An Investigation of Colostral IgG in the Neonatal Bovine Intestine from Birth to "Gut Closure"

by

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Abstract

IgG absorption from colostrum is essential for transfer of passive immunity in the neonatal calf; despite best practices for colostrum feeding – administering 3L of a colostrum with >50 mg/mL IgG content within 6 hrs of life - some calves still have failure of transfer of passive immunity as defined by a serum IgG content of <1000 mg/mL. The aims of this thesis were to investigate IgG absorption at the tissue level in order to better understand the phenomenon of "gut closure", or the cessation of macromolecule absorption in the calf that occurs around 24 hrs of life. Kinetics and mechanisms of IgG transport have not been investigated at the intestinal tissue level in the neonatal calf, and labeling methods for IgG have not enabled researchers to distinguish between colostrum derived IgG and experimentally supplemented IgG. A novel ELISA system was developed to distinguish between biotin-labeled IgG and non-labeled IgG when they are held in the same ratio. This ELISA system has the potential to be applied to proteins other than IgG. For animals, Holstein × Angus calves were assigned to one of 6 treatment groups, n = 6, 1) non-fed animals euthanized at 1 hr of life, 2) non-fed animals euthanized at 24 hr24 hrs of life, 3-6) animals fed colostrum at 1 hr of life and euthanized at 6, 12, 18, or 24 hrs of life. Intestinal tissue samples from all calves were used in ex-vivo kinetic experiments to determine IgG transport rate and capacity, and additional samples were analyzed for histological features. IgG was measured in the luminal contents, intestinal tissue, via kinetic experiments, and colocalization to the IgG receptor, FcRn, was performed to analyze the passage and absorption of IgG in the neonatal calf from birth to 24 hr24 hrs of life. Data showed that IgG bioavailability in serum reaches levels indicating transfer of passive immunity has been achieved by 12 hrs of life in fed calves. The lumen of the intestine had high IgG concentrations at 6 hrs of life (5 hrs post feeding) but the concentrations dropped close to 0 by 12 hrs of life (11 hrs post

feeding), showing that one feeding of colostrum passes through the intestinal tract, away from the absorptive sites in the small intestine, by 12 hrs of life. In the tissue itself, IgG concentration was highest in the proximal and distal jejunum and 6 and 12 hrs of life, corresponding with increased serum IgG data. However, data showed that IgG transport capacity did not differ between birth and24 hrs of life with animals that were fed colostrum, a novel finding, as the gut has previously been considered to be "closed" by 24 hrs of life. Non-fed animals at24 hrs of life lost the capacity to transport IgG through the intestinal tissue, suggesting that age rapidly decreases absorptive capacity, and that feeding retains the absorptive capacity for IgG. In conjunction, 1 hr and 24 hr old non-fed animals exhibited IgG-negative vesicles in the intestinal villi, while 24 hr old fed animals exhibited IgG-positive vesicles in the intestinal villi. The presence of empty intestinal vesicles did not correspond with intestinal transport of IgG, and thus IgG-negative vesicle number, thought to indicate transport capacity due to physically available space for absorption of IgG, does not have an effect on transport capacity. In the intestinal villi, IgG was colocalized to FcRn. FcRn was strongly colocalized to lymph vessels and weakly to blood vessels, showing that IgG is preferentially transported into the lymphatics in the calf instead of directly into serum, as has been previously reported in other species. These data suggest that "gut closure", historically defined as the cessation of macromolecular uptake, does not occur at 24 hrs of life in the neonatal calf. This novel finding challenges previous thinking about the timing of colostrum feeding and the benefits of feeding several colostrum meals past 24 hrs of life.

Preface

The research conducted for this thesis forms part of an international research collaboration between Zinpro Inc (A. Geiger), Sunalta Farms (J.P. Brouwer), and University of Alberta (B. Hiltz, A. Laarman). All animal work was completed following the guidelines by Agriculture Animals in Research and Teaching (FASS, 2010) and the Canadian Council on Animal Care (2009) and were approved by the University of Alberta Animal Care Committee (protocol 3884). Analysis of harvested samples completed at University of Alberta was deemed category A invasiveness by the University of Alberta Research Ethics Office.

I was responsible for data collection, laboratory analysis, and statistical analysis of chapter 2, 3, 4, and 5 of this thesis. I was also responsible for experimental design, execution, live animal data collection, animal husbandry and sample collection, writing and editing results and manuscripts. Geiger was responsible for technical support and supplement delivery. Laarman was responsible for training, experimental design, and editing of manuscripts.

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1.0 Transfer of Passive Immunity in Neonatal Calves – A Review From Birth to "Gut Closure"

1.1 Introduction

Neonatal bovine calves are particularly at risk for developing disease in early life if they do not receive transfer of passive immunity by drinking colostrum. Ungulates are arguably more at risk than other mammals, even though their immune system is fully functional at birth (Godden et al., 2019), because they do not acquire maternal antibodies in utero (Baumrucker and Bruckmaier, 2014). According to the National Animal Health Monitoring Survey, at two weeks old, a calf with adequate transfer of passive immunity (TPI) has a 15% chance having some sort of clinical morbidity, but a calf with failure of transfer of passive immunity (FTPI) has a 25% chance of having a morbidity. These differences widen with age – a 60 day old calf with adequate TPI has a 25% chance of morbidity but a calf with FTPI has a nearly 50% chance of having some sort of morbidity (Godden et al., 2019; NAHMS, 2021). Among these, 50.9% of clinical morbidities are digestive illness (NAHMS, 2021). In the USA, 19.2% of calves were found to have FTPI, with 40.7% of farms having at least one calf with FTPI (Beam et al., 2009), and mortality was 3.1% (Sutter et al., 2023). These rates equate to one in every two calves that do not get adequate maternal antibodies from colostrum having a morbidity before weaning – on a small farm of 100 calves per year, that equals about 8 calves a year with morbidity and 3 calves a year dying from FTPI alone.

The structure of the bovine placenta is different from that of other mammals and presents the neonatal calf with unique challenges – due to the separation of blood supply between dam and calf, the calf does not receive maternally derived antibodies before it is born (Yang J, 2019). The calf must therefore drink and absorb antibodies from colostrum to obtain TPI; without colostrum, it is not until 4-7 weeks of life that a calf can mount a response to a vaccine immune challenge by producing specific antibodies against the vaccine target (Haessig et al., 2007; Nonnecke et al., 2012). Calves that do not receive adequate TPI have lower body weights (Windeyer et al., 2014), higher veterinary costs, overall higher incidence of respiratory disease (Raboisson et al., 2016), lower average daily gain and even lower milk production for the first

two lactations (Faber et al., 2005). Without colostrum, calves are at an increased risk for disease, lack of performance, and death, making colostrum essential for the newborn calf.

To successfully transfer passive immunity, calves should have at least 15 - 25 g/L of serum IgG by 24-48 hrs of life to have adequate maternal immune coverage and the best chance of survival (Urie et al., 2018; Godden et al., 2019; Lombard et al., 2020); new categories have been added to the recommendations, stating that "excellent" TPI is a calf with serum IgG > 25 g/L, which is increased from the previously recommended 10 g/L of serum IgG. While the effectiveness of TPI against disease does depend upon the specificity of antibodies being passed to the calf (Kirkpatrick et al., 2019), it has been found that any increase in serum IgG levels lowers the risk of mortality in calves (Urie et al., 2018). Ensuring transfer of passive immunity is therefore of utmost importance to calf health and survival for the first weeks of life (Godden et al., 2019), and for production later on.

Factors influencing transfer of passive immunity are split between the dam (quantity, quality), the producer (administration), and the calf (absorption; McGuirk and Collins, 2004). While most dam and producer management factors can be controlled, factors that improve physiological absorption mechanics cannot yet be consistently manipulated. In 2000, it was estimated that 35% of calves had FTPI (Weaver et al., 2000); in 2014, that number was 32% (Windeyer et al., 2014) and in 2017 a study in British Columbia saw a range of 3% to 39% across 18 farms (Atkinson et al., 2017). In many cases, FTPI can be prevented by administration of a sufficient quantity of maternal antibodies through colostrum, suggesting that in a production setting, not all calves are receiving enough good quality colostrum. Nevertheless, even when all of the recommended management practices are followed, calves can still have FTPI. An in-depth understanding of the mechanisms behind passive transfer failure and the variation between calves requires further investigation, especially for protection of the vulnerable neonate.

1.2 Principles of Colostrum Feeding and Management

Our knowledge of transfer of passive immunity in calves is mostly based on studies that have used blood sampling to assess immunoglobulin status while manipulating colostrum quality, quantity, or feeding frequency in a variety of species (Erhard et al., 2001; Cabral et al., 2014; Desjardins-Morrissette et al., 2018), and tissue physiology studies were conducted over 40 years ago (Staley et al., 1972; Carlsson et al., 1980). With best colostrum management practices,

a sufficient amount of good quality colostrum can be obtained to feed to calves (Meganck et al., 2015). The core objectives of colostrum management are summarized as the 5 Q's– quality, quantity, quantification, quite clean, and quickness (Geiger, 2020).

Quality colostrum begins with collecting the colostrum from the dam. For collection, timing is equally as important as it is in feeding the calf (Moore et al., 2005; Morin et al., 2010); with every hour after calving that the dam is not milked, colostrum IgG concentration decreases by about 3.7% (Moore et al., 2005). Colostrum amount and quality, as defined by IgG concentration (Stott et al., 1979a) can vary from cow to cow – in general, the more colostrum a cow produces, the lower quality it will be due to the effect of dilution (Moore et al., 2005; Morin et al., 2010). It is important to collect quality colostrum to feed to calves, despite the variation seen between cows and collection times. One study found a range of 0.5 – 30.4 kg colostrum produced by dams, depending on the time they were first milked (Morin et al., 2010), with cows producing more colostrum having lower colostral IgG concentrations; this emphasizes the need for timely collection to ensure sufficient quality.

Quantity of colostrum produced by each dam is variable, but in this case, quantity refers to the quantity of IgG in the colostrum, not the quantity of colostrum itself. An average-sized calf (43 kg birthweight) should receive between 150 – 200g IgG in its first colostrum feeding (Godden et al., 2009), regardless of the volume of colostrum required. Just one change in colostrum management practices can positively increase the rate of adequate passive transfer in the calf by 10% (Atkinson et al., 2017), likely due to the sensitivity in timing when it comes to colostrum collection, storage, and administration to the calf.

Quite clean refers to colostrum storage conditions, and two important factors in cleanliness are the milking procedure and storage conditions (Stewart et al., 2005). Proper preparation of the udder prior to milking results in very little bacterial contamination when samples are taken directly from the udder (<100,000 CFU/mL), while dirty milking and feeding equipment can lead to much higher levels of contamination (36% of samples >100,000 CFU/mL; Stewart et al., 2005). Fresh colostrum should be stored at 4°C if being stored for more than 2 hrs, and at -20°C if being stored long-term (more than 7 days; Cummins et al., 2017). Cummins et al (2017) observed that while IgG content does not change in colostrum stored at 20°C over a course of 72 hrs, bacterial content increases rapidly as compared to colostrum stored at 4°C. Pasteurization is another method to prevent bacterial build-up, but it will also affect IgG

concentrations and cellular components of colostrum (McMartin et al., 2006). Freezing also destroys certain components of colostrum that are made of cells and thus, refrigerating colostrum for up to one week may be beneficial for FTPI rates (Chigerwe et al., 2012). Much less is known about the benefits of the components of colostrum that are made of cells than is known about the benefits of IgG concentrations (Gonzalez and Santos, 2017), especially considering the link between a calf being fed their own dams colostrum or that of another dam.

Quickly is how fast colostrum should be administered to the calf. Even if clean, good quality colostrum is collected, bovine calves have a relatively short window to absorb colostral components; according to literature, calves have until approximately 24 hrs of life to absorb colostrum and the window for efficient absorption is even shorter (up to 12 hrs of life; Stott et al., 1979c). Ideally, good quality colostrum should be administered to the calf within four hrs of life to take advantage of the period of maximal absorption (Stott et al., 1979b; Godden et al., 2019). If the calf must be fed later, more IgG can be fed to help override the negative effects of improper timing (Stott et al., 1979a; b; c), but feeding more IgG can also saturate a calf's absorption potential and lead to lower apparent efficiency of absorption (AEA; Besser et al., 1985). Still, early feeding is the best practice to ensure maximal IgG absorption from colostrum (Weaver et al., 2000; Godden et al., 2019).

Quantification of IgG is necessary for giving calves the best chance to obtain TPI. While colostrum administration is often measured in liters, the quantity of IgG within the colostrum meal is most important. Apparent efficiency of absorption refers to the amount of IgG out of what was fed that is passed into blood circulation. At a maximum, calves are able to uptake up to 60% of the IgG administered in colostrum into the blood, though the average is closer to 30% (Halleran et al., 2017a). The apparent efficiency of absorption must be taken into account when calculating how much IgG the calf should be fed – a common recommendation is 150 – 200g IgG in the colostrum feeding (Geiger, 2020), which would yield 90 – 120g IgG total transported into serum. The size of the calf should also be taken into account, as larger calves will require more IgG to obtain the same circulating levels as a smaller calf. Without quantification of the IgG in colostrum, usually via a colostrometer or a refractometer (Bartier et al., 2015), it is impossible to tell if the quantity of IgG is sufficient for the calf being fed.

Adequate TPI in calves begins with clean, quality colostrum administered quickly. After feeding, the rest of the work is up to the calf to complete the absorption process in the intestines.

The apparent efficiency of absorption is, on average, less than 50% and therefore colostrum quality is of great importance. Intestinal absorption of colostrum is a multifaceted process that we do not yet understand fully, nor are able to control.

