width, and villi width (Kvidera et al., 2017), the ileum results indicate that surface area enlargement was driven by crypt width and villi width in the ileum of calves fed colostrum at12 h of life. At ultrastructural level, no qualitative differences were detected between treatments in the ultrastructure of enterocytes.

Two mechanisms that may explain our results in which increased villi height and surface area were observed in the calves that fed colostrum later are: 1) compensatory growth, and 2) timing of the initial colostrum feeding. Compensatory growth is the phenomenon in which the animals that experienced the period of nutrient deprivation show accelerated growth during the period of nutrient provision (Winter et al., 1976; Kamalzadeh et al., 1998). Intestine length and weight increases were reported as reflections of compensatory growth in sheep subjected to dietary changes (Kamalzadeh et al., 1998; Winter et al., 1976); however, differences in small intestine weight and length were not observed in our study. Compensatory growth of villi in the jejunum of weaning piglets was observed when a higher provision of nutrients was preceded by a low energy density diet (low protein) (Levesque et al. 2012). In the ileum, the same study observed a compensatory crypt depth increase in the proximal ileum but not in the jejunum (Levesque et al. 2012). These reports support the idea that compensatory growth might have occurred in our calves in the form of increased villi height, although our crypt results in the ileum did not show significant differences among treatments.

Our findings may have also been influenced by the timing of the colostrum feeding, and the nutrient level discrepancy between the colostrum and milk replacer. Since all samples were collected at 51 h of life (3 hours after the seventh meal), 0 h calves had longer period of feeding with milk replacer (lower energy) following the initial colostrum meal compared to 6 h and 12 h calves. Furthermore, in a study where 27 wk old veal calves were fed milk and 20:80 mixture of roughage (50% corn silage and 50% wheat straw), the calves showed approximately 63.9% of the total tract passage rate at 48 h after the meal (Berends et al., 2015). Based on the limited passage rate data in calves one can speculate that calves fed colostrum at 6 h and 12 h most likely still had the colostral component in the intestinal tract at 51 h of life (euthanasia and sample collection). Consequently, calves who received colostrum at 6 h and 12 h of life may have had the trophic effect of colostrum still affecting their intestine growth later into the trial compared with calves that received colostrum at 0 h of life.

The serum concentration of IGF-1, which is also an indirect indicator of intestinal growth, represented as total area under the curve (AUC), were not affected by treatments (Figure 2-3). Such findings are in agreement with a recent study from our group that measured the gut growth hormone GLP-2 in plasma from the same trial, in which no differences among treatments were observed (Inabu et al., 2018a). The IGF-1 measured in this study are most likely from the endogenous production, as colostral IGF-1 absorption is minimal in the neonatal intestine and does not affect the serum IGF-1 concentration (Hammon and Blum, 2002). Our results, along with that of Inabu et al. (2018a), suggest that the treatments of this experiment do not impact the endogenous production of IGF-1 and of GLP-2.

To our knowledge, this study is among the first to characterize the effect of delayed colostrum feeding on the ultrastructure of neonatal calf enterocytes. It is also the first study to

look at the effect of delayed initial colostrum feeding on small intestine growth. Transmission electron microscopy (TEM) showed characteristics of fetal enterocytes such as apical canaliculi and apical mitochondria at 51 h of life. Our results suggest that the fetal enterocytes of neonatal calves are not replaced by adult-type enterocytes until 51 h of life. In future studies, collecting samples from different time points in the first days of life will aid in discerning the exact time period of fetal enterocyte replacement.

In conclusion, histological analyses of neonatal intestinal samples of calves from our study suggest that villi height, crypt depth, and surface area in the proximal jejunum and ileum are affected when feeding is delayed by as little as 6 h. Increased villi height and surface area were observed in calves that had colostrum feeding delayed until 6 h and 12 h of life, which are likely due to two mechanisms: 1) compensatory growth, and/or 2) the transitory residual effect of colostrum on intestinal growth. Based on our trial design, we could not conclude whether the increased growth represents a short- or long-term effect; therefore we cannot determine if the increased intestinal growth observed in specific segments in this study in 6 h and 12 h calves is beneficial for calf health. Further studies with longer trial durations are necessary to clarify the potential effects of delaying colostrum feeding on calf health.

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2.5 Figures

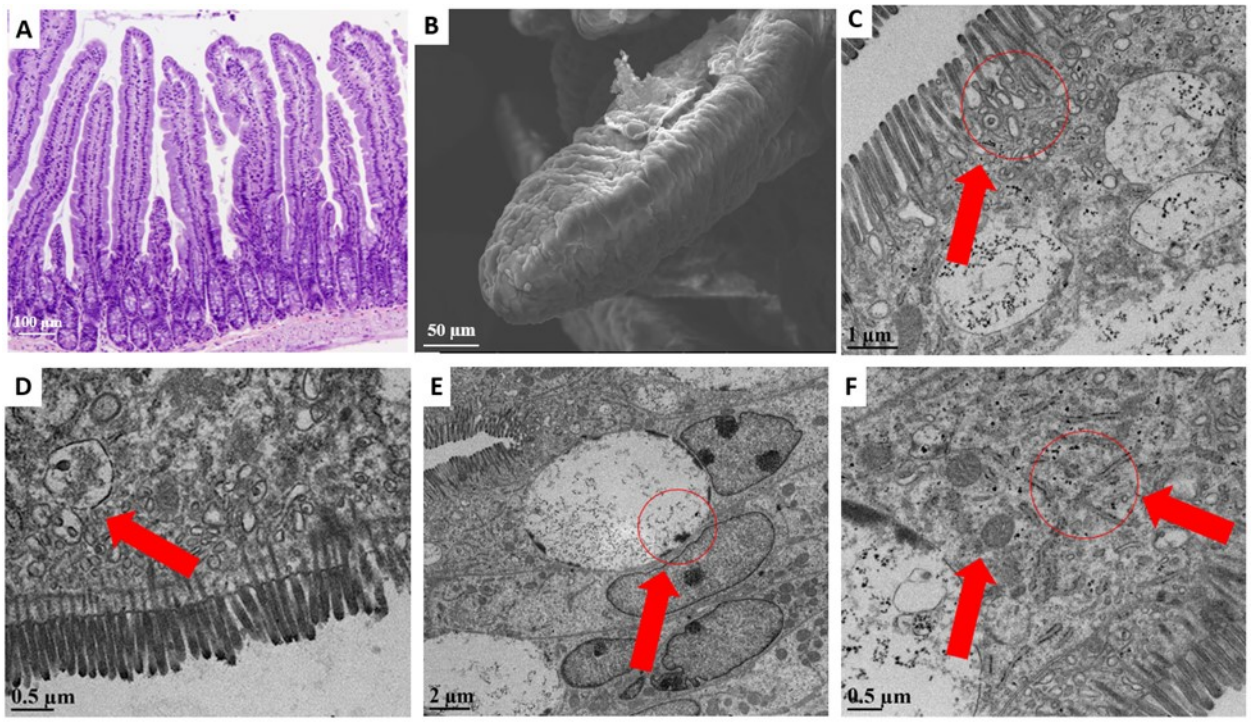

Figure 2-1. Histological images of neonatal calf small intestine indicating unique features of neonatal enterocytes

(A) brightfield microscope image of proximal jejunum (100 ×), (B) SEM image of ileum villi, (C) pinocytosis (14000×), (D) vesicle formation in ileum (14000×), (E) large vacuole formation with electron-dense material in ileum (5600 ×), and (F) apical canalicular system & apical mitochondria in ileum (18000×).