1.3 Anatomy and Physiology of Transfer of Passive Immunity in the Small Intestine

To fully understand IgG absorption in the neonatal calf, it is important to understand the structure and function of the intestinal epithelium. The columnar intestinal epithelium is made up of a singular layer of cells and is constantly developing throughout the life of the organism, differentiating from stem cells (De Santa Barbara et al., 2003). The epithelium is made up of absorptive cells like enterocytes, mucous-producing cells like goblet cells, secretory cells like neuroendocrine cells and Paneth cells, and immune cells like macrophages (Figure 1.1). In the small intestine, protrusions called villi increase the surface area for absorption and invaginations called crypts contain intestinal stem cells that mature and differentiate as they move up the crypt. The intestine of the calf is an ideal environment for the cross-talk between nutrients like those in colostrum entering the system and the absorptive cells that make up the intestinal epithelium. Indeed, a mucous barrier secreted by goblet cells protects the epithelium from coming in direct contact with the entirety of the host of microbes and nutrients passing through the gut (Okumura and Takeda, 2017; Allaire et al., 2019); though some contact between colostral components and the epithelium is needed for initial stimulation of the neonatal intestine. Immunoglobulins interact with commensal and pathogenic microbes in the gut, and both signal homeostatic mechanisms from microbial interactions, and are modulated themselves due to microbial signaling (Hill and Artis, 2010). Thus, immunoglobulins provide immune support to the brush border membrane, but also provide immune coverage when transferred into circulation in lymph and serum.

The epithelial cell is polar, which has an effect on transport of molecules through the cells. Cell membrane components are arranged according to this polarity, and indeed their function depends upon the polarity of the cell (Handler, 1989). The basolateral membrane is often the site of hormonal and growth factor receptors, nutrient and ion transporters, and the point of attachment to the basement membrane (Handler, 1989). The apical side contains receptors such as the Fc receptor of the neonate (FcRn, involved in IgG transport), nutrient and

ion transporters, and mucous-secreting goblet cells. Transcytosis, which moves substances from one side of the cell to the other, is mediated by this polarity and regulates the movement of immunoglobulins across the membrane. IgG moves from the apical side to the basolateral side during transcytosis on its way to the bloodstream (Handler, 1989). Overall, the intestinal epithelium is a balance between defense and nutrition (Brandtzaeg, 2013), and the mechanisms involved in absorption are critical to that balance.

Macromolecule absorption in the calf into the tissue has two proposed mechanisms – one, occurring without the aid of a receptor (non-selective) or two, occurring with the aid of a receptor (receptor mediated; Jochims et al., 1994c). The mechanism of large amounts of nonselective IgG absorption has been demonstrated in ruminants and piglets but not in mice, rats, or humans (Mehrazar et al., 1993).

In the calf, reference to non-selective transport of molecules generally indicates IgG transport via pinocytosis. Pinocytosis, in which the cell performs endocytosis on molecules and fluid from the intestinal lumen, has been described in the calf intestine at birth (Kaup et al., 1996). However, pinocytosis, by definition, forms vesicles that are < 100 nm in diameter (Alberts et al., 2002), which is smaller than some of the vesicles that are observed in the small intestinal villi in the calf (Jochims et al., 1994c). Uptake of large molecules such as polyvinyl pyrrolidone, which is of similar molecular weight to IgG, appear to be dependent on the presence of vesicles in the intestinal epithelial cells, (Clarke and Hardy, 1970), though polyvinyl pyrrolidone is too large to be absorbed via traditional defined pinocytosis.

The second mechanism of macromolecule absorption is selective transport, which is mediated by the FcRn receptor in most mammals. The FcRn receptor functions as a salvage and transport system for IgG and for albumin (Challa et al., 2014), and has only recently been identified in the neonatal calf intestine (Zhu et al., 2020). It is unknown whether FcRn contributes to transcytosis of IgG like it does in other mammals (Rojas and Apodaca, 2002). It is also unknown whether the vesicles described in non-selective absorption/pinocytosis contain FcRn in calves – presence of FcRn in the vesicles may imply selective absorption of IgG in the bovine calf, despite decades old claims of non-selectivity. One study found that the vesicles in the neonatal epithelium contain clathrin (Jochims et al., 1994c), which is historically found in vesicles that form due to signaling from membrane-bound receptors such as FcRn. Though transporter-based absorption mechanisms are still being studied in bovine calves, we do not

know the extent to which FcRn is involved in IgG uptake and transcytosis, nor do we know how the cessation of that absorption functions.

Despite the lack of knowledge on the exact mechanism of uptake, the fact that the neonatal gut is open and able to absorb molecules non-selectivity emphasizes the importance of gut "closure"; the gastrointestinal tract, when mature, has millions of microbes that can be pathogenic or commensal (Turula and Wobus, 2018). The barriers, whether physical or chemical, that are present in the mature epithelium exist to protect the calf from unwanted invasion of pathogens or unwanted absorption of molecules. Secretory immunoglobulins, such as IgA and IgM, can modulate the commensal microbial populations and pathogenic microbes (Turula and Wobus, 2018), but the extent of this modulation in the neonate is unknown. If the gut is open to non-selective absorption there must be a balance between adequate absorption of IgG and other colostral components and timely closure so that pathogens are not free to invade the epithelium.

1.4 Gut Closure

Bovine calves have only the first day of life in which to absorb essential IgG from colostrum before "gut closure" occurs. Despite the limited timeframe, importance of colostrum feeding for calves, and depth of research on serum IgG levels, information regarding the mechanisms of gut closure timing are limited. Gut closure is defined as a "multifactorial event comprising the replacement of the fetal intestinal epithelial cells by more mature populations, the initial cessation of transport at the basal and lateral cell membrane of the absorptive enterocytes, and an increase in the intracellular proteolytic activity by lysosomes" (Jochims et al., 1994a). This is a detailed definition that takes into account the different components of gut closure, rather than just the cessation of macromolecule uptake.

Replacement of the fetal enterocytes with a mature population appears to occur gradually after birth, with nutritional stimulation, and with age. In piglets, the decline of large macromolecule absorption happens over the course of about the first three days of life, coinciding with the estimated time needed to completely replace the ileal epithelial cells (Clarke and Hardy, 1969). In calves, after colostrum feeding but before 24 hrs of life, filled vesicles are visible in the intestinal epithelial cells (Comline et al., 1951) but are not visible several days after colostrum administration or if colostrum is not administered, which is consistent with nutritional stimulation and timing of gut closure (Clarke and Hardy, 1969). The same timing to gut closure

occurs even in piglets that are removed by hysterectomy 3 days before their due date (Mehrazar et al., 1993); it appears that maternal factors are key to keeping the gut "open" and able to receive macromolecules. Once the maternal factors are removed, maturation progresses with age and gut closure will occur upon replacement of the fetal enterocytes. In calves, closure time is delayed up to 36 hrs if no colostrum is fed, but closure will happen regardless of nutritional input (Stott et al., 1979b; Pyo et al., 2018). Maturation of the neonatal intestine is stimulated by colostrum feeding, and as soon as the animal is born the count-down to gut closure begins.

It is unknown which components of colostrum stimulate the commencement of gut closure. In bovine calves fed colostrum with supplemental sodium butyrate, which is known to increase cell proliferation, FTPI occurred even though all calves were fed an adequate amount of IgG (Hiltz and Laarman, 2019). Factors that accelerate cell maturation and thus cessation of absorption are also present in the whey of the colostrum of the cow, sow, goat, and ewe (Hardy, 1969a) and must be absorbed via the gut to induce closure, though the specific factors have not been identified. Large molecules such as polyvinyl pyrrolidone are not absorbed if administered in water or salt water, indicating that there is something else in colostrum that stimulates the uptake of macromolecules (Hardy, 1969b). Nutritional stimulation by some component of colostrum influences the time to gut closure and stimulates maturation of the intestinal epithelium.

Cessation of immunoglobulin absorption has been measured in different ways. One strategy is to call the point of "closure" the time at which the immunoglobulin concentration in the blood is at the highest peak (Burton et al., 1989). The issue with this method is that most experiments do not sample blood as frequently as others, and this peak could be inaccurate by several hrs depending upon the frequency of sampling. The peak method defines gut closure at 24-36 hrs of life (Burton et al., 1989), and this definition is still widely assumed today (Meale et al., 2017).

A second strategy for measuring gut closure timing is by using a "join point" (Stott et al., 1979b), which takes the peak of IgG concentration in the blood and uses the intersection of the increasing and decreasing slopes of the peak to determine a closure time. The accuracy of the join point is again dependent on the frequency of blood sampling in the calf. The join point method has determined no difference in closure time for the three immunoglobulin classes (IgG, IgM, and IgA), which points to macromolecule absorption being non-selective in the calf (Stott

et al., 1979b). The join point method places gut closure in calves at a median time of 24 hrs of life.

The variability between methods is due to the frequency of sampling used, and also due to the uniqueness of each calf's absorption potential. The join point method and the peak method both depend upon the time of maximum circulating IgG to determine closure. Neither focus on the actual transepithelial flux of IgG, which may much improve characterization of the decline and ultimate cessation of absorption capacity.

1.5 Conclusion

Despite the available methods for determining gut closure, most studies that assess IgG uptake in dairy calves take a single serum sample after 24 hrs of life to quantify IgG concentration in blood (Tyler et al., 1999; Windeyer et al., 2014; Cummins et al., 2017). Using serum IgG concentrations taken at or after gut closure has allowed researchers to assess different qualities, timing, and administration methods for colostrum and their impacts on overall TPI (Godden, 2008; Windeyer et al., 2014; Desjardins-Morrissette et al., 2018).

There are, however, limitations to measuring serum IgG concentrations to analyze immunoglobulin uptake. First, immunoglobulin appearance in blood does not give insight into what is happening at a tissue level in terms of absorption – only immunoglobulins that have successfully transferred into the blood are measured. Second, IgG appearance in the blood at 24 hrs of life does not indicate what the rate of absorption is into or out of the tissue. Thirdly, serum IgG appearance does not further our understanding of the mechanisms involved in the uptake of IgG into the bloodstream. Future research should focus on signaling pathways, receptor mediated transport, and assessment of immunoglobulin uptake from the lumen to the tissue. The more we understand about gut closure, immunoglobulin uptake, and transfer of passive immunity, the closer we are to reducing rates of FTPI and having healthier calves.

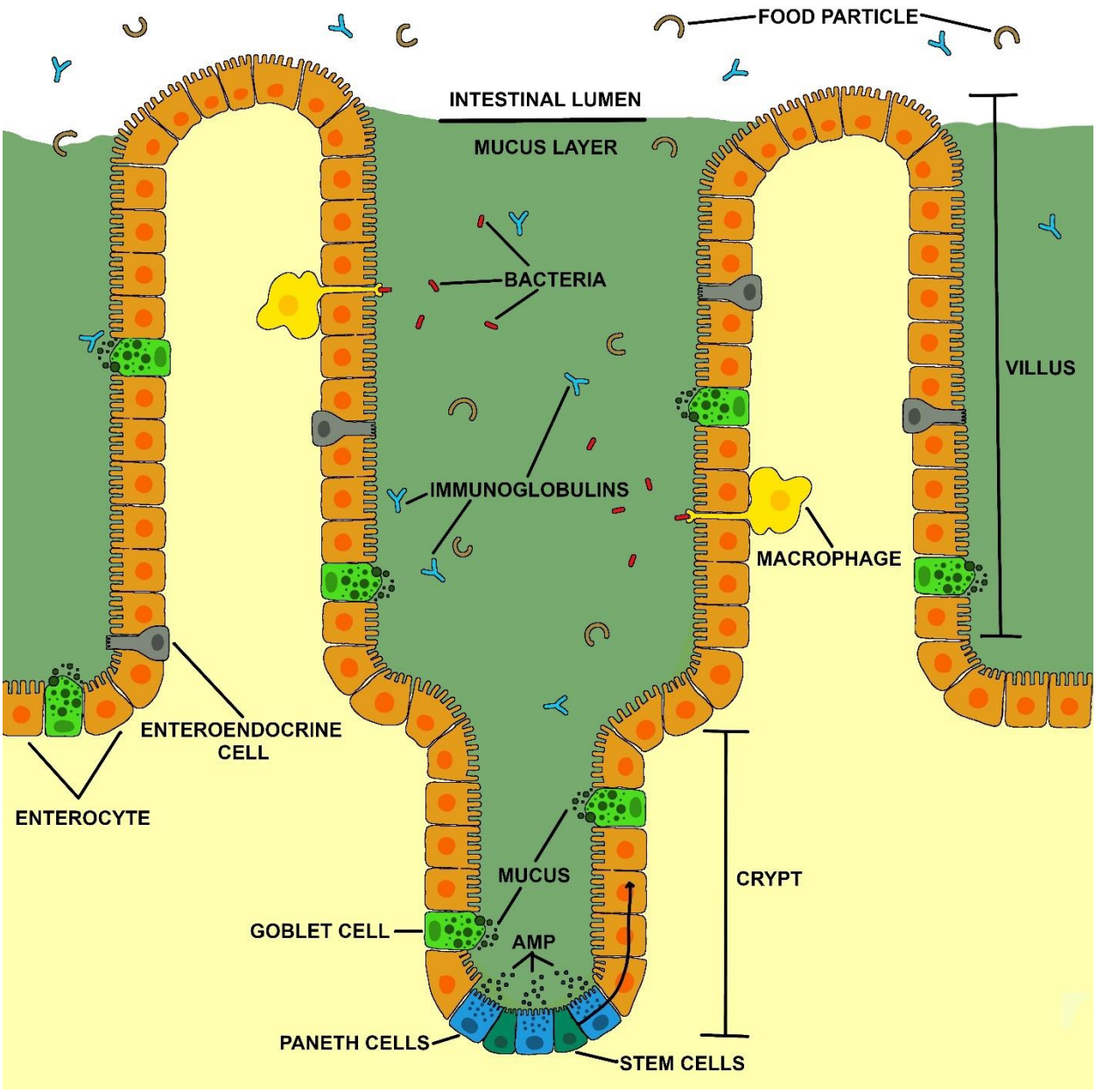


Figure 1.1 The small intestine and its constituents, including various cell types, the mucus layer, and villi and crypts.

2.0 “Shortened” Sandwich ELISA for Detection of Biotinylated IgG in Biological Samples

2.1 Abstract

Labeling proteins is a critical part of many experiments, but one limitation is that radioisotope labels cannot be used in typical in-vivo experiments in an animal production setting. Biotin, a small protein, is non-radioactive and therefore the chosen label for our in-vivo experiments in dairy calves. Biotinylation is routinely utilized to detect or purify proteins by taking advantage of the biotin-avidin bond; however, the 4'-hydroxyazobenzene-2-carboxylic acid (HABA) assay commonly used to quantify biotinylation of a target is not compatible with biological samples, as the complexity of biological samples interferes with the colorimetric properties of the assay. The objective of this study was to evaluate a sandwich enzyme-linked immunosorbent assay (ELISA) for detecting biotinylated IgG (BIgG) in biological samples. Biotinylated IgG standards and samples from Ussing chamber experiments were used to assess the specificity, detection range, and sensitivity of the modified assay. The assay has a linear detection range of 25,000 ng/mL – 500 ng/mL, with a sensitivity of 50 ng/mL. The specificity of the assay was assessed via a range of samples spiked with biotin, pure IgG, or biotinylated IgG. This sandwich ELISA method for detecting biotinylated proteins in biological samples has the potential for many different applications both within and outside the dairy industry.

2.2 Introduction

Immunoglobulin G (IgG) constitutes 65-95% of the immunoglobulins in bovine colostrum (Puppel et al., 2019), however little is known about the kinetics or transport of IgG at the intestinal level, particularly in the neonate. IgG detection and labeling has been identified as critical to understanding the mechanisms of intestinal transport of immunoglobulins in the neonate. Current methods to determine immunoglobulin transport pathways use radionuclide labels, however these are not highly sensitive to inform on cellular localization or transport mechanisms (Sasaki et al., 1977). In addition, radionuclide labels have the complication of safety concerns in a commercial farm setting for both staff and research animals. Biotin is a 244 Dalton molecule that can be used to label proteins and can offer more sensitivity without the cost associated with radioactivity required for in-vivo and ex-vivo experiments (Ginel et al., 1996). The proposed ELISA system offers an alternative labeling and detection method for biotin-labeled IgG and has the potential to be applied to many other protein models as well.

ELISAs are commonly used to detect analytes in low abundance. The biotin-avidin system has been extensively utilized in ELISAs as a method of detection (Tokiwa et al., 1990; Mohsin et al., 2023), as a marker in cell biology (Elia, 2008; Go et al., 2021), and in protein purification (De Boer et al., 2003; Raducanu et al., 2020). Due to its relatively small size biotin typically does not interfere with the biological activity and physical characteristics of the protein to which it is bound (Wilchek and Bayer, 1990). In addition, the biotin-avidin bond is particularly strong, with an affinity of up to a million times greater than an antibody-ligand interaction (Hantowich et al., 1987; Diamand and Christopoulos, 1991).

Most commonly in a sandwich ELISA system, the secondary “detection” antibody will be biotinylated (Figure 2.1A). Streptavidin-Horse Radish Peroxidase (HRP conjugated to streptavidin, a bacterial form of avidin with increased affinity for biotin conjugates), can be used to detect the amount of secondary antibody via a colorimetric reaction with the substrate tetramethylbenzidine (TMB). While this system takes advantage of the biotin-avidin bond, it does not allow for accurate quantification of biotinylated proteins within a sample because it binds to the protein or antigen regardless of biotinylation status.

Once the biotin-avidin bond is established, there are several methods utilized to quantify the degree of biotinylation (the average number of biotins that are bound to each protein molecule). In an aqueous protein solution, the dye 4’-hydroxyazobenzene-2-carboxylic acid

(HABA) is utilized to determine the ratio of biotin to protein (Delgadillo et al., 2019). The degree of absorbance change between HABA alone, and HABA combined with a biotinylated sample can be used to quantify the degree of biotinylation in the sample (Green, 1970). However, the HABA assay is subject to interference in complex protein samples like serum or chamber fluid from Ussing experiments, as these samples contain other proteins that shift the absorbance without being related to the biotin/HABA binding. Biotinylated proteins can be quantified via mass spectrometry (Schiapparelli et al., 2014) or with direct biotin detection via an ELISA, though these methods do not account for the degree of biotinylation in the sample.

We hypothesized use of a "shortened" version of a standard sandwich ELISA would be able to provide quantification of biotinylated antibodies in a mixture of unmodified IgG and biotinylated IgG in chamber fluid from Ussing chamber experiments. The ELISA system proposed in this technical note offers an alternative labeling and detection method for biotin-labeled IgG and has the potential to be applied to many other protein models as well. The shortened ELISA proposed here is more cost effective and higher throughput than mass spectrometry or a traditional biotin ELISA.

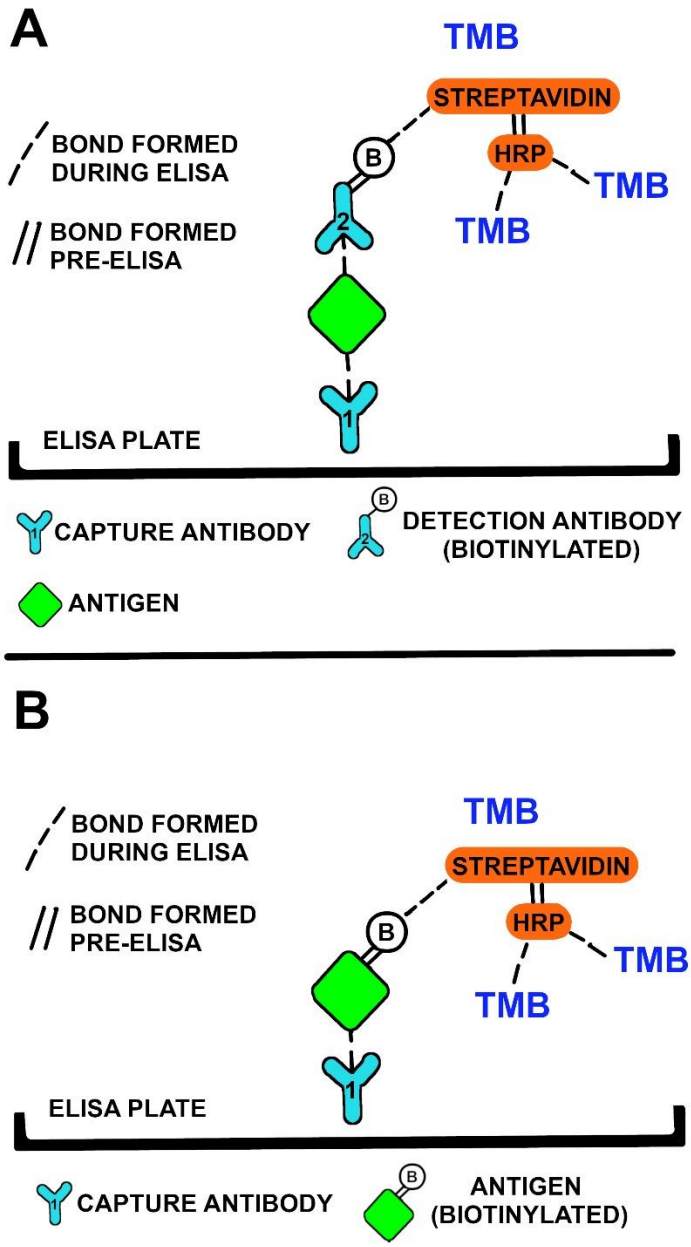


Figure 2.1 A) A diagram of a standard sandwich ELISA using the biotin-avidin bond to enhance the detectable signal of the assay. The antigen, in this case IgG, is "sandwiched" between a pair of antibodies. The antigen itself is not biotinylated. B) A diagram of the shortened sandwich ELISA system. The antigen is biotinylated IgG; it can be directly detected using streptavidin-Horseradish Peroxidase (HRP) and Tetramethylbenzidine (TMB).

2.3 Materials and Methods

The shortened ELISA protocol is based on the total IgG ELISA protocol (Mabtech, ELISAflex Bovine IgG HRP), but did not use a separate detection antibody (Figure 2.2). First, IgG was biotinylated using EZ-Link NHS-Biotin (Thermofisher, procedure performed according to manufacturer instructions). It was then passed through three successive rounds of dialysis (10K MWCO Snakeskin dialysis tubing, Krebs' buffer (Ungell et al., 1992)) to remove free biotin. The HABA assay was performed on dialyzed biotinylated IgG to determine the biotin:IgG ratio (Pierce Biotin Quantitation kit, Thermofisher). This ratio is required in order to convert the outcome of the shortened ELISA from biotin quantification to protein quantification (see equation below), as the shortened sandwich ELISA directly detects the amount of biotin, not the amount of IgG. After the ratio of biotin:IgG is determined, the biotinylated protein can be used for its intended application (feeding to a calf or adding to the mucosal side of an Ussing chamber, in our case). All samples used in the development of this assay were obtained via procedures that were approved by the Animal Care and Use Committee of the University of Alberta, Edmonton, AB (AUP #00003673).

$$\frac{mol\ biotin}{mol\ IgG} \times \frac{1\ mol\ IgG}{MW\ IgG\ (150{,}000\ g)} \times \frac{244.31\ g\ biotin}{1\ mol\ biotin} = \frac{g\ biotin}{g\ IgG}$$

Samples used in the development of this assay were chamber fluid from the mucosal and serosal sides of an Ussing chamber experiment. In the experiment, samples were set up to have IgG concentrations similar to that of bovine colostrum (130 mg/mL IgG), with 10 mg/mL being biotinylated IgG and 120 mg/mL being unmodified IgG. After samples were obtained, each sample was run on an ELISA designed to detect total IgG (Mabtech; ELISA Flex for bovine IgG, HRP conjugated). The results from this assay indicated the total amount of IgG (both biotinylated and unmodified) in each sample. As the capture antibody of our shortened sandwich ELISA binds IgG regardless of biotinylation status, there was competition for binding sites between biotinylated and unmodified IgG. To be able to account for the competition, all standards were produced to have the same ratio of BIgG:IgG as the samples (1:12).

Standards for the shortened sandwich ELISA were created by serially diluting a stock solution of biotinylated (10 mg/mL) and unmodified (120 mg/mL) IgG 1:1, creating a dilution range of 10 mg/mL to 0.15 mg/mL BIgG and 120 mg/mL to 1.8 mg/mL unmodified IgG.

Standards and samples were diluted in Kreb's buffer (Ungell et al., 1992), as that was the physiological buffer used in the Ussing chamber experiments.

For the shortened sandwich ELISA protocol, each well on a high-binding 96-well microtiter plate (Corning; product number 3367) was coated with 100 µL of a solution of unconjugated anti-bovine IgG antibody (1 µg/mL) diluted in phosphate buffered saline (capture antibody, Mabtech MT134; PBS, pH 7.4) and incubated overnight at 4°C. The next day the plate was washed five times with a solution of PBS + 0.05% Tween20. A wash consisted of filling each well in the plate with 300 µL of the PBS/Tween20 solution, letting it stand for 2 mins, and then inverting the plate and removing the wash buffer with a vigorous shaking motion. After five washes were completed, the plate was tapped upside down onto a paper towel to remove excess wash buffer. If any bubbles or liquid remained in the wells, they were aspirated with a pipette. Next, 100 µL of each sample, standard, or blank (Krebs buffer for Ussing samples) was added to the appropriate wells and incubated with a plastic sealing tape cover at room temperature for 2 hrs (tested temperature range of 22-30°C). After two hrs of incubation the plate was again washed five times with PBS + 0.05% Tween20 and tapped out on a paper towel as described above. Then, 100 µL of streptavidin-HRP (Thermofisher, product number S911; [150 ng/mL]) was added to each well and incubated with a plastic sealing tape cover at room temperature for 1 hour. Following this incubation, the plate was again washed five times with PBS + 0.05% Tween20 and tapped out on a paper towel as described above. Next, 100 µL/well of TMB substrate (Pierce TMB Substrate Kit, Thermofisher, product number 34021) was added to each well and incubated for five mins at room temperature. After five mins the reaction with TMB had reached its peak, and a stop solution was added to stop the reaction (100 µL/well 1N HCl). The plate was then read at 450 nm and 600 nm on a plate reader (Spectramax Plus 384; 450 nm chosen as absorbance spectrum for TMB substrate, 600 nm used as a blank according to Mabtech ELISAFlex Bovine IgG HRP).

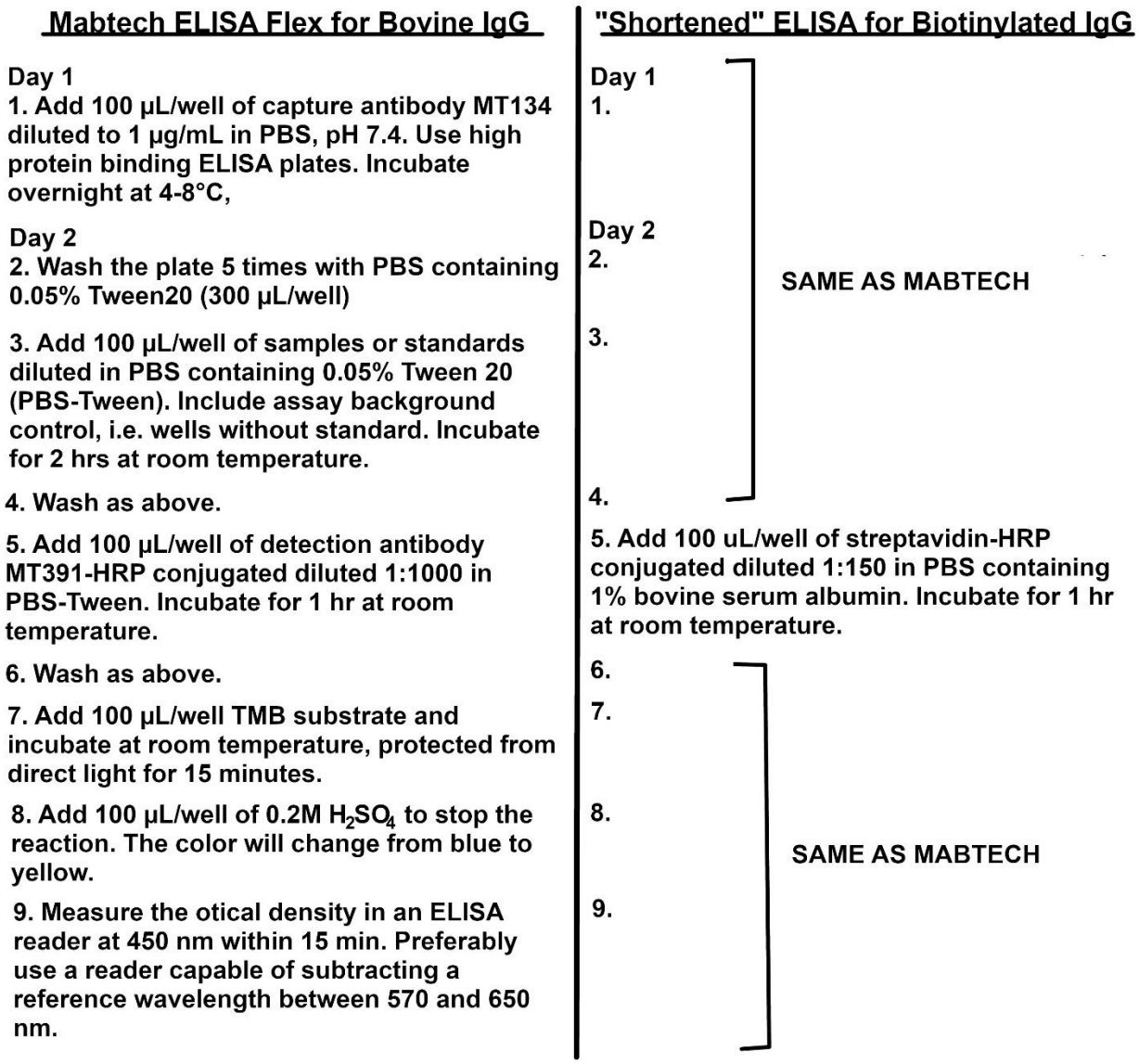


Figure 2.2 A diagram of the Mabtech ELISA Flex protocol and how the "shortened" ELISA protocol differs. Only step 5 is different between the two protocols.