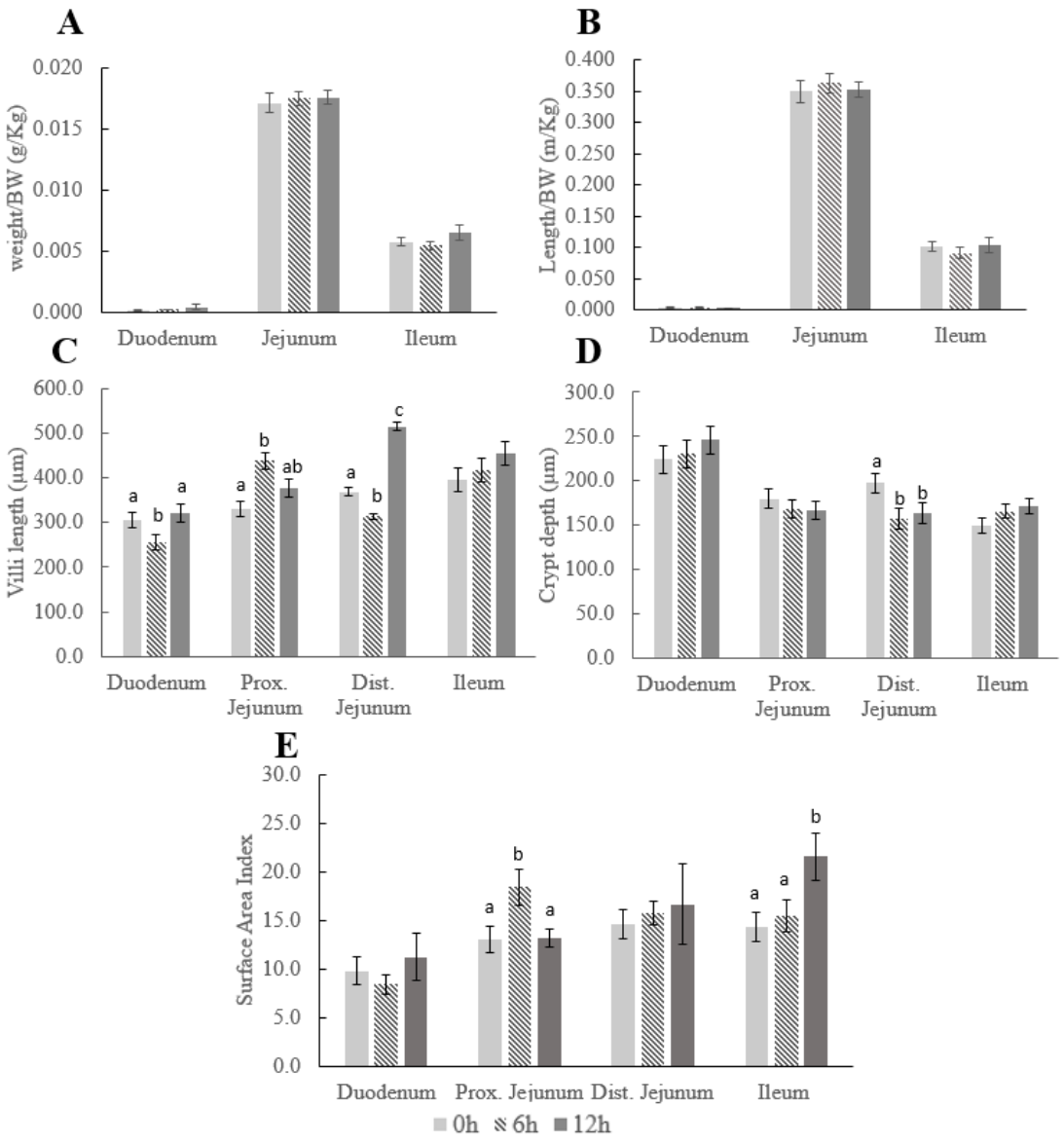

Figure 2-2. Comparisons among colostrum feeding treatment groups (at 0, 6, or 12 h of life) for variables obtained through histology analyses of each small intestine compartment in 27 Holstein bulls calves:

(A) proportion of body weight represented by small intestine tract weight; (B) proportion of body weight represented by small intestinal tract length; (C) villi length, (D) crypt depth, and (E) surface area index. ^{a,b,c} *Different superscripts indicates significant differences* ($P \leq 0.05$) *among treatment groups within a same compartment. All values are presented as LSM ± SE.*

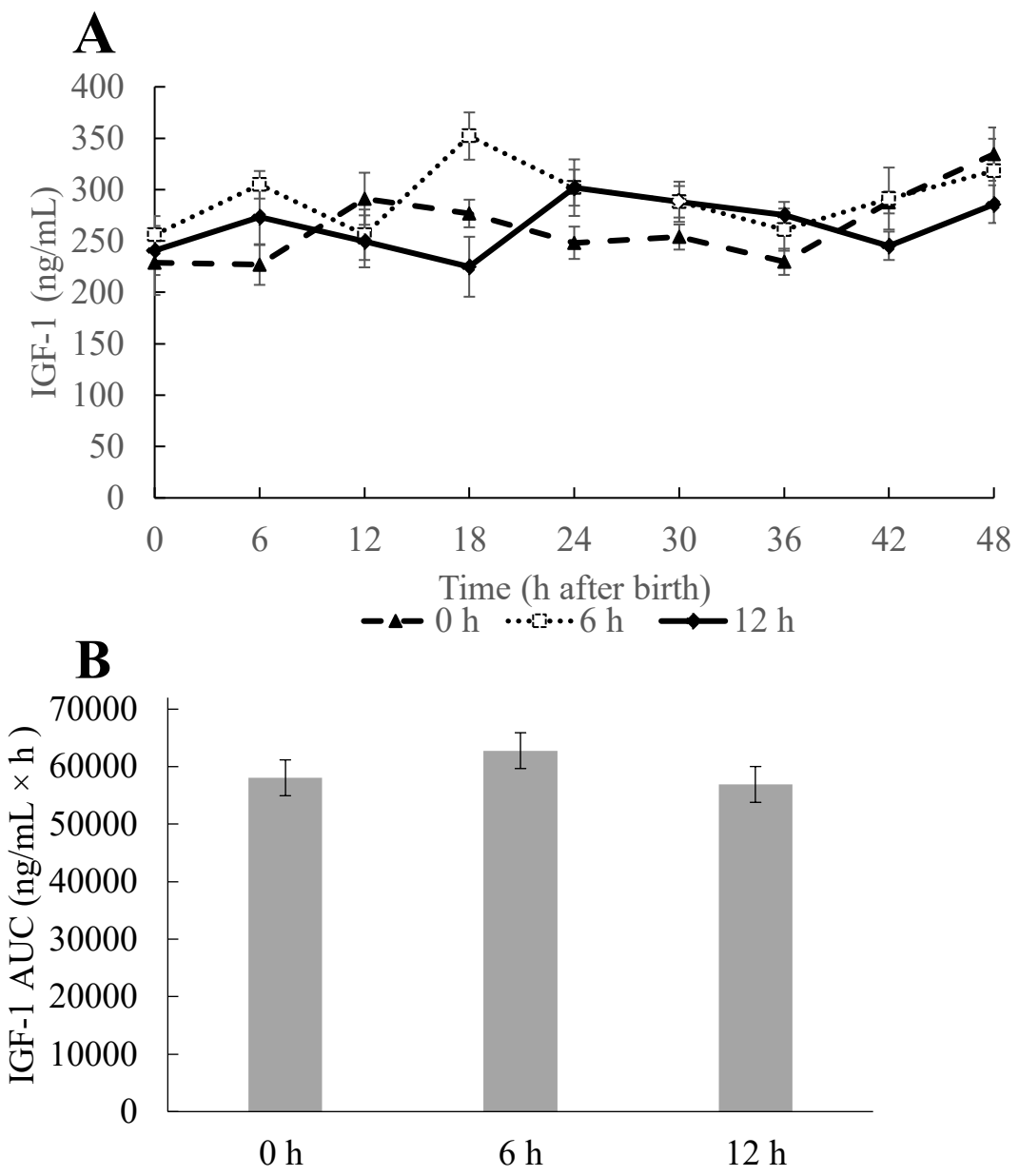

Figure 2-3. Comparisons among colostrum feeding treatment groups (at 0, 6, or 12 h of life) for insulin-like growth factor 1 (IGF-1) concentrations in 27 Holstein bull calves:

(A) Average serum IGF-1 level concentrations during the first 48 h of life; of treatment groups over time. (B) The total area under the curves (AUC) of the insulin-like growth factor 1 (IGF-1) IGF-1 measured from 0 h to 48 h among colostrum treatment groups (colostrum given at 0, 6, or 12 h of life). No differences were observed among treatment groups ($P > 0.10$). All values are presented as LSM ± SE.