2.4 Results

Using the biotin:IgG ratio determined by the HABA assay at the beginning of the procedure, results from the shortened sandwich ELISA (presented in ng/mL biotin) were used to calculate the concentration of biotinylated IgG of each sample. When the amount of biotinylated IgG was determined, it was subtracted from the total IgG value to quantify the unmodified IgG concentration.

The precision of the assay was evaluated, and within-plate CV values were less than 10%; between-plate CV values were less than 15%. Selectivity in this assay was a challenge as the capture antibody binds both pure IgG and biotinylated IgG, creating competition. However, by keeping the BIgG:IgG ratio of the standards the same as the samples, the competition is predictable and fits a 4PL curve (Figure 2.3). Sensitivity of the assay is 50 ng/mL biotin, with the lower range of detection at 500 ng/mL biotin. Linearity of dilution was achieved by diluting samples with high BIgG concentration through the range of the assay.

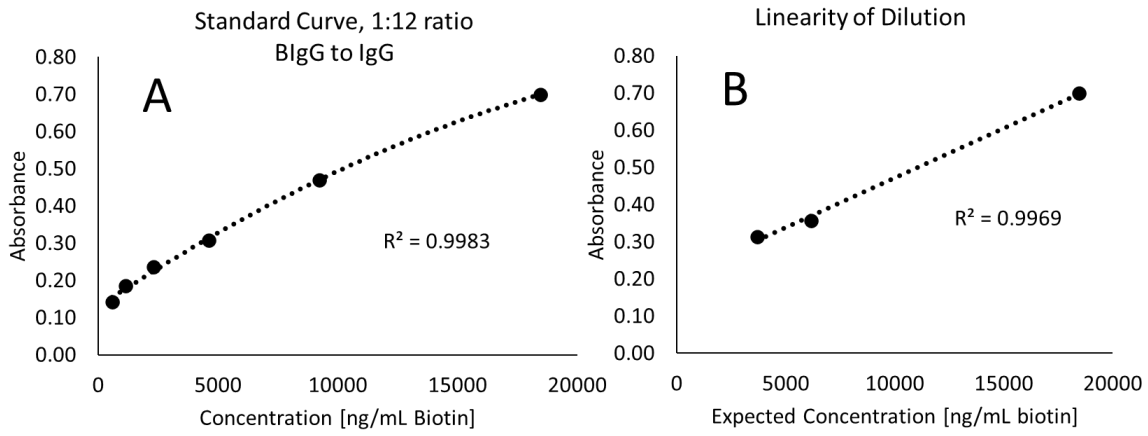


Figure 2.3 A) Standard curve with a 1:12 ratio of biotinylated IgG to IgG. Trendline is a 4PL curve with an R^2 value of 0.9983. B) Linearity of dilution of high BIgG sample in 1:12 ratio, with an R^2 of 0.9969. Each data point represents 4 biological replicates.

In conclusion, this shortened ELISA method provides an effective way to quantify the amount of biotinylated protein in a biological sample which may include multiple components and other complex proteins that interfere with other quantification methods. The use of this ELISA method to measure biotinylated IgG and differentiating it from pure IgG can be applied to quantifying proteins in complex biological samples in the dairy nutrition field including colostrum and milk, and in other fields to determine intestinal tissue transport of IgG.

3.0 Influence of Feeding Status on IgG Transport Kinetics and Histology in the Neonatal Bovine Small Intestine

3.1 Abstract

While colostrum is of utmost importance to the neonatal calf, it is unclear what effects nutritional stimulation via colostrum has on small intestinal transport of IgG. This is important to understand because neonatal ungulates require absorption of colostral IgG to obtain transfer of passive immunity, and current knowledge states that the neonatal ungulate only has a 12-24 hr period for absorption of ingested immunoglobulins. The study aim was to examine the effects of the feeding of colostrum on IgG transport kinetics and histological morphology in the small intestine in neonatal calves. Holstein × Angus bull calves (n = 18) were assigned to one of three treatment groups in a randomized complete block design: two groups not fed colostrum and euthanized at 1 or 24 hrs of life (1NC and 24NC), and one group fed colostrum within an hour of life and euthanized at 24 hrshrs of life (24C). These treatment groups allowed for us to examine the effect of age (1NC vs. 24NC) without colostrum independent of the effect of feeding colostrum in the neonate (24C vs. 24NC). At euthanasia, intestinal tissue samples were collected for IgG kinetics and histology, and blood and digesta samples were collected to examine IgG concentration and digesta pH. A Periodic Acid-Schiff's (PAS) stain was utilized to investigate intracellular vesicle quantity and size, while FITC-dextran (4 KDa) was used to determine tissue permeability in Ussing chambers and paracellular transport. Mucosal IgG % influx, defined as the % of total loaded IgG that was absorbed into the tissue, did not differ between treatments, indicating that IgG uptake did not change with feeding of colostrum or age within the first day of life, which is surprising considering the central dogma among livestock researchers that IgG uptake declines quickly after birth in the calf. Mucosal to serosal FITC-dextran efflux was greater in the proximal intestine while IgG efflux was greater in the distal intestine for 1NC calves, indicating selective intestinal uptake sites for small and large molecules in the neonate. PAS-positive vesicles increased in quantity in response to colostrum feeding and the distal jejunum had the greatest number of PAS-positive vesicles out of all intestinal segments. Overall, the small intestine of the neonate has the capacity to absorb IgG whether the calf has been fed colostrum or not and regardless of age at 1 hr or 24 hr. However, efflux of IgG in the distal

jejunum and ileum increases with feeding status and age, and associated PAS-positive vesicle count also increases with age in the first day of life.

3.2 Introduction

The ingestion and absorption of colostral immunoglobulins is essential for passive immunity and protecting the newborn dairy calf from disease (Weaver et al., 2000; Windeyer et al., 2014; Godden et al., 2019). The amount (Stott et al., 1979a; Jaster, 2005), timing (Stott et al., 1979b; Morin et al., 1997; McGuirk and Collins, 2004), and administration method (Laestander, 2016) of colostrum on serum IgG levels are well established. Historically, the principal measure of immunoglobulin absorption is serum IgG concentration, as measured at 24 – 48 hrs of life in the neonatal calf (Fidler et al., 2011; Godden et al., 2019). A calf is considered to have failure of transfer of passive immunity (FTPI) if serum IgG concentration is <1000 mg/dL, and recent re-categorization have labeled <1000mg/dL as "poor", 1000 to 17.9 mg/dL as "fair", 1800 to 24.9 mg/dL as "good", and >2500 mg/dL as "excellent". (Lombard et al., 2020). Best practices for colostrum feeding do exist – bovine colostrum with at least 50 g/dL IgG should be fed to a calf within 4 hrs of life, equaling 150 to 200 g total IgG fed via a nipple bottle or esophageal feeder (Godden et al., 2019; Lombard et al., 2020). The issue at hand is that even when best practices are followed, the apparent efficiency of absorption, a measure of the percentage of IgG that is absorbed into the blood from colostrum, remains below 40% in most calves (Halleran et al., 2017b); this is considered "low" in the calf industry, where the measure of a successful colostrum feeding program focuses on serum IgG concentrations. (Windeyer et al., 2014; Godden et al., 2019). To our knowledge, the dairy industry has never considered tissue or lymph IgG concentrations to be a measure of passive transfer, even though it is likely that most of the "missing" IgG remains in the intestinal tissue and lymphatic circulation. Dairy producers need quick, easy, measurable ways to assess calf passive transfer in an on-farm setting, which is why serum IgG concentrations have been the predominant method. Despite this, in order to improve colostrum feeding practices there needs to be further understanding of the apparent efficiency of absorption and the physiology of immunoglobulins in the neonate calf, mode of antibody uptake, and epithelial closure to transport of antibodies and immunoglobulins, even if some of these parameters cannot be measured by a producer on-farm. At the present time, the role of these factors in IgG absorption remains unclear.

IgG intestinalkinetics have not been assessed in neonatal calves. Jones and Waldmann (1972) investigated IgG uptake in the intestine in 12-14d old neonatal rats using intraduodenally administered radioisotopes to assess protein transport into circulation; Jochims et al. (1994a)

investigated colostral IgG uptake via immunoelectron imaging in newborn calves and described large vesicles in the ileum enterocytes, but these data do not tell us anything about the kinetics of IgG transport. Jochims et al. surmised that the absorption of IgG is likely non-selective via pinocytotic vesicles at the apical luminal membrane of the enterocyte, and this was supported by a lack of evidence for paracellular transport using gold-labeled IgG. In addition, Jochims et al. did state that from their data it was unclear whether a receptor-mediated transport pathway was used alongside or as part of the pinocytotic vesicle transport mechanism. Vesicular transport in the gut is associated with lymphatic permeability (Dobbins and Rollins, 1970), and indeed the permeability of the neonatal gut is affected by colostrum consumption within 2 hrs of life (Araujo et al., 2015), suggesting that intestinal permeability and potentially vesicular transport in the neonate are altered by feeding status.

Currently, the cessation of IgG uptake and efflux from the enterocyte or "gut closure", lacks a defined physiological mechanism, and lacks a clear definition but is thought to occur at approximately 24 hrs of life (Stott et al., 1979a). Stott et al. (1979b) defined gut closure as the point at which "cells in the intestine have matured to a point where macromolecules can no longer transfer into the blood"- an arguably vague definition. Meale et al. (2017) defined gut closure as the point at which "large macromolecules can no longer be absorbed by the gut". It has also been described that gut closure, related to immunoglobulin transport is the point at which serum IgG peaks (Stott et al., 1979c; Burton et al., 1989), These definitions do not inform on the physiological mechanisms of gut closure or reduced uptake of IgG in the enterocyte. In the bovine, where transfer of passive immunity does not occur in utero, understanding the mechanisms involved in transporting IgG from the lumen of the intestine into systemic circulation is important as it is indicative of calf resistance to disease and calf survival (Windeyer et al., 2014). This thesis chapter will examine the interaction between age of the calf and colostrum feeding status with regards to uptake and transport of IgG.

FITC-dextran (4 KDa) is used as a marker to indicate paracellular transport of molecules such as water and small solutes (less than 7KDa in diameter, Yun et al., 2013) rather than large macromolecules such as IgG (150 KDa). We used FITC-dextran as a quality control measure in the Ussing chamber because it has been observed to not cross the intestinal epithelium in large quantities unless the epithelium has been damaged (Hubbard et al., 2014; Bzik and Brayden, 2016; Liu et al., 2021). The use of FITC-dextran as a permeability maker is a tool that allows for

examination of both transcellular and paracellular transport in the neonate (Cereijido et al., 1993; Liu et al., 2021). To date, the role of paracellular transport in the neonatal bovine intestine is unknown.

Timing of the acidification of the abomasum is unknown in neonatal calves and may be an important factor in the timing of feeding of the first or second colostrum meal. If a calf is fed and then the abomasal pH decreases, additional feedings of colostrum could be degraded and digestedbefore reaching the small intestine. This could explain why the quality and quantity of the first feeding are so important in the calf (McGrath et al., 2016). Abomasal pH has been measured 2 hrs post colostrum feeding (average pH 5.5, only four calves measured, Miyazaki et al., 2017), and the pH of amniotic fluid has been measured (pH 7.6 to 8.1, Javed and Wright, 1990) but to our knowledge, stomach and intestinal digesta pH has not been measured in 1 hr old non-fed calves nor at 24 hrs hrs of life.

The objectives of this study were to i) examine the effect of age (1NC vs 24NC) and colostrum feeding status (24NC vs 24C) on intestinal IgG transport and subsequent serum levels of IgG in four defined segments in the small intestine, ii) examine the mechanism of IgG uptake (selective vs. non-selective) and transport in neonatal calves, and iii) to measure the timing of abomasal acidification over the first 24 hrs of life. We hypothesized that colostrum feeding and age would modify intestinal IgG influx and efflux, and IgG serum concentrations, and that IgG would be transported across the intestinal tissue via vesicle transport. In addition, we hypothesized that abomasal pH would decrease with age in the first 24 hrs of life, potentially affecting the absorption of some components of colostrum.

3.3 Materials and Methods

3.3.1 Calves and Study Design

Between October 2021 and March 2023, 18 Holstein × Angus bull calves were born on a single commercial farm. At birth, calves were weighed and assigned to one of three treatment groups (n=6); 1. not fed colostrum and euthanized at 1 hr of life (1NC), 2. not fed colostrum and euthanized at 24 hrs of life (24NC), and 3. fed colostrum within one hr of life (45.2 ± 2.6 min.) and euthanized at 24 hrs of life (24C). The three treatment groups allowed for two comparisons – firstly, in non-fed neonates to observe the effect of age (1NC vs. 24NC), and secondly in colostrum fed or non-fed neonates at 24 hrs of life to observe the effect of feeding (24C vs.

24NC). Animals were matched for birth weight for each group. All calves were allowed to be cleaned by their dam but were removed from the dam before the calf could stand and suckle. After removal, calves were weighed and assigned to a treatment group, and then placed in an individual hutch bedded with straw until the time of euthanasia. All calves were euthanized via captive bolt and exsanguination. Blood samples were collected at exsanguination into blood collection tubes containing a clot activator (BD Vacutainer, silicone-coated interior, Fisher Scientific). All animal procedures were approved by the University of Alberta (AUP #3673).

3.3.2 Colostrum Treatment

A commercial colostrum replacer containing 135 g IgG in 1.3 L of warm water (104g IgG/L) was provided via an esophogeal feeding tube (Premolac, Zinpro Inc.). The colostrum replacer in question only contains the whey fraction of colostrum and is devoid of fat and casein, which would naturally be removed during the clotting process in the abomasum of the calf (Hocquette and Bauchart, 1999).

3.3.3 Intestinal Dissection and Digesta pH

After euthanasia, digesta (stomach or intestinal lumen fluids) were collected from the abomasum, duodenum, proximal jejunum, distal jejunum, ileum, and cecum. Intestinal tissue samples from the same segments were rinsed with phosphate buffered saline (PBS; Fisher Scientific) and placed in ice-cold Krebs buffer for intestinal permeability using Ussing methods; additional small intestinal tissue samples were washed with PBS and then preserved in 10% buffered formalin, or snap frozen and stored at -80°C until later histological and biochemical analysis. Digesta pH was measured from the abomasum, each section of the intestine, and the cecum to assess abomasal acidification and its effects on the downstream intestinal digesta pH.

3.3.4 Intestinal and Vesicle Histology

Periodic Acid-Schiff's staining method was used to identify glycoproteins in intestinal segments (Pluta et al., 2011). After 24 hrs in 10% buffered formalin, tissue samples were transferred to a 70% ethanol solution until they were processed, encased in paraffin, and 5µm thick sections were mounted on slides (Al-Sabawy et al., 2021). The slides were then stained with Periodic Acid-Schiff's stain, as follows - slides were first de-waxed in two rounds of toluene and then rehydrated with a progression of ethanol dilutions (100%, 90%, 70%, 50%, and

tap water). After rehydration, slides were added to a solution of Periodic Acid (Fisher Sci, reconstituted in nitric acid) for 5 mins, placed in tap water for 1 min, and then added to fresh Schiff's reagent (Thermofisher) for 10 mins. After the Schiff's reagent, slides were rinsed with running tap water for 5 mins, at which point the slides were counterstained with Hematoxylin Gill III (Leica) for 50 seconds and then were rinsed with running tap water for 12 mins. Finally, coverslips were applied with DPX mountant and cured overnight in a 37° incubator. PAS staining revealed the presence of PAS-positive vesicles (identified by their bright pink color) in the intestinal tissue, and one image was taken of three separate villi per slide, and vesicles were counted and measured. PAS-positive vesicles were distinct from other features and cell types in the small intestine, as similarly shaped structures such as goblet cells or nuclei dyed a dark purple to blue color (Figure 3.2). Counting was performed using an assistive software for manually counting objects in an image (DotDotGoose, American Museum Of Natural History). Briefly, DotDotGoose displayed a 200 x 200 pixel grid over the selected image. A 3 x 3 section of the grid was selected and the PAS positive or PAS negative vesicles inside were counted. As each vesicle was clicked on, the software recorded the number of clicks, and added a dot so that vesicles were not counted twice (Figure 3.1).

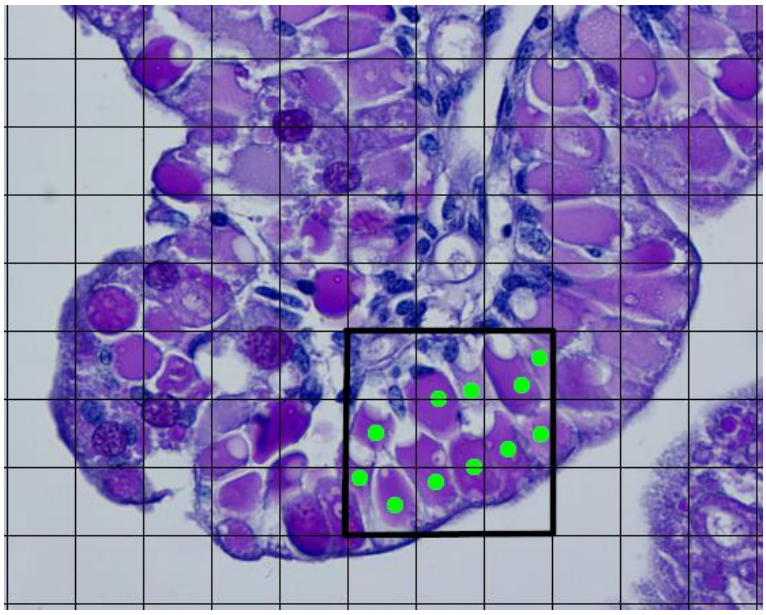


Figure 3.1 Vesicle counting in the jejunal villus. A PAS-stained image, using DotDotGoose 3x3 grid to count vesicles/20 μm^2 in each villus. The program prevented double-counting of the same vesicle, and provided a consistent grid size in which to count the vesicles.

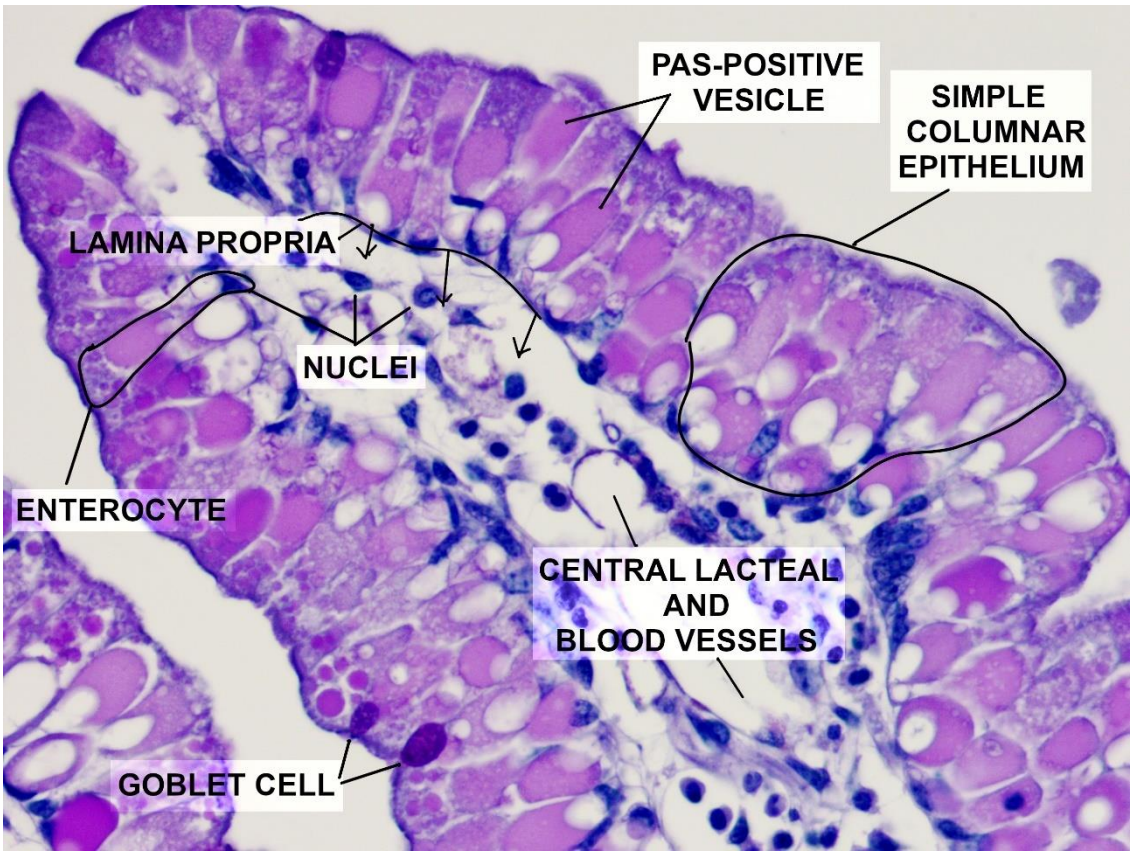


Figure 3.2 A PAS-stained jejunal villus indicating the staining pattern for PAS-positive vesicles (bright pink), goblet cells (dark purple), and nuclei (blue), and identifying structural landmarks of the villus.

3.3.5 Bioavailability of IgG

Following euthanasia, whole blood samples were allowed to clot at room temperature before being centrifuged at 2000 × g for 20 mins at 4°C, after which the serum was removed, aliquoted, and frozen at -80°C. For analysis, samples were plated on a radial immunodiffusion plate with antigens against bovine IgG (RID; Triple J Farms Bovine IgG RID Plates) according to manufacturer's instructions. Plates were incubated for 18 hrs at 22°C before being read with a jeweler's loupe capable of measuring 1/10 of a millimeter. Apparent efficiency of absorption (AEA), a measure of the percentage of IgG that is absorbed and transported to the systemic circulation from the colostrum-fed neonatal calf, was calculated according to (Halleran et al., 2017a). The equation for calculating AEA is as follows:

$$AEA\ \% = \left\{\frac{serum\ IgG\ concentration\ \left(\frac{g}{L}\right) \times\ body\ weight\ (kg) \times [0.7(estimated\ \%\ blood\ volume)]}{colostral\ IgG\ concentration\ \left(\frac{g}{L}\right) \times volume\ colostrum\ administered\ (L)}\right\} \times 100$$

3.3.6 Intestinal Permeability Experiment

Intestinal samples were mounted in Ussing chambers (NaviCyte System, Harvard Apparatus Inc.), approximately 2.5 hrs after euthanasia. Sections of each intestinal tissue type (duodenum, proximal jejunum, distal jejunum, ileum) were mounted chambers with three replicates per tissue type. As neonatal tissues are very delicate and thin, outer serosal and tunica muscularis layers were not peeled as to not damage the epithelium, lamina propria and serosal layers before mounting. Tissues were equilibrated for 20 mins at 37°C before initial samples were taken. Initial samples represent the beginning of the Ussing chamber experiment and thus act as a baseline measurement. The mucosal side of the chamber was filled with carbogenated Krebs' Buffer supplemented with 130 mg/mL (780 mg total) bovine IgG and 1 mg/mL (6 mg total) FITC-dextran (4 KDa; used as a permeability marker of paracellular transport), at a pH of 7.4; the serosal side of the chamber was filled with plainKrebs' buffer at a pH of 7.4 that was gassed with carbogen (95% O_2 and 5% CO_2). Osmolality was equal for both the mucosal and serosal buffers. Samples (600uL) were taken from the donor mucosal chamber at 0, 2 and 4 hrs, and from the receival serosal chamber at 0, 1, 2, 3 and 4 hrs. After a sample was taken, it was replaced with the IgG/FITC-dextran-supplemented Krebs buffer (mucosal chamber) or non-supplemented Krebs buffer (serosal chamber). Mucosal samples were used to assess appropriate loading of IgG and FITC-dextran. Serosal samples were used to assess IgG intestinal transport flux (efflux defined as mucosal to serosal flux) from the mucosal to serosal chamber.

3.3.6.1 Ussing Chamber Fluid Sample Correction for Serial Dilution

During the 4hr Ussing experiment, 600 µL of sample was removed for analysis and 600 µL of buffer was replaced in the chamber at each sampling timepoint. This resulted in a serial dilution of samples over the course of the experiment. The mucosal replacement buffer contained 130 mg/mL IgG and 1 mg/mL FITC-dextran in Krebs buffer. The serosal replacement buffer was plain Krebs buffer. To correct for the serial dilution, the following equations were used:
Mucosal Correction:

$$mucosal\ coefficient = \frac{(read\ sample\ concentration)(chamber\ volume - sample\ volume) + (return\ buffer\ concentration)(volume\ to\ return)}{\frac{chamber\ volume}{read\ sample\ concentration}}$$

$$corrected\ mucosal\ concentration = t_n \times mucosal\ coefficient\ of\ t_n$$

Serosal Correction:

$$serosal\ coefficient = \frac{chamber\ volume - sample\ volume}{chamber\ volume}$$

$$corrected\ serosal\ concentration = \frac{t_n}{serosal\ coefficient^n}$$

Where t_n = sample concentration reading at timepoint n. The mucosal coefficient accounts for the addition of 600 µL of 130 mg/mL IgG and 1 mg/mL FITC-dextran to the mucosal chamber each time a sample is taken, which is why the corrected concentration is multiplied by the coefficient. The serosal coefficient accounts for the addition of 600 µL of plain Krebs buffer which dilutes the serosal chamber, which is why the corrected serosal concentration is divided by the coefficient.

3.3.6.2 FITC-Dextran Flux as a Permeability and Paracellular Transport Marker

FITC-dextran serosal efflux values were used to assess tissue integrity and to measure paracellular transport across the neonatal calf intestine. To measure FITC-dextran flux, mucosal and serosal samples were read at an excitation of 485 nm and an emission of 520 nm on a plate reader (BioTek Synergy H1, Aligent). The concentration of FITC-dextran was determined using a standard curve prepared at the same time as the mucosal buffer to minimize differences from FITC degradation. In addition, samples were read immediately following the Ussing experiment to minimize FITC degradation. For FITC-dextran mucosal to serosal flux, serosal concentration thresholds were set based on natural break points in the data, where unexpectedly high concentrations of FITC-dextran identified damaged tissues (Figure 3.3, Aguanno et al., 2021). These thresholds were used to determine which tissues were viable during the experiment and which had potential degradation or other permeability issues.