3 Effect of Extended Colostrum Feeding Duration on Gastrointestinal Tract Growth of Holstein Bull Calves

3.1 Introduction

In bovine placenta, the maternal blood stream is separated from the fetal blood stream and therefore the dam is unable to provide passive immunity *in utero* to fetus (Wooding, 1992). Consequentially, neonatal calves rely on the passive transfer of Ig, especially the immunoglobulin G (IgG) in colostrum for their immune protection (Stott et al., 1979). Due to the importance of IgG in calf health, ensuring successful passive transfer has been the focus of colostrum feeding, management and research. However, other roles and benefits of colostrum have been relatively neglected, such as its contribution to intestinal growth and health. This is surprising when the high neonatal calf mortality (7.8% - 11%) in the Canadian dairy industry is considered, of which the majority (55%) of mortality is associated with digestive diseases (OMAFRA, 2018). Guilloteau et al. (2009) and Sanglid et al. (2003) suggested that the high rate of digestive disorders in pre-weaning calves is likely due to poor functional growth and adaption of the digestive tract.

In addition to IgG, colostrum also contains high levels of nutrients and bioactive molecules that stimulate growth and development of the digestive tract in neonatal calves (Odle et al., 1996, Hammon and Blum, 2000). Compared to mature milk, colostrum contains higher levels of fat, protein, and growth factors such as insulin-like growth factors 1 & 2 (IGF-1, IGF-2), minerals, hormones, and cytokines (Campana and Baumrucker, 1995; Hammon and Blum, 2000; McGrath et al., 2016; Odle et al., 1996). In nature, levels of colostral bioactives decline in a time-dependent manner, producing transition milk after colostrum and eventually mature milk

(Bastian et al., 2001; Yang et al., 2015). On the contrary to the gradual transition of nutrient levels in nature, many producers in Canada feed 1 or 2 meals of colostrum, followed by milk or milk replacer, subjecting the calves to the abrupt transition from the colostrum to a lower energy diet (Vasseur et al., 2010). Sanglid et al. (2003) suggested that the calf intestine during the neonatal period experiences an intense structural and developmental stress similar to what the intestine would experience during the weaning period, which may contribute to the digestive disorders observed in neonatal calves. Despite this, information regarding the impact of an abrupt transition in nutrient level during the neonatal period in calves is limited.

A study by Bühler et al. (1998) demonstrated that calves fed colostrum 6 times during the first 3 d of life had increased villi length and crypt depth compared to calves fed only milk replacer. The trophic effects of colostrum can benefit the overall health and growth of calves as the increased growth of the digestive tract (particularly the small intestine) leads to improved absorption of nutrients and other bioactive molecules. Furthermore, an increased growth in small intestine and subsequent improvement in glucose absorption can also benefit calves by allowing them to overcome hypoglycemia and negative energy balance which is a commonly seen in newborn calves (Steinhoff-Wagner et al., 2014).

The trophic effect of colostrum is exerted by both colostral nutrients and growth hormones (Hammon and Blum, 2000). There are several growth factors in colostrum, and one of the most abundant growth factors is insulin-like growth factor 1 (IGF-1) (Marnila and Korohnen 2002). Insulin-like growth factor 1 is an important promoter of intestinal and overall organismal growth, with a broad spectrum of physiological roles (Georgiev et al., 2003). Another important growth factor, glucagon like peptide 2 (GLP-2) also plays a key role in intestinal growth. While it has not been identified in colostrum, endogenous production of GLP-2 is stimulated by the ingestion

of nutrients which can be found within colostrum (Dube et al., 2006). The effects of GLP-2 on small intestinal growth are well defined, including villi and crypt growth, proliferation, as well as controlling intestinal permeability, motility, and blood flow in numerous animal models but ruminants (Dube et al., 2006). Both GLP-2 and IGF-1 actions are closely related to each other. In fact, several studies have shown that the intestinal trophic effect of GLP-2 is potentially modulated by the intermediary action of IGF-1 (Dube et al., 2006; Yusta et al., 2000).

An appropriate colostrum feeding strategy is one of the key factors to counteract the high digestive disease associated mortality in neonatal calves. However, little is known about the ideal duration of colostrum feeding and similarly, there is a lack of studies regarding the trophic effects of extended colostrum feeding on the calf intestine. Thus, in this current study, we hypothesized that extended colostrum feeding increases intestinal growth and elevates serum IGF-1 and plasma GLP-2 concentration in blood. Overall, the aim of this study was to investigate the effect of extended colostrum feeding duration on small intestine development of neonatal calves utilizing histological parameters as well as blood GLP-2 and IGF-1 concentrations.

3.2 Material and Methods

3.2.1 Animals

The experiment was conducted at the Dairy Research and Technology Center (DRTC), University of Alberta and approved by the Animal Care and Use Committee (AUP00001595). Bull calves (n = 24), from both multi- and primiparous-cows, were removed from the dam within 10 minutes after birth, weighed, and transferred into the individual calf pens bedded with fresh shavings and straw. Only single-birth calves weighing between 35kg-55 kg were used. Calves were dried and stimulated thoroughly using clean towels for 20 min after birth. Calf navels were

dipped in a 7% iodine solution to sterilize the navel within the first hour of life. All calves were fed pooled and pasteurized colostrum (Saskatoon Colostrum Company Ltd., Saskatoon, SK, Canada) 2 h after birth at 7.5% of their BW. Following the first colostrum feeding calves were randomly placed in one of three treatments: colostrum only (COL; n = 8), 50:50 mixture of colostrum and pooled and pasteurized milk (MIX; n = 8), or milk only (MILK; n = 8). The colostrum had a composition of 4.6% fat and 13.1% protein while the milk had 5.2% fat and 3.0% protein. The first treatment meal was fed 10 h after the first colostrum meal (12 h postpartum) and every 12 h thereafter at 5% of BW until the last meal at 72 h of life. Each meal was fed in the bottle after thawed and heated in water bath to 39°C. If calves did not consume meals within the first 30 min, the rest of the meal was fed via an esophageal tube. We excluded calves who did not entirely consume two consecutive treatment meals from this study.

3.2.2 Blood Sampling

A 16-gauge I.V. catheter (Terumo Medical Corporation, NJ, USA) was placed in the jugular vein of the calves at 1 h postnatal and a baseline blood sample was taken. The maximum blood collection for this approved protocol was 1% of BW per day. Samples were taken at 1, 2, 3, 6, and 9 hours postnatal. Two intensive postprandial sampling periods followed the 12 h and 60 h meals (second and sixth meal of life) when the blood samples were taken more frequently. Between 30 h and 60 h, samples were collected at 6 h intervals. Immediately after blood sampling, the catheter tubing was flushed with 6mL of saline and 1.5 mL of heparinized saline (20 IU/mL of heparin) to prevent blood clot formation in the catheter. Individual samples were transferred into serum and plasma tubes (Becton Dickinson, NJ, USA) immediately after collection. Plasma tubes were immediately centrifuged at 3,000 ×g at 4°C for 20 min, whereas serum was allowed to clot for 3 h prior to centrifugation to facilitate serum collection. Both

serum and plasma supernatants were aliquoted into microcentrifuge tubes then stored at -20°C until analysis.

3.2.3 Euthanasia and Intestinal Tissue Collection

Calves were euthanized 75 h postpartum with Euthanyl injection (Vetoquinol, Lavaltrie, Quebec, Canada) at 1.25 mL/10 Kg of euthanasia BW via jugular catheter. Exsanguination was performed after the calves reached a surgical plane of euthanasia. The digestive tract was immediately removed from the body cavity with excising incisions performed at the esophagus and rectum. The digestive tract was placed on a disinfected tray and forestomach, spleen, and liver samples, in addition to 10 cm segments of small intestine were removed from the specific locations. A 10 cm section of duodenum was collected immediately after the abomasal pyloric sphincter; proximal jejunum samples were collected from 100 cm distal from abomasal pyloric sphincter; distal jejunum was collected from 30 cm proximal to the cranial mesenteric artery; and ileum samples were collected from 30 cm proximal to the ileo-cecal junction as described by Malmuthuge et al. (2015). Intestine samples were immersed into 15 mL of formaldehyde for brightfield microscopy sample preparation. Samples intended for brighfield microscopy were transferred to Histocore facility (University of Alberta, AB, Canada) to be prepared for histomorphometric analysis.