We acknowledge that there are limitations to eliminating samples in this way, but in this experiment voltage measurements and histological samples were not taken throughout the Ussing experiment, and thus FITC-dextran efflux is the best quality control measurement of integrity for these intestinal tissue samples. Integrity of the tissue was considered to be low when the initial serosal concentration of FITC-dextran was above 0.005 mg/mL. The break-point of

0.005 mg/mL was chosen based on analysis of the entire sample set – there was a natural break-point in the data between 0.005 mg/mL and 0.01 mg/mL FITC-dextran for initial (0hr) serosal efflux samples. In addition, integrity of the tissue was considered to be low when the difference between sequential samplings (ex. corrected concentration of FITC-dextran at hour 2 – corrected concentration of FITC-dextran at hour 1) was above 0.005 mg/mL. The break-point of 0.005 mg/mL was again chosen based on analysis of the entire sample set – there was a natural break-point in the data between 0.005 mg/mL and 0.01 mg/mL FITC-dextran for the difference between sequential samples (ex. 2 hr – 1 hr). The difference between sequential samples indicated that, for example, FITC-dextran concentration spiked unexpectedly high between hrs 1 and 2, and thus the tissue at hr 2, 3, and 4 was degraded or otherwise compromised. Samples were eliminated from the overall dataset (for both FITC-dextran and IgG data points) based on the FITC-dextran thresholding, resulting in the following n per treatments (Table 3.1). FITC-dextran efflux data was unavailable for 5 calves due to a plate reader malfunction; those samples were removed from IgG and FITC-dextran flux analysis due to inability to assess the tissue integrity.

Table 3.1 Number of samples removed from analysis due to FITC-dextran thresholding or due to lack of FITC-dextran data, by treatment group.

Treatment Group	n samples removed due to FITC thresholding	n samples removed due to unavailable FITC-dextran data	Total n removed	Total n used in analysis
1NC	81	0	81	279
24NC	11	96	107	253
24	55	48	103	257

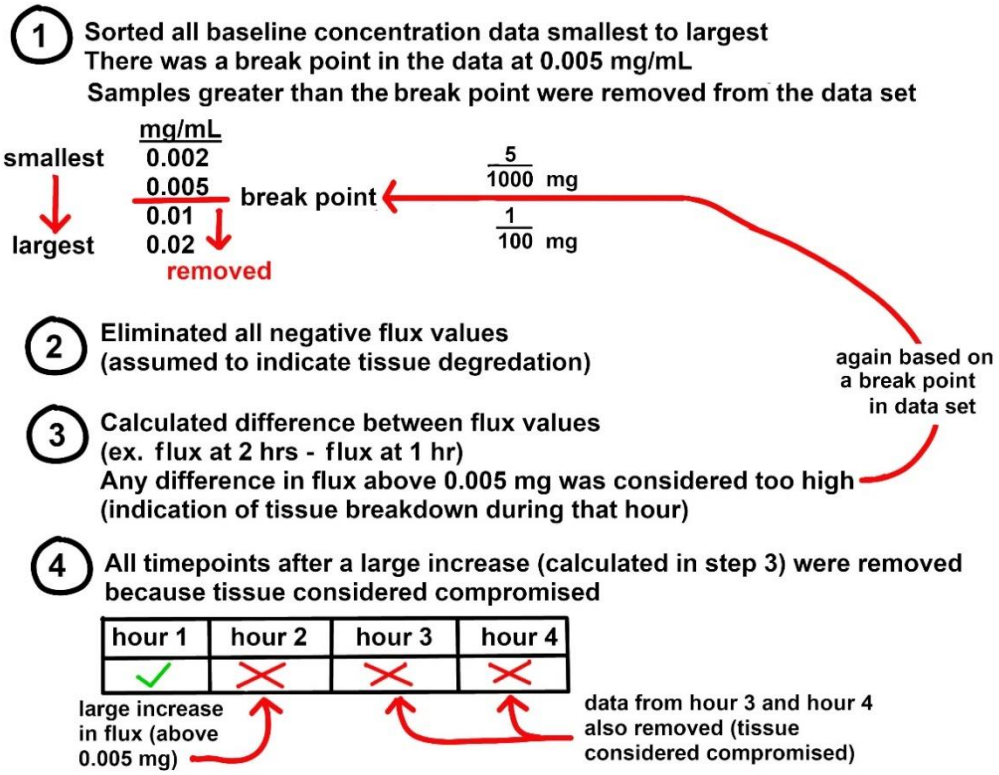


Figure 3.3 A diagram of how FITC-dextran samples were eliminated from the data set. 1) Baseline concentrations were used to determine which tissues were compromised from the beginning of the experiment. Break points in the data were used to determine thresholds for intact vs. compromised tissue 2) negative flux values were also removed, mostly from data in the last hour of the experiment 3) a large increase in flux (as determined by breakpoints) indicated tissue breakdown 4) all timepoints after the tissue was compromised were removed from the dataset.

3.3.6.3 IgG Efflux in Neonatal Intestinal Segments

IgG efflux from intestinal tissue segments in Ussing experiments allowed us to assess IgG permeability with and without nutritional stimulation in the neonatal calf. Previously, serum IgG has been used to measure timing of gut closure, defined as the point at which serum IgG peaks (Stott et al., 1979b), but IgG absorption has not been investigated at the intestinal tissue level. IgG concentrations in mucosal and serosal samples were measured using a commercial ELISA kit for bovine IgG according to manufacturer's instructions (Mabtech Bovine IgG ELISAflex, HRP conjugated), with in-house standards of $1x10^{-1}$ mg/mL, $1x10^{-2}$ mg/mL, $1x10^{-3}$

mg/mL, $1x10^{-4}$mg/mL, and $1x10^{-5}$ mg/mL IgG. Briefly, a standard sandwich ELISA was run using a matched antibody pair specific to bovine IgG. The quantity of detection antibody, conjugated to horseradish peroxidase, was detectable using the substrate tetramethylbenzidine (TMB) when samples were read on a plate reader at 450 nm.

3.4 Statistical Analysis

Using six animals per treatment allowed us to detect, with 80% power, a difference of 20% between treatment means for all variables when the coefficient of variation within treatments is 10% (Berndtson, 1991). Data were analyzed using a one-way ANOVA to examine difference between treatment groups (digesta pH, serum IgG) or a two way ANOVA to examine the difference between treatment groups and the difference between intestinal segments, as well as the interaction between treatment × intestinal segment (PAS stained and PAS-negative vesicle count, villi height, crypt depth, villi height:crypt depth ratio). Tukey post-hoc tests were used to determine, when there was a significant effect in the ANOVA, the difference between treatment groups or intestinal segments. Data were analyzed using R (RStudio 2023.03.1+446 "Cherry Blossom").

FITC-dextran and IgG in permeability experiments were analyzed by repeated measures ANOVA over the course of the 4hr experiment to account for the same chamber being sampled at multiple timepoints. In the repeated measures analysis there were no three-way interactions of treatment x intestinal tissue segment x time. Tukey post-hoc tests were run to analyze the difference between treatments, intestinal tissue segments, or time individually. Outliers were removed from analysis by using a 1.5 interquartile range test (25^{th} – 75^{th} percentile).

Data were analyzed according to the following model:

$$Y = \mu + T_i + \varepsilon_{ij}$$

where Y is the response, μ is the overall mean, T_i is the treatment effect, and ε_{ij} is the residual error. Significance was declared at $P < 0.05$ and tendencies were declared at $0.05 < P \leq 0.10$. Numbers displayed are least squared means ± standard error of the mean, unless otherwise indicated as is standard in agricultural research.

3.5 Results

3.5.1 Digesta pH

The pH of liquid stomach contents and intestinal lumen contents (digesta) was measured to assess the effect of colostrum feeding on acidification of the abomasum, and the effect of abomasal acidification on the pH of the digesta in other intestinal segments. Though immunoglobulins are relatively acid-stable (Ejima et al., 2007), the pH of the abomasum could affect the ability of other colostral components to pass through the stomach intact; abomasal pH in an adult cow is about 2, while protein can denature between pH 2 and pH 5. Digesta pH was not different among treatments for the stomach or intestinal segments ($P = 0.16$). There was no significant effect of age (1NC vs. 24NC) or colostrum feeding status (24NC vs. 24C) in the abomasum, but calves in the 24C group had the lowest pH (2.7± 0.85) and calves in the 1NC and 24NC groups had a 1.5 to 2-fold higher pH (pH 4.19 ± 0.66 and 5.28 ± 0.85, respectively, Figure 3.4).

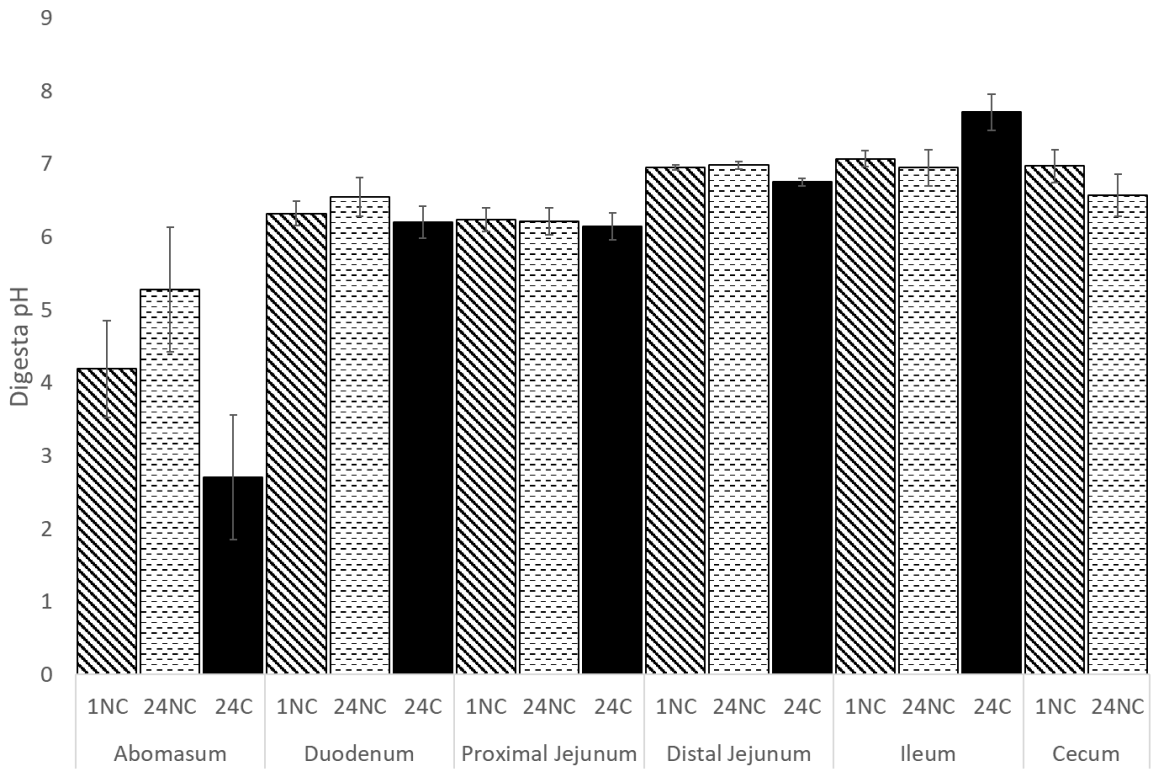


Figure 3.4 The digesta pH in intestinal segments in non-fed neonatal calves at 1 hr and 24 hr, and colostrum treated calves at 24 hr. Data are presented as mean ±SEM, $P = 0.16$, n = 6/treatment. Most tissues (duodenum, proximal jejunum, distal jejunum, ileum, cecum) had

consistent pH regardless of feeding status. There was no significant effect of age (1NC vs. 24NC) or feeding status (24NC vs. 24C) on abomasal pH.

3.5.2 Bioavailability and Apparent Efficiency of Absorption of IgG

Serum IgG was used to determine the bioavailability of IgG from colostrum as shown in Figure 3.5 (Hernandez et al., 2016). Levels of serum IgG were 1207-fold higher in 24C colostrum fed calves as expected, compared to no-colostrum fed groups (1NC, 0.00 ± 0.00 24NC, 0.00 ± 0.00 mg/dL, 24C, 1207 ± 171; $P < 0.01$, Figure 3.5). On average calves had >1000 mg/mL serum IgG in the 24C group, indicating transfer of passive immunity was achieved. One calf in the 1NC group had 82 mg/dL serum IgG at birth but was not fed colostrum, and this is not an uncommon finding based on similar studies (Hiltz and Laarman, 2019), but is considered an outlier in this study. The apparent efficiency of absorption showed similar results to serum IgG, with the colostrum fed group exhibiting a "normal" absorption percentage (Halleran et al., 2017b) compared to the non-fed groups with no serum IgG detected (1NC, 0.00 ± 0.00 24NC, 0.00 ± 0.00 , 24C, 30.1 ± 3.32; %, $P < 0.01$, Figure 3.5).

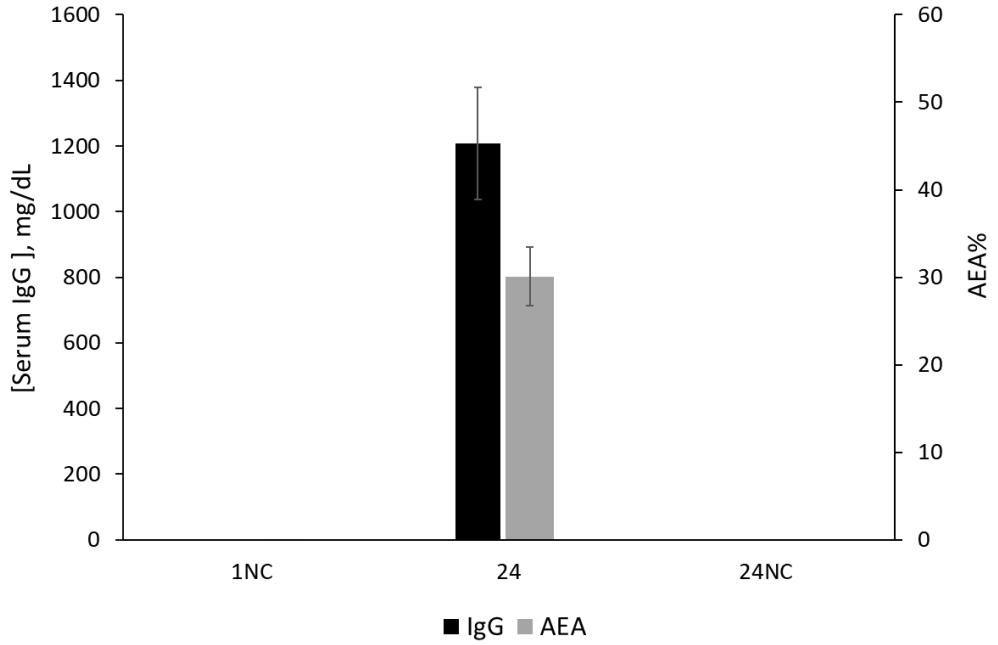


Figure 3.5 Serum IgG and apparent efficiency of absorption (AEA)% in non-fed neonatal calves at 1 hr and 24 hr and colostrum treated calves at 24 hrs of life. Data are presented as mean ±

SEM, P < 0.01 (IgG), P < 0.01 (AEA), n = 6/treatment. One calf in the 1NC group had 82 mg/mL circulating IgG at birth but was not fed colostrum, and thus was removed as an outlier.