3.2.4 Brightfield Microscopy and Measurements

Histology samples were dehydrated overnight with series of ethanol solutions. After dehydration, the samples were submerged in paraffin wax and solidified to section the samples (5 µm slice per section) using microtome (Leica, Wetzlar, Germany). After the hematoxylin and eosin staining, slides were analysed using Zeiss Brighfield Axio Scope at Biological Sciences

Microscopy unit (University of Alberta, AB, Canada). ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used to measure the villi height and crypt depth in duodenum, ileum, proximal and distal jejunum. For all histological measurements, an auto-generated grid was overlaid on each image for visual accuracy and consistency. Villus was measured from the fully attached epithelial cell to the villus-crypt junction, and crypt was measured from the villus-crypt junction down to the tip of the crypt (Wongdee et al., 2016). A minimum of 2 measurements from 5 different images per slide (minimum of n = 10 per compartment) were taken under 100× magnification. The ratio of villi:crypt was calculated by dividing villi length over the crypt depth using Excel (Microsoft, Redmond, Washington, USA). Widths were measured perpendicularly from the mid-section of 2 fully attached villi and crypts per image, from 5 images (n = 10) from duodenum, proximal and distal jejunum, and ileum of individual calves for surface area measurements, as indicated by the equation presented by Kvidera et al. (2017). The equation follows:

$$M = \frac{\left[(v.\ width \times v.\ length) + \left(\frac{v.\ width}{2} + \frac{c.\ width}{2}\right)^2 - \left(\frac{v.\ width^2}{2}\right)\right]}{\left(\frac{v.\ width}{2} + \frac{c.\ width}{2}\right)^2}$$

In formula: M = mucosal-to-serosal amplification ratio, v. width = villi width, v. length = villi length (height), and c. width = crypt width.

3.2.5 Proliferating Cell Nuclear Antigen (PCNA) Quantification

Proliferating cell nuclear antigen (PCNA) samples were prepared from the same paraffin embedded blocks of proximal jejunum and ileum as previously described. Blocks were sectioned in 5 µm sections, which were rehydrated on Leica CV 5030 autostainer program 2 (Leica, Wetzlar, Germany). Antigen (Ab18197-Rabbit anti PCNA) (Abcam Inc., Toronto, Ontario,

Canada) was diluted with 20% normal goat serum (NGS; Cedarlane, Burlington, Ontario, Canada) to 1/2000 dilution, and the diluted antigen was applied to the samples. Antigen retrieval was performed with citrate for 16 min at 80%, then cooled for 30 min. The sections were then washed twice in PBS, followed by quenching for 6 min in 10 mL solution of 50% H_2O_2 in 40 mL 100% MeOH. Sections were rinsed with distilled water for 5 min, then 1 min in phosphate buffered saline (PBS). Antibodies were blocked with 20% NGS for 60 min. Normal goat serum were aspirated off, and primary rabbit anti-PCNA antibodies were added for 60 min. Biotinylated Goat Anti Rabbit (BGAR) diluted to 1/200 dilution with NGS was applied as a secondary antibody for 40 min, then washed with PBS three times. Avidin-biotin complex (ABC; Cedarlane, Burlington, Ontario, Canada) was added for 40 min, followed by PBS washing three times before 3,3'-Diaminobenzidine (DAB; Cedarlane, Burlington, Ontario, Canada) solution application with a pipette (to cover the sample). When adequate color intensity was reached, sections were washed with distilled water, then counterstained with hematoxylin. Proliferating cell nuclear antigen samples were quantified using the Zeiss Brighfield Axio Scope (Zeiss, Oberkochen, Germany) at Biological Sciences Microscopy unit (University of Alberta, AB, Canada). From each slide, five images were taken. The ratio of PCNA+ cells/total cells from maximum of two villi and crypts were measured per image under 400 × magnification (n=10 per compartment). For all PCNA stained images, only the cells that are opaque and fully stained were counted as PCNA+ cells. For individual images, color intensity thresholds were set manually under RBG option on ImageJ program to include only the fully stained cells, which were counted over total cell numbers.

3.2.6 Analysis of Plasma Glucagon-like Peptide 2 (GLP-2) and Serum Insulin-like Growth Factor 1 (IGF-1)

Plasma GLP-2 levels were measured using solid phase competition assay. Human GLP-2 labeled with Europium (Eu) (Peptide institute Inc, Osaka, Japan), polyclonal anti-rat GLP-2 serum, and goat-anti-rabbit-γ-globulin coated polystyrene microtiter strips (Yanaihara Institute Inc, Shizuoka, Japan) were used to target the N-terminal of GLP-2, as described by Inabu et al. (2017). The intra- and inter-assay coefficients of variance were 3.85 and 4.42% respectively. For IGF-1 analysis, serum samples were frozen at -20°C and shipped overnight on ice to Western College of Veterinary Medicine Endocrine Lab (WCVM, Saskatoon, SK, Canada). Serum IGF-1 concentration was analysed using an automated solid-phase chemiluminescent immunoassay on Immulite/Immulite 1000 systems machine (Siemens AG, Munich, Germany). Immulite/Immulite 1000 systems was calibrated following the instructions provided by the manufacturer. The intra- and inter-assay coefficients of variance for IGF-1 analysis were 5.0 and 4.1% respectively.

3.2.7 Calculation of the Area Under the Curve for GLP-2 and IGF-1

Area under the curve (AUC) was calculated from the GLP-2 and IGF-1 data using the equation described by Inabu et al. (2018a). The formula is described below:

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

In formula: a = GLP-2 or IGF-1 (ng/mL) concentration at time point 1, b= GLP-2 or IGF-1 concentration (ng/mL) at time point 2, c = total duration of time (h).

For GLP-2, two postprandial sampling periods ($AUC_{12\text{-}24}$ and $AUC_{60\text{-}72}$), which were taken at 12 h – 24 h of life (first treatment meal) and 60 h – 72 h of life (6th treatment meal)

were calculated separately as these periods have increased sampling frequency. For IGF-1, total AUC was calculated from 12 h postnatal (the first treatment meal) for every 6 h until 75 h.

3.2.8 Statistical Analysis

Statistical analyses were performed using the MIXED function procedure of SAS 9.4 (SAS Institute, Cary, NC) for small intestinal weight, length, villi height, and crypt depth, GLP-2 and IGF-1 AUC measurements. The group mean of each dependent variables for small intestinal regions (duodenum, proximal and distal jejunum, and ileum) were compared with the mixed effects ANOVA, including the fixed effect of treatment (COL, MIX, and MILK). For histological analyses with multiple observations, calf was used as random effect, and post-hoc Tukey-Kramer test was used to correct the multiple comparisons. For GLP-2 and IGF-1 measurements over time, calf was included as the repeated measure subject to control for the variability of multiple measurements. For each model, the covariance structure (compound symmetry, unstructured, autoregressive, or Topelitz) with the lowest Bayesian information criterion (BIC) was chosen. The repeated measures data GLP-2 and IGF-1 AUC results were corrected using Bonferroni test. Least squares means (LSM) by time point were assessed using the SLICE command, which allows for a partitioned analysis of the LSM for an interaction. Normality and homoscedasticity of the residuals were assessed graphically using standardized residuals. All values were presented as Least Square Means (LSM) LSM ± standard error (SE).

Statistical significance was defined as $P \leq 0.05$ and tendency as $0.05 \leq P \leq 0.1$.

3.3 Results

3.3.1 Intestinal Morphometrics

Length and weight for all small intestine segments were not affected by treatments. MILK calves had shorter villi height than MIX calves in all compartments except for ileum (Table 3-1). In addition to ileum, MILK calves showed no difference ($P = 0.22$) compared to COL calves in duodenum, but in both proximal and distal jejunum MILK calves had shorter villi (260.6 ± 31.3 and 304.6 ± 36.7 μm, respectively) than COL calves, which had proximal jejunum villi height of 469.3 ± 31.3 μm and 463.3 ± 39.3 μm in distal jejunum. There was no difference in villi height between MIX and COL calves in all regions. This trend can be visually exemplified in Figure 3-1 in which there are three micrographs displaying of the visual impact of the treatments on villi length in the proximal jejunum segment. There was no difference in crypt depth between treatments in all compartments of the small intestine (Table 3-1). The villi:crypt ratio results were similar to the villi results (Table 3-1). Comparing the proximal jejunum and ileum, MIX and COL calves had larger villi:crypt ratio than MILK calves, but there was no difference between MIX and COL calves ($P = 0.84$ and $P = 1.00$, respectively). In duodenum, MILK (1.6 ± 0.2) calves tended to have smaller ratio compared to both MIX and COL calves (2.29 ± 0.2, $P = 0.07$ and 2.27 ± 0.2, $P = 0.08$ respectively).

In all compartments except duodenum, MIX calves had larger surface area than MILK calves (Table 3-1). In proximal jejunum COL calves had larger surface area than MILK calves (14.9 ± 1.9 and 7.5 ± 0.9 respectively). There was no difference between MIX calves and COL calves, except in distal jejunum where MIX calves had a larger surface area than COL calves (18.7 ± 1.3 and 12.8 ± 1.4 respectively).

3.3.2 Cellular Proliferation

Proliferating cell nuclear antigen (PCNA) showed no treatment effect on cell proliferation except in ileal crypts (Figure 3-2). The MIX calves showed increased proliferation over MILK calves (0.43 ± 0.01 vs. 0.38 ± 0.01 PCNA+/total cells; $P = 0.03$; Figure 3-2). The COL treatment group showed no difference when compared with MIX or MILK treatment groups.

3.3.3 Growth Factors

Plasma GLP-2 levels did not differ across the treatments during the first postprandial sampling period ($AUC_{12\text{-}24}$). However, during the second postprandial sampling period ($AUC_{60\text{-}72}$), MILK calves had a smaller AUC than COL calves by the difference of 348.8 ± 142.4 ng/mL × h ($P = 0.04$) (Figure 3-3). There was a treatment, time, and treatment × time effects for GLP-2 $AUC_{12\text{-}24}$ and $AUC_{60\text{-}72}$ ($P < 0.001$). In all treatments, AUC increased from d 1 ($AUC_{12\text{-}24}$) to d 3 ($AUC_{60\text{-}72}$). For MILK treatment, $AUC_{12\text{-}24}$ increased by 619.7 ± 122.8 ng/mL × h, for MIX treatment, by 985.2 ± 122.8 ng/mL × h, and for COL treatment, by 1143.1 ± 122.8 ng/mL × h. The total AUC of IGF-1 tended to be greater in COL calves (1323.3 ± 64.7 ng/mL × h) than MILK calves (1095.8 ± 84.2 ng/mL × h; $P = 0.10$; Figure 3-4), while there was no difference between MIX and the other groups (COL and MILK).

3.4 Discussion

Neonatal calves rely on colostral IgG for early immune protection, as the bovine placenta prevents the transfer of IgG *in utero* (Wooding, 1992; Weaver et al., 2000). Due to this reason, research has focused heavily on how to maximize the passive transfer of IgG (Vasseur et al., 2010). Colostrum however also contains nutrients and bioactive molecules such as growth factors that will benefit calf health and growth, by promoting growth of the intestine (Hammon

and Blum et al., 2000). Notwithstanding the high mortality rate of pre-weaning calves due to the digestive diseases (OMAFRA, 2018), a limited number of studies are available on the trophic effect of colostrum in neonatal calf intestine. Moreover, there is a paucity of information regarding the impact of abrupt transition from the colostrum to milk or milk replacer on neonatal calf intestine. Therefore, this current study investigated the effect of extended colostrum with different nutritional transition schedule on neonatal calf gut, especially on the small intestine where the majority of nutrients are absorbed (Hof, 1980; Meale et al., 2017). Additionally, to our knowledge this study is one of two studies that have investigated GLP-2 concentration in blood – a potent trophic hormone in calf intestine, along with the study by Inabu et al., (2017), which is an important growth factor involved in numerous critical functions and pathways for neonatal calf health and growth (Conner et al., 2016; Drucker et al., 1996).

3.4.1 Villi Height, Crypt Depth and Intestinal Surface Area

Overall, villi height was increased in COL and MIX calves compared to MILK calves demonstrating that diets higher in nutrient density (COL and MIX) have more trophic effects than milk alone (Figure 3-1). Surface area results agreed with the villi results only in proximal jejunum, where both MIX and COL calves had larger surface area than MILK calves (Table 3-1). However, only the MIX calves had larger surface area compared to MILK calves in other compartments (distal jejunum and ileum), and even COL calves in distal jejunum. These results were not expected, as in our study the higher levels of nutrients and bioactive molecules such as in the COL group were not directly correlated with increased intestinal surface area. The unexpected result of MIX treatment in inducing greater trophic effect in calf small intestine can be explained by these three potential mechanisms:

1) Maximizing the absorptive capacity of enterocytes. The high level of nutrients provided by the colostrum treatment may have overwhelmed the digestive and absorptive capacity of the small intestine (Hof, 1980), and that this may have imposed a limitation on the trophic effect of the COL diet. However, this only accounts for the villus height result where MIX and COL calves had the same level of trophic effect, not the surface area results where MIX calves had larger surface area in distal jejunum and ileum.

2) High level of total solids disturbing the absorptive function of the gut. This explanation can account for the result of larger surface area in MIX calves than COL calves. High level of osmotically active molecules in lumen beyond the absorption capacity will lead to simultaneous excretion of water and electrolytes in feces (Michell, 1974). According to Hof (1980), the osmotically active molecules include inorganic solutes such as sodium and soluble organic components such as amino acids and fatty acids. Additionally, many of the inorganic solutes, such as sodium, also determine the absorption of the amino acids and monosaccharides via coupled transport (Sepulveda et al., 1982), further affecting the absorption dynamics. There also may be a pathological consequence of the hyperosmolar feeding for an extended time, such as direct damage to mucosa observed in the infants who were fed hyperosmolar formula (750 mOsm per L) (Santulli et al., 1975). To our knowledge, no study has been conducted in neonatal ruminants to test the effect of high levels of osmolality on growth. Thus, further studies are required to investigate whether the high osmolality of colostrum affect the nutrient absorption in neonatal calves.

3) The benefits of gradual transition of nutrients and energy level. As previously mentioned, calves in nature experience a gradual transition from colostrum to milk. The decline of nutrients

and bioactive molecules in transition milk is faster than the fixed level within the MIX treatment of this study (Hammon and Blum, 2000). Nonetheless, the MIX treatment still provide the most physiologically available model of gradual transition of the colostral contents compared to COL and MILK treatments in our study. Future studies should be conducted to investigate the effect of transitional diet modelling the transition milk more accurately.

Unlike villi height, the crypt depth results differed from a previous study in which the authors observed crypt depth increase calves that were fed colostrum for 3 d followed by milk replacer up till d 7 when compared with calves that were fed only the milk-based formula for 3 d then milk replacer up till d 7 (Blättler et al., 2001). However, the lack of treatment effect in crypt in our study can be explained with the findings of Cummins et al. (2006), in which they observed crypt fission, the increase in the number of the crypt, as the main mode of crypt growth in the neonatal mice. Similarly, the crypts of the neonatal calves in our study may have employed the crypt fission as the main mode of crypt growth, therefore showing no treatment effects in crypts in our study.

3.4.2 Insulin-like Growth Factor-1 and Glucagon-like Peptide 2

Both IGF-1 and GLP-2 are major trophic factors in intestine promoting proliferation, villi and crypt growth (Drucker et al., 1996; Taylor-Edwards et al., 2011). Hammon and Blum (1997) identified that colostral IGF-1 plays a role in initiating endogenous production of IGF-1 via modulating other pathways such as GH, insulin, and prolactin, instead of inducing a direct trophic effect in the intestine. In addition, Vacher et al. (1995) demonstrated that orally ingested IGF-1 does not enter the bloodstream, suggesting that the IGF-1 levels detected in our study are most likely derived from endogenous IGF-1.