3.5.3 Tissue Integrity and Paracellular Permeability

FITC-dextran flux was used to indicate tissue integrity in Ussing experiments and is also used as a marker of paracellular transport (Hubbard et al., 2014; Bzik and Brayden, 2016, (Thomson et al., 2019). The linear regression of the flux of FITC-dextran over time was used as an indicator of tissue integrity (Aguanno et al., 2021). Our data showed linearity of FITC-dextran over time in a constant manner with no co-efficient variance overtime and thus concentration values were assessed at each time point instead of regression analyses. There were no differences in intestinal segment type (P = 0.82) on mucosal FITC-dextran concentrations.

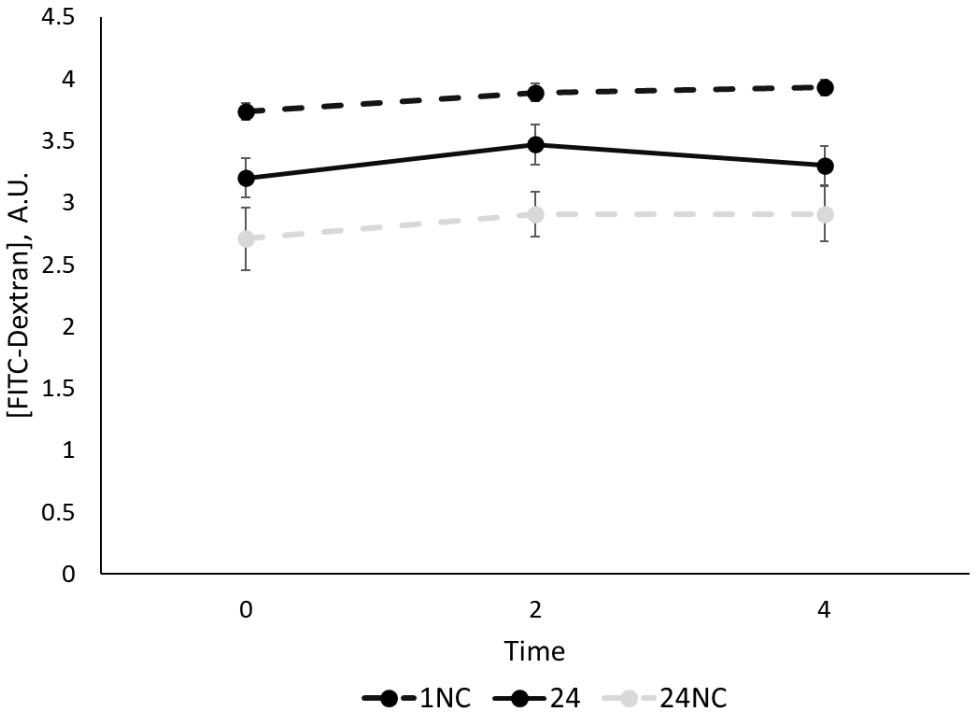
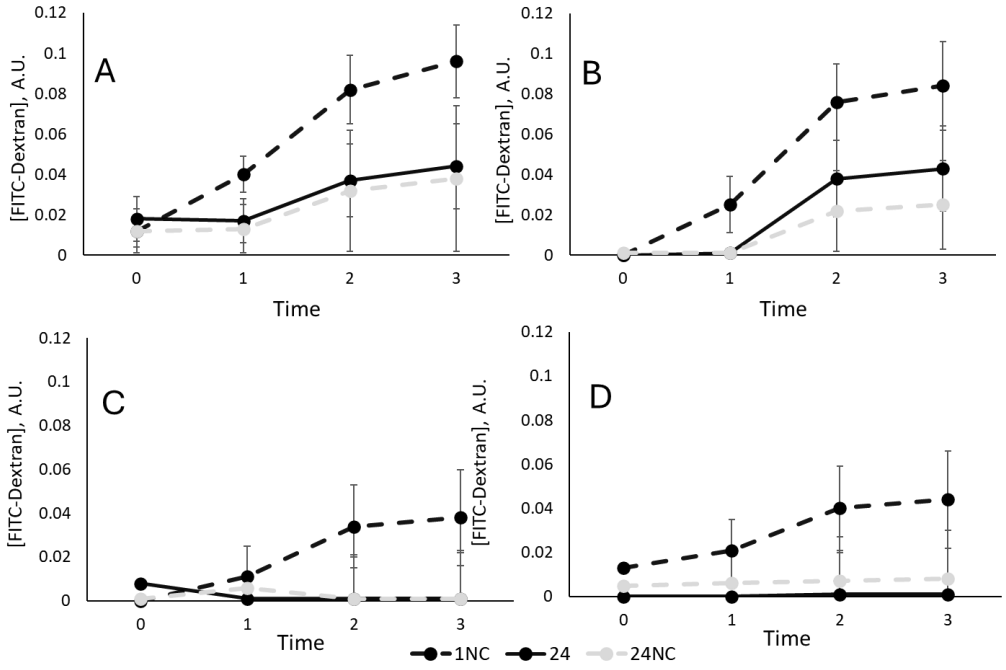


Figure 3.6 FITC-dextran mucosal concentration in non-fed neonatal calves at 1 hr and 24 hr and colostrum treated calves at 24 hrs of life. Data are presented as mean ± SEM, P = 0.03, n = 6/treatment. Linearity of FITC-dextran indicates proper loading of the mucosal chamber.

Total tissue transport or serosal efflux of FITC-dextran was 61% and 71% higher in 1NC calves compared to 24NC and 24C groups, respectively (1NC, 0.039 ± 0.01; 24NC 0.011 ±

0.003; 24C, 0.015 ± 0.003; A.U., $P = 0.01$, Figure 3.7), indicating greater paracellular transport in unfed calves at 1 hr.This demonstrates that at 1 hr with no colostrum feeding paracellular transport is greatest compared to 24 hr calves regardless of feeding status, suggesting reduced permeability of paracellular pathways at 24 hrs.. The 24 hr colostrum fed group permeability of FITC-dextran was similar to the 24 hr nonfed group, indicating reduced permeability of paracellular pathways at 24 hrs and that colostrum feeding did not affect these pathways at 24 hr



post-partum.

Figure 3.7 Serosal FITC-dextran efflux in non-fed neonatal calves at 1 hr and 24 hr and colostrum treated calves at 24 hrs of life. Data are presented as mean ± SEM, $P = 0.03$, n = 6/treatment Intestinal tissue segments are represented in A) duodenum, B) proximal jejunum, C) distal jejunum, and D) ileum. There is greater FITC-dextran transport in the 1NC group than in other treatment groups, and there is greater transport in the proximal intestine than the distal intestine, indicating an effect of age, feeding status, and intestinal tissue segment on paracellular transport.

Overall, paracellular transport pathways tended to have higher FITC-dextran efflux observed in the proximal intestinal segments (duodenum, 0.041 ± 0.006; proximal jejunum, 0.031 ± 0.006; distal jejunum, 0.011 ± 0.004; ileum, 0.015 ± 0.004 A.U., $P = 0.05$) compared to

the distal jejunum and ileum. The proximal intestinal segments had 2 to 4 fold higher FITC-dextran efflux than the distal intestinal segments, indicating an effect of intestinal tissue segment on paracellular transport. There was also a treatment × time interaction ($P = 0.02$), suggesting a combined effect of age, feeding status, and paracellular transport over time on FITC-dextran efflux. Additionally, there was an intestinal segment type × time interaction ($P = 0.03$), indicating that intestinal segment type was not solely responsible for the changes seen between the proximal and distal intestine for FITC-dextran efflux. The combined effects of age, feeding status, and intestinal tissue type demonstrate that paracellular transport is greatest in the 1 hr old, unfed calf in the proximal small intestine, and that the least amount of paracellular flux is in 24 hr old fed calves in the distal small intestine. Though paracellular flux does not cease at 24 hrs of life, reduced flux could indicate "gut closure" mechanisms – closure of tight junctions - and a reduction of permeability in the day-old calf.

3.5.4 IgG Transport

Influx and efflux of IgG from intestinal tissue segments were measured to assess IgG transport capacity in relation to age and colostrum feeding. There was no difference in transport of IgG with colostrum feeding compared to the non-colostrum fed groups at 1 hr or 24 hr, as indicated by % total mucosal influx of IgG into the intestinal tissue (1NC, 55.1 ± 3.06; 24NC, 62.5 ± 5.09, 24C, 59.3 ± 5.84; %total mucosal influx of IgG, $P = 0.48$, Figure 3.8). Since 130 mg/mL IgG (780mg total) was loaded into the mucosal donor chamber, this represents an uptake of 373 mg/ cm^2 of tissue for 1NC, 423 mg/cm^2 of tissue for 24NC, and 401 mg/cm^2 of tissue for 24C over a 2 hr period, which is equivalent to approximately 50-62% total uptake. This appears to be a large capacity for uptake into the intestinal tissue however it should be noted that this could also represent adhesion to the chamber plastic and mucosa brush border, as well as uptake into the epithelium and tissue.

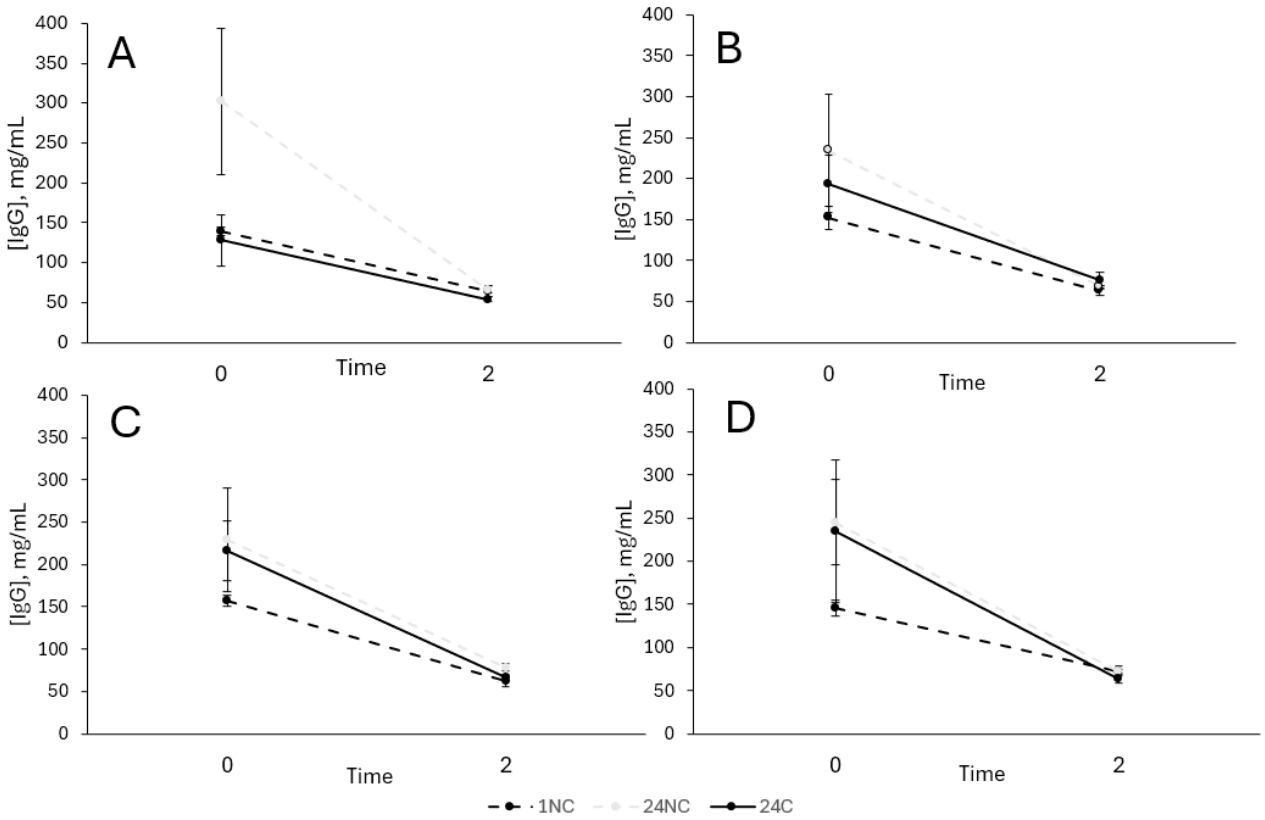


Figure 3.8 Mucosal IgG influx into intestinal tissue in non-fed neonatal calves at 1 hr and 24 hr, and in colostrum fed calves at 24 hr. Data are presented as mean ± SEM**,** $P = 0.48$**,** n = 6/treatment. There were no differences between A) duodenum, B) proximal jejunum, C) distal jejunum, or D) ileum for mucosal influx of IgG.