Unlike IGF-1, GLP-2 has not been identified in colostrum (Inabu et al., 2017), thus it is assumed that all the GLP-2 is derived from the endogenous production. The lack of difference between treatments during the first meal postprandial sampling (AUC_{12-24}) may be caused by the timing of the sampling, which coincided with the first treatment meal feeding (at 12 h of life), could also have been too early to observe any treatment effects. Based on the passage rate of milk replacer with roughage measured by cobalt (Co) recovery rate of 27.3% in small intestine after 4 h of feeding (Berends et al., 2015), it was very likely that the treatment meals were still in the intestine affecting the GLP-2 results during this sample period. The higher AUC values for COL calves compared to MILK calves during the second postprandial sampling (AUC_{60-72}) agrees with the literature (Figure 3-3), in that the presence of nutrients in lumen leads to GLP-2 production (Burrin et al., 2001; Dube et al., 2006; Martin et al., 2005). The study by Dube et al. (2006) has demonstrated that the IGF-1 is also a mediator of the GLP-2 driven tropic effect, thus explaining the similar result of GLP-2 and IGF-1 levels in which COL calves had higher level of both growth hormones during the second postprandial sampling periods. It has to be noted that there are numerous trophic pathways in the neonatal intestine. Though IGF-1 and GLP-2 are two major growth promoters, other trophic agents (such as growth hormone and epidermal growth factor) may also play a role in growth. Future studies are warranted to investigate the other pathways that may have impacted small intestine growth in our study.

3.5 Conclusion

Our findings revealed that feeding colostrum (COL) for extended duration, even at 50:50 colostrum and milk mixture (MIX) improved the small intestinal growth in the neonatal calf compared to the milk only (MILK) treatment. Between the COL and MIX treatments, MIX treatment demonstrated the largest intestinal trophic effect. Therefore, supplementing milk with

colostrum to provide a gradual transition from colostrum to milk or milk replacer or feeding transition milk may benefit small intestinal growth of neonatal calves.

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3.7 Figures and Tables

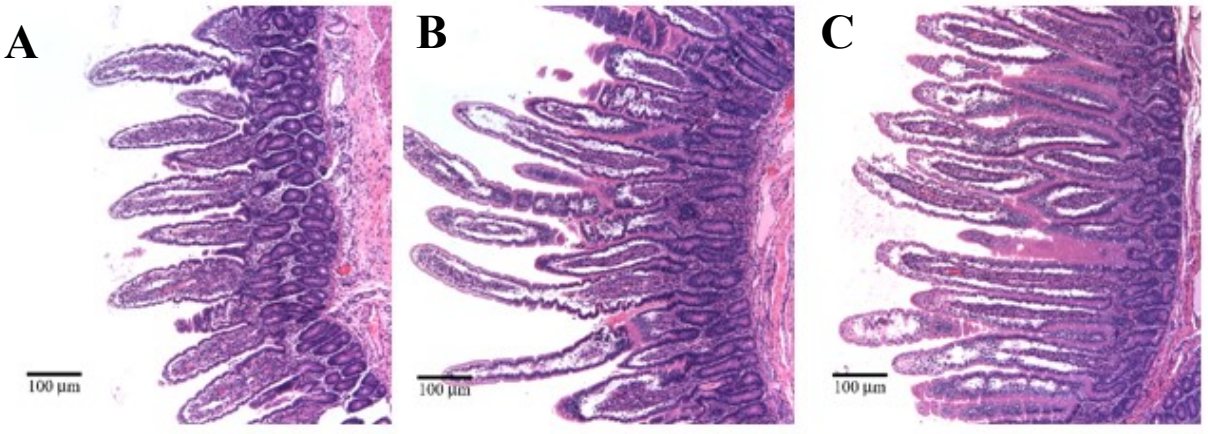


Figure 3-1. Proximal jejunum samples processed with hematoxylin and eosin stain.

A) MILK calf B) MIX Calf C) COL calf. Images were taken under 200× magnification.

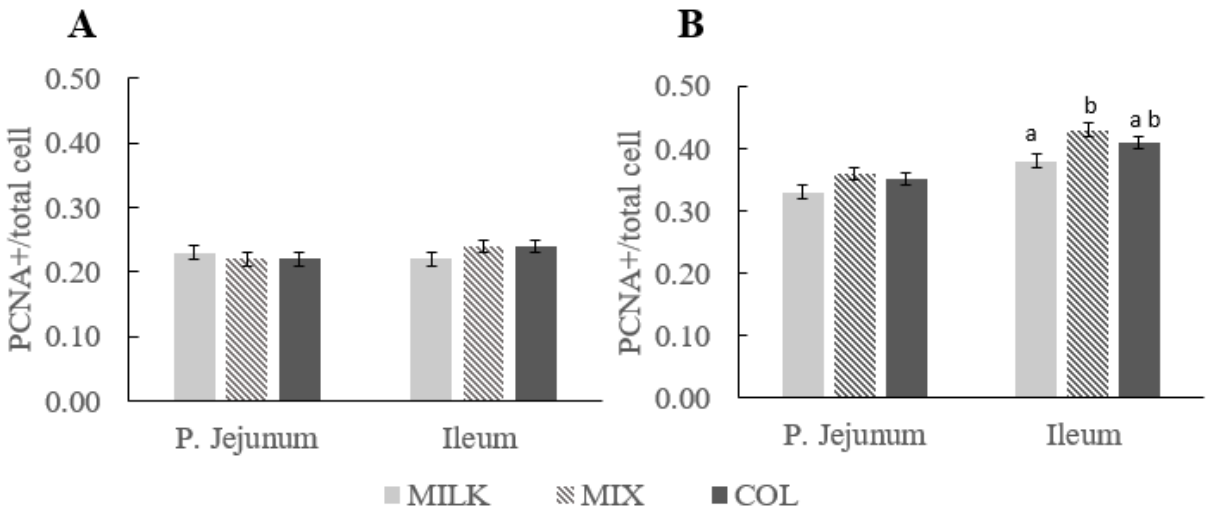


Figure 3-2. Proliferating cell nuclear antigen (PCNA) stain.

A) Comparison of PCNA+ cell/Total cell ratio in villi. B) Comparison of PCNA+ cell/Total cell ratio in crypt. Significance was denoted by **a** and **b**.

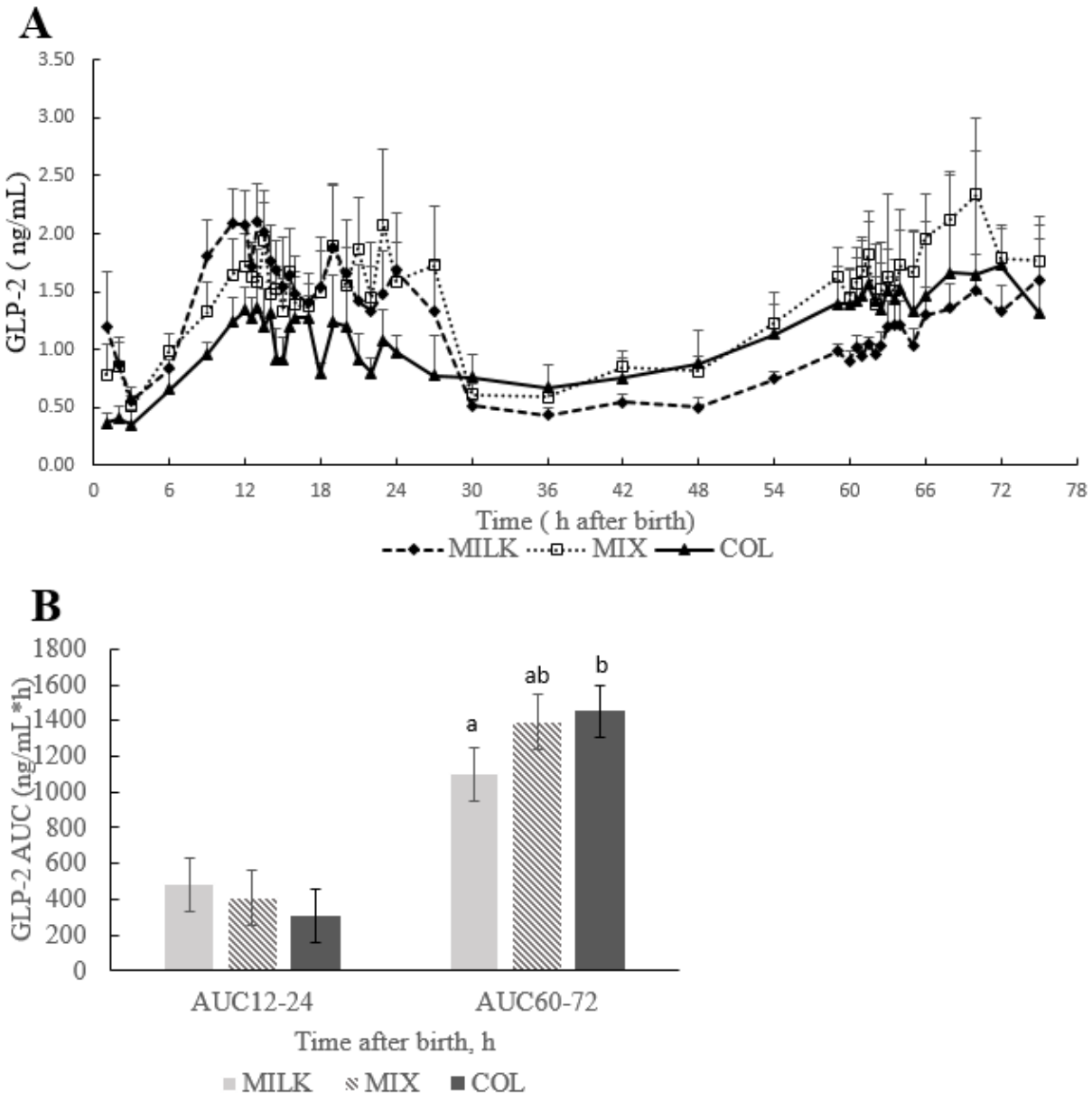
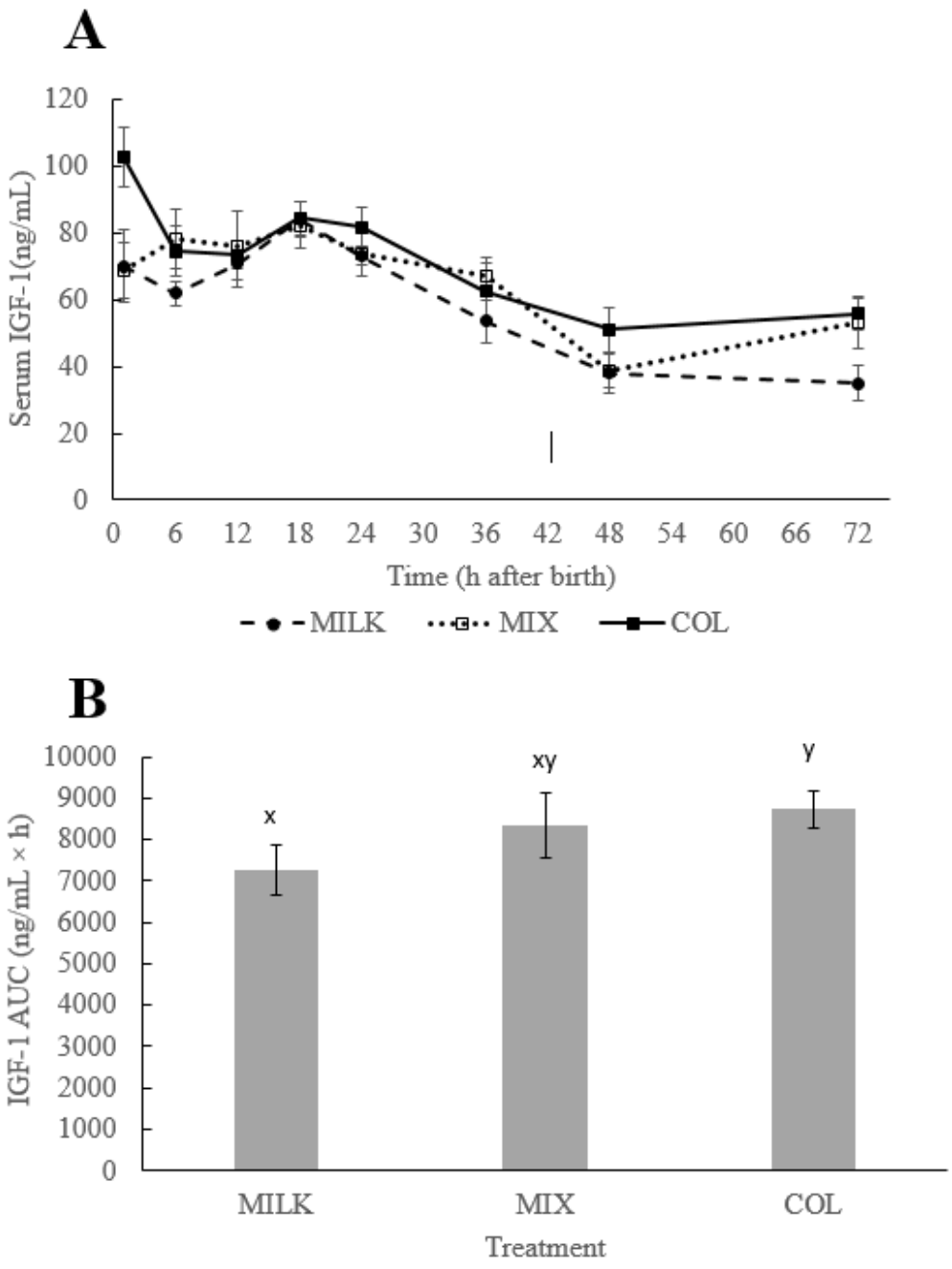


Figure 3-3. Glucgon-like peptide 2 levels during extended colostrum feeding of neonatal Holstein calves.

A) Plasma glucagon-like peptide 2 (GLP-2) level (n=24; ng/mL) of calves that were fed milk only (MILK), milk and colostrum mixture (MIX), and colostrum only (COL) at 5 % body weight every 12 h followed by the initial meal of pooled colostrum at 7.5% of body weight. B) AUC of two postprandial sampling periods at 12h of life and 60 h of life (AUC_{12-24} & AUC_{60-72}). Significance was denoted by **a** and **b**.



<u>Figure 3-4</u>. Insulin-like growth factor 1 levels during extended colostrum feeding of neonatal Holstein calves.

A) Serum Insulin-like grow factor I (IGF-1) level (n=24; ng/mL) of calves that were fed milk only (MILK), milk and colostrum mixture (MIX), and colostrum only (COL) at 5 % body weight every 12 h followed by the initial meal of pooled colostrum at 7.5% of body weight. B) Total AUC of IGF-1. Tendency was denoted by **x** and **y**.

Table 3-1. Histological Analyses for calves fed milk (MILK), colostrum mixture (MIX) and colostrum (COL).

	Treatment			P-Value for treatment effect		
Item	MILK	MIX	COL	MILK vs MIX	MILK vs COL	MIX vs COL
Villi height (µm)						
Duodenum	295.61 ± 13.37[a]	440.76 ± 17.01[b]	395.03 ± 15.70[ab]	0.041	0.219	0.706
Proximal Jejunum	260.63 ± 9.28[a]	480.74 ± 16.87[b]	469.28 ± 17.71[b]	< .001	< .001	0.964
Distal Jejunum	304.59 ± 10.88[a]	466.40 ± 15.55[b]	463.29 ± 19.57[b]	0.005	0.010	0.990
Ileum	335.79 ± 10.36[x]	454.66 ± 18.96[y]	428.76 ± 21.20[xy]	0.076	0.227	0.890
Crypt depth (µm)						
Duodenum	211.17 ± 7.78	201.01 ± 7.70	189.08 ± 6.54	0.924	0.688	0.880
Proximal Jejunum	202.70 ± 6.00	211.36 ± 5.74	195.46 ± 5.88	0.901	0.930	0.704
Distal Jejunum	177.45 ± 5.15	203.45 ± 6.64	194.26 ± 5.07	0.220	0.530	0.830
Ileum	191.25 ± 5.48	178.59 ± 6.38	195.91 ± 7.62	0.776	0.968	0.643
Villi : Crypt ratio						
Duodenum	1.60 ± 0.24[x]	2.29 ± 0.20[y]	2.27 ± 0.20[y]	0.070	0.080	1.000
Proximal Jejunum	1.36 ± 0.23[a]	2.41 ± 0.23[b]	2.59 ± 0.23[b]	0.004	0.001	0.84
Distal Jejunum	1.86 ± 0.27	2.52 ± 0.27	2.54 ± 0.29	0.200	0.210	1.000
Ileum	1.88 ± 0.29[a]	2.86 ± 0.29[b]	2.39 ± 0.31[ab]	0.050	0.460	0.530
Surface Area [1]						
Duodenum	7.31 ± 2.12	12.63 ± 1.90	10.76 ± 1.99	0.186	0.476	0.786
Proximal Jejunum	7.50 ± 0.87[a]	15.06 ± 1.74[b]	14.88 ± 1.88[b]	0.008	0.007	0.996
Distal Jejunum	9.40 ± 1.32[a]	18.74 ± 1.32[b]	12.75 ± 1.41[a]	< .001	0.21	0.01
Ileum	9.83 ± 1.98[a]	19.96 ± 1.98[b]	15.38 ± 2.12[ab]	0.005	0.120	0.280

[1]Surface area = calculated using the equation presented in Kvidera et al. (2017)

4 General Discussion

4.1 Significance of Research

In Canada, neonatal dairy calf mortality is a significant welfare and economic issue, with recent reports noting rates of 11% - 55% of which is associated with digestive diseases (OMAFRA, 2018). Part of the reason for these alarming statistics is due to improper management of colostrum on dairy farms (Vassuer et al., 2010). Beyond the passive transfer of Ig, the impact of colostrum feeding strategies on intestinal health and development has not been studied. Since digestive diseases are such a large contributor to health problems in early life, it is possible that we can still improve our colostrum feeding strategies on farm. One of the key findings from this thesis (Chapters 2 and 3) is that the abrupt adaptation from colostrum to milk may have a large impact on gut development, health and growth during the neonatal phase. This is because in addition to Ig, colostrum also contains an abundance of nutrients and bioactive molecules that are beneficial for calf gut growth and health (Campana and Baumrucker. 1995; McGrath et al., 2016) after the first milking, which is something that most commercial farms are not providing to their calves.

Despite the importance of colostrum and its effect on small intestinal growth, limited information is available around the effect of timing and duration of colostrum feeding on the calf's small intestine. Thus, the studies summarized in this thesis were designed to provide the industry with more knowledge and understanding about the effect of both timing and duration of colostrum feeding on the calf's small intestine, which may benefit calf health and growth and further highlight the importance of colostrum beyond the passive transfer of Ig.

To our knowledge, our studies were unique as they utilized highly controlled colostrum sources (all the same) and feeding times (to the minute relative to birth) and combined the characterization of effects in organs, tissues, cells and hormonal levels. The first study also provided more in depth observations of the structure and ultrastructure of the small intestine using a variety of microscopes. To our knowledge this is also the first attempt to investigate the impact of delayed initial colostrum feeding on intestinal structure and ultrastructure of intestinal cells in a neonatal calf.

The second experiment was conducted to determine the effect of extended colostrum feeding on small intestine growth. The uniqueness of this study comes from the three treatments: full colostrum, supplemented milk with colostrum (50% each), and milk. As an introductory study demonstrating the supplementation of milk with colostrum, a relatively high value of 50% colostrum supplementation was used. Another way of looking at this treatment is to consider it as a step-down treatment, similar to what is done for weaning (Khan et al., 2007). To our knowledge this is also the first study to use pooled colostrum and milk with the controlled component level and accompanied with highly frequent blood sampling (45 samples per animal in the first three days of life) to access gut peptides.

4.2 Effect of Delaying Colostrum Feeding on the Small Intestine

The objective of the second chapter was to determine the effect of delaying the first colostrum meal on small intestinal growth, as well as characterizing the small intestinal structure and ultrastructure. The results from this study highlighted that delaying the colostrum dose affects the growth of the small intestine, though not in the direction we expected – for calves that were fed delayed colostrum at 6 and 12 h of life showed greater small intestinal growth. The limitation of this study was the short duration of the trial (51 h), and due to this, we could not conclude

whether the unexpected increase in growth in 6 and 12 h calves in our study was a transient short-term effect or a long-term effect on small intestinal growth.

Further investigation with a longer trial duration is necessary to determine whether the larger growth response we observed in 6 and 12 h calves does benefit future calf and calf intestinal growth. It is also equally important to consider the advantages of passive transfer and healthy intestinal bacteria found in 0 h calves compared to calves that were fed colostrum on a delayed schedule (Fischer et al., 2018). Therefore it is still recommended to feed the colostrum within the optimal window of within 4 h after birth (Stott et al. 1979).

4.3 Effect of Extended Colostrum Feeding on the Small Intestine

The objective of chapter 3 was to determine the effects of extended colostrum feeding on small intestine structural growth, from both a histological and growth hormone perspective. Despite the differences in nutrient levels between MIX (50% colostrum and 50% milk) and COL (colostrum) treatments, villi height, surface area, and proliferation rate measured by PCNA staining collectively suggest similar, and in some cases increased, growth and proliferation in the MIX treatment. This suggests that nutrient contents alone do not determine the growth response. However, growth factors showed different response patterns to treatments as compared to structural growth response, in that COL calves had higher serum IGF-1 and plasma GLP-2 concentrations. The difference between structural response and growth factor response reaffirms that colostral trophic effect is multi-factorial and involves a synergistic effect of both nutrients and growth factors (Odle et al., 1996; Hammon and Blum, 2000).

In this study, the similar and in some case increased growth response of intestinal villi and surface in MIX vs. COL may have been influenced by factors, such as: 1) saturation of the

absorption capacity of the enterocytes, and 2) high osmolality of the colostrum depressing the structural growth. In order to test these potential mechanisms, future studies should investigate the plausibility of each mechanism. One of the methods to test whether the absorptive capacity was exceeded in both MIX and COL calves is using radiolabelled nutrients and ussing chambers, similar to the measurement of glucose absorption capacity measurements in the small intestine of mice (Debru et al., 2001). Furthermore, by comparing the digestibility of the MIX and COL diet, the absorption capacity of the small intestine in response to different diets can be determined. For total tract digestibility, supplementing Chromium oxide in the diet can be effective (Berends et al., 2015). A bomb calorimeter is another possible method to measure the gross energy of fecal samples from different treatments (Radostits and Bell, 1968). For amino acid digestibility, ileal digesta is preferred due to the possibility of hind gut fermentation affecting the amino acid levels in fecal digesta (Zhang and Adeola, 2017). To test the effect of osmolality in intestinal absorption, osmolality measurements of each treatment meal (COL, MIX, MILK) can be performed.

Based on our histological results, MIX treatment showed the largest trophic effect in small intestinal structure. Therefore, feeding transition milk, or supplementing milk with colostrum in the first days of life, like in our study, may be beneficial for the dairy industry. However, it may be difficult to implement on farm as it will require processing and storing of transition milk separately for feeding or adding colostrum to whole milk or milk replacer in the first days of life. Furthermore, supplementing milk with colostrum at 50% could be economically burdensome, as it would cost $17.00 CAD per meal of 4 L, compared to $2.00 CAD per 4 L of milk replacer or milk. If calves are fed 6 supplemented meals, similar to the study design of chapter 3, it would cost a total of $102.00 CAD per calf, not including the initial colostrum meal and the sub-

sequential milk replacer meals. The limitation of this study was that only one level of colostrum supplementation of 50%, which is cost ineffective in practice, was tested. This level of colostrum supplementation also provides exceeding levels of colostral bioactive molecules compared to the transition milk occurring in nature (Hammon and Blum, 2000 For example, by the second milking after parturition, the level of crude fat and crude protein are decreased to 0.06 % w/w and 0.09 % w/w respectively (modified from Hammon and Blum, 2000), while the crude fat and crude protein level of MIX meal in the current study were 4.8 % w/w and 8.0 % w/w respectively. To address this, further investigation of different levels of colostrum supplementation at various time points in the first days and weeks of life is necessary to establish the most effective and practical conditions.

4.4 Conclusion

The goal of this thesis was to demonstrate the impacts of varied colostrum feeding schedules on the small intestinal growth of neonatal calves. Overall, it was determined that delaying and extending colostrum feeding impacts small intestinal growth mechanisms and surface area. This research highlighted that the neonatal calf intestine is unique and in a state of transition during the first days of life and these changes can be impacted by colostrum feeding regimens. However, and as learned from these two studies, extending the neonatal diet period – e.g. colostrum supplemented milk (MIX treatment) – showed a greater impact on small intestinal growth than delaying the colostrum feeding. Additional studies with more short-term and long-term sampling points are required to determine the economic viability of colostrum supplementation in milk.

In conclusion, this thesis provided new insights into how colostrum can impact not only passive transfer, but also gut physiology, and further supports why more neonatal calf research needs to consider the gut physiology.

4.5 Literature Cited

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