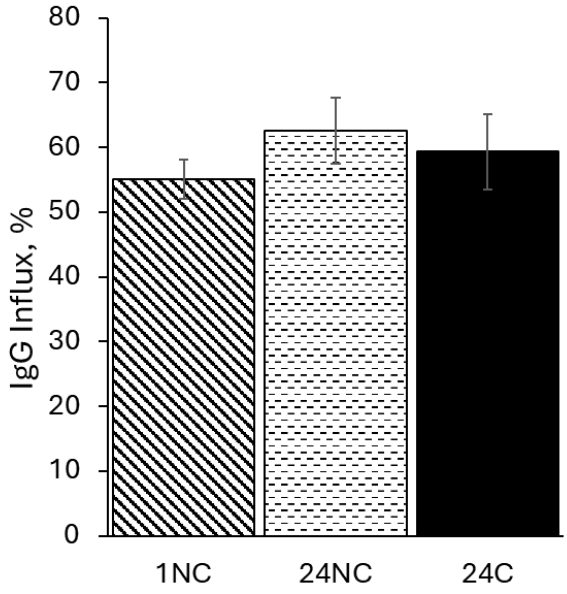


Figure 3.9 Percent total mucosal IgG influx in non-fed neonatal calves at 1 hr and 24 hr, and in colostrum fed calves at 24 hr. Data are presented as mean ± SEM, $P = 0.48$, n = 6/treatment. The initial measurement was considered 100%, and the measurement at 2 hrs was subtracted from 100% to obtain the % influx. There were no differences between treatments for mucosal IgG influx %, indicating a large capacity to intake IgG regardless of age or feeding status.

In the proximal small intestine (duodenum and proximal jejunum) 24C calves had greater % total IgG efflux over 2 hrs than 1NC and 24NC calves by 31-32% and by 28-33%, respectively (Figure 3.9). This represents a colostrum feeding effect in the proximal intestine and represents total tissue transport. Total tissue transport includes influx of donor IgG, and efflux of both donor and existing tissue IgG from colostrum feeding at the time of euthanasia. In the distal small intestine (distal jejunum and ileum, Fig 3.9), total IgG efflux over 2 hrs was 30% higher in both 1NC and 24C calves compared to 24NC calves (Figure 3.9). This demonstrates that non-fed calves at 1 hr and fed calves at 24 hrs had similar total efflux of IgG despite differences in nutritional stimulation. Colostrum feeding, therefore, did not have an effect on the transport of IgG, at least in the ex-vivo setting of the Ussing chamber. In contrast, non-fed calves at hrs show

comparatively very low efflux of IgG, indicating tissue age or neonate age without feeding colostrum significantly lowers IgG transport.

Intestinal total efflux was 2-fold and 4-fold higher in the colostrum-fed group at 24 hr, compared to both non-colostrum fed groups (1NC, 8.55 ± 2.32; 24NC, 4.3 ± 0.57, 24C, 19.1 ± 3.27 mg/cm^2, $P < 0.01$, Figure 3.10), as expected as the other two groups did not have previous exposure to the IgG substrate. IgG efflux increased and peaked at 2 hrs and then declined at 3hrs in all intestinal segments. This could indicate IgG reuptake into the tissue at 3 hrs, or simply less uptake at that timepoint. To ensure measurement of IgG at 3 hrs was accurate, total protein measurements were taken of 3 hr samples (data not shown), and there was indeed a decrease in IgG content rather than a denaturing or inability of the ELISA method to detect IgG in those samples.

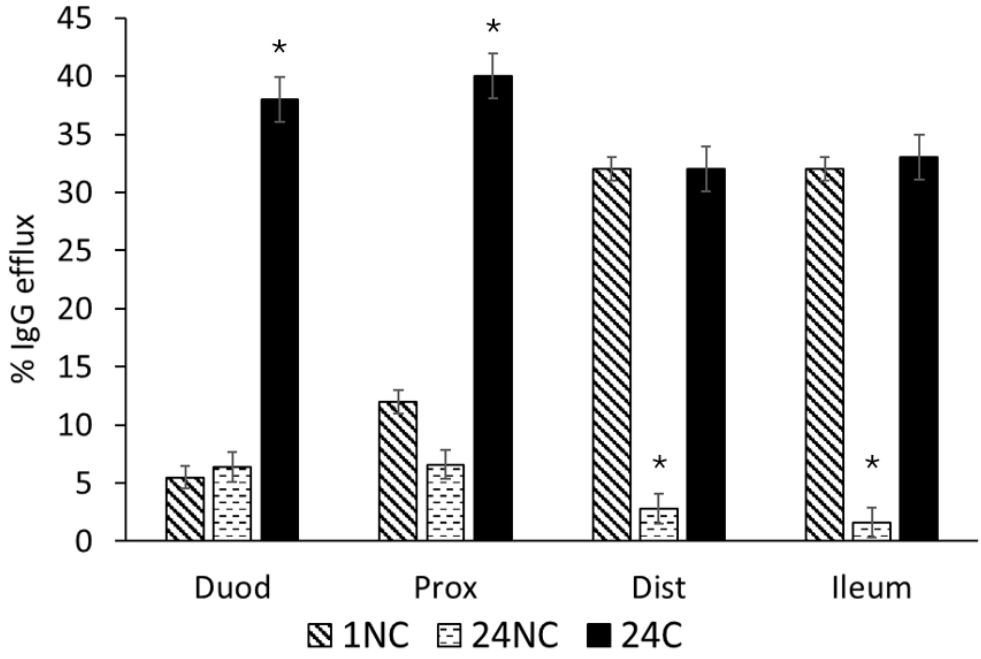


Figure 3.10 IgG efflux over 2 hrs as a percent of the total loaded IgG (780 mg) in non-fed neonatal calves at 1 hr and 24 hr, and in colostrum fed calves at 24 hr. Data are presented as mean ± SEM, n = 6/treatment. Feeding colostrum influenced IgG transport in the proximal intestine but did not influence IgG transport in the distal intestine. 1NC calves were able to transport the same amount of IgG as 24C calves, despite differences in age and feeding status. 24NC calves had consistently low transport rates compared to 1NC and 24C calves.

The overall differences in IgG efflux were associated with intestinal segment type (P = 0.03), and tissue transport of IgG over time ($P < 0.01$), and their interaction ($P < 0.01$, Figure 3.10) An interaction of intestinal segment type and time demonstrates that neither intestinal segment type nor tissue transport of IgG over time were completely responsible for the variation between treatments, but instead were both involved in the variation seen in IgG efflux. It appears that the distal intestine (distal jejunum and ileum) has a larger capacity for tissue transport in the 1 hr old calf than the proximal intestine (duodenum and proximal jejunum). This finding is in line with the PAS-positive vesicle counts, which indicate a greater number of glycoprotein-containing vesicles in the distal intestine, and suggest a greater capacity for IgG absorption in those intestinal sections. Several factors may influence the site of absorption, including vesicle number and size, and decreased motility at the distal end of the small intestine (Ma and Lee, 2019).

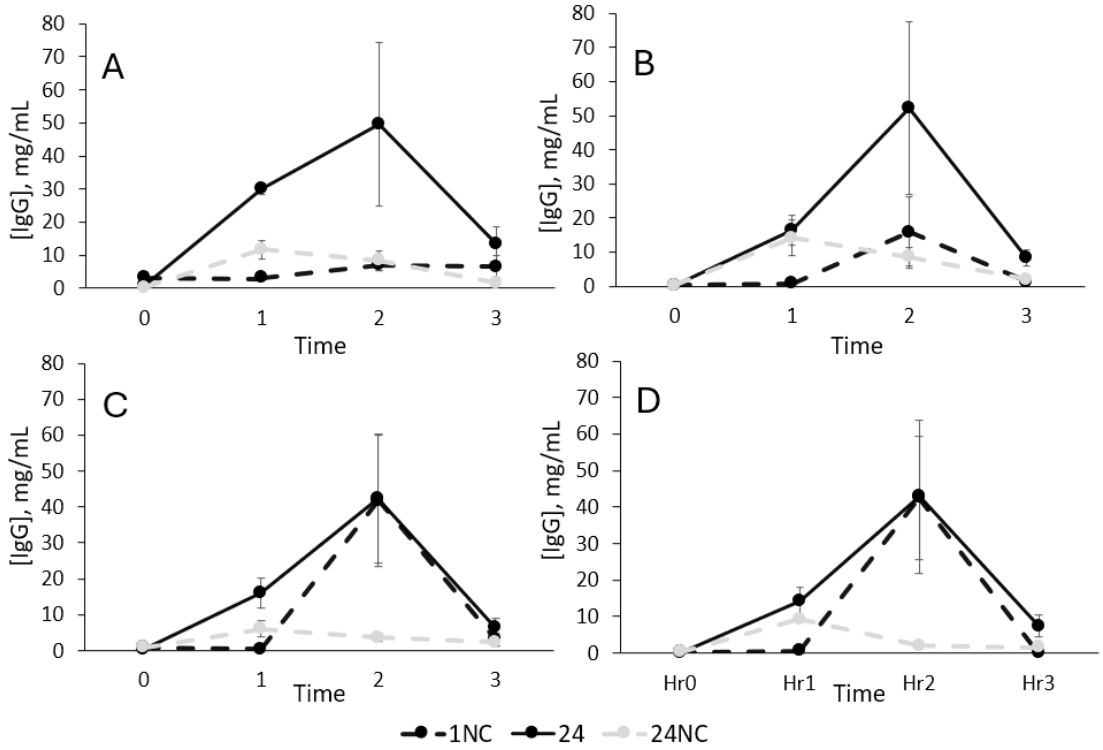


Figure 3.11 Intestinal efflux of IgG in non-fed neonatal calves at 1 hr and 24 hr, and colostrum treated calves at 24 hr. Data are presented as mean ± SEM, P < 0.01, n=6/group. Four tissues were measured A) duodenum, B) proximal jejunum C) distal jejunum, and D) ileum. Fed calves

(24C) retained the ability to transport a relatively large amount of IgG in all tissues while non-fed calves (24NC) did not. Younger calves (1NC) were able to transport IgG in the distal jejunum and ileum as effectively as older, fed calves (24C).

3.5.5 Intestinal Morphology

Villi height did not change among treatment groups (1NC, 525 ± 21.7; 24C, 466 ± 19.0; 24NC, 495 ± 22.1 μm, $P = 0.16$, Table 3.2). Due to the relatively short timeframe of this study, it is not surprising to see no change in villi height, even with colostrum vs. no colostrum feeding.

Villi height did not differ by intestinal segment type ($P = 0.44$) but crypt depth did differ by intestinal segment type, where the distal jejunum had the shortest crypt depths (duodenum, 165 ± 10.2; proximal jejunum, 140 ± 5.72; distal jejunum, 118 ± 4.53; ileum, 131 ± 6.62, $P < 0.01$, Table 3.3), as expected due to normal physiology.

Table 3.2 Villi height, crypt depth, and villi height:crypt depth ratio (V:C ratio) in non-fed neonatal calves at 1 hr and 24 hr, and colostrum treated calves at 24 hr. Data are presented as mean ± SEM. n=6/treatment. Villi height and crypt depth did not differ by treatment group, indicating no effect of age or feeding status.

	1NC	24NC	24C	*P*-Value
Villi height (μm)	525 ± 21.7	495 ± 22.1	466 ± 19.0	ns
Crypt depth (μm)	149 ± 7.43	134 ± 5.06	132 ± 7.89	ns
V:C ratio	3.72 ± 0.27	3.78 ± 0.21	3.72 ± 0.22	ns

Villi height:crypt depth ratio is a parameter often reported in agricultural experiments to assess intestinal integrity, proliferation, and absorptive capacity (Wilson et al., 2018) due to its ability to inform on the microanatomy and thus surface area of the intestine. Colostrum-fed calves have the highest VHC ratios at 7 d of life, which indicates the increased proliferative and absorptive capacity effect with feeding (Yang et al., 2015) but, to our knowledge, VHC ratios have not been reported in calves before 1d of life. In this study, there was no effect of colostrum feeding at 24 hrs on villi height:crypt depth ratio ($P = 0.89$) nor was there any effect on VHC with age (at 1 hr or 24 hr) with no-colostrum feeding.

The villi height:crypt depth ratio differed overall by intestinal segment type(duodenum, 3.36 ± 0.27; proximal jejunum, 3.39 ± 0.21; distal jejunum, 4.40 ± 0.28; ileum, 3.88 ± 0.23, $P =$

0.01), the same as previously reported in non-fed neonatal calves, though the ratios were smaller than those previously reported (Yang et al., 2015). From these data it appears that our calves displayed normal physiology at 24 hrs and the intestine is reported to take several days to one week undergo adaptive growth and increase villi height:crypt depth ratio in response to colostrum feeding (Yang et al., 2015).

Table 3.3 Overall villi height, crypt depth, and villi height:crypt depth ratio (V:C ratio) by intestinal tissue type, with P-values and significance indicated by asterisks, n=6/treatment.

	Duodenum	Proximal Jejunum	Distal Jejunum	Ileum	*P* -Value
Villi height (μm)	521 ± 27.8	461 ± 21.4	507 ± 25.2	493 ± 23.3	ns
Crypt depth (μm)	165 ± 10.2	140 ± 5.72	*118 ± 4.53	131 ± 6.62	< 0.01
V:C ratio	3.36 ±0.27	3.39 ± 0.21	*4.40 ± 0.28	*3.88 ± 0.23	0.01

3.5.6 Vesicle Transport of IgG

As expected, the PAS-positive vesicle count, indicating presence of vesicles containing glycoproteins in the villus-epithelium (stained pink), was greatest in fed calves (24C) and is consistent with large amount of glycoproteins present in colostrum (1NC, 0.05 ± 0.05; 24NC, 1.90 ± 0.53; 24C, 4.94 ± 1.46 PAS-positive vesicles per 20 μm^2, $P < 0.01$, Figure 3.11) . Across all treatment groups, there were more PAS-positive vesicles in the distal jejunum than in other tissues (duodenum, 0.00 ± 0.00; proximal jejunum, 4.83 ± 2.74; distal jejunum, 11.7 ± 3.22; ileum, 1.80 ± 0.53 full vesicles per 20 μm^2, $P < 0.01$, Figure 3.10). The dominant glycoprotein in bovine colostrum is IgG and therefore we assume that the vesicles indicated contain IgG; in PAS stained tissue, PAS-positive vesicles are distinguished from glycoprotein-containing goblet cells by a difference in staining intensity. The goblet cells are a dark purple color, while the vesicles are a bright pink. Interestingly, 24NC calves exhibited the presence of a small number of PAS-positive vesicles, although they had approximately 50-70% fewer vesicles than the 24C calves. It is not clear why 24NC calves had some PAS-positive vesicles when they were not fed colostrum, though there may be an effect of age in 24 hrs on the presence of the PAS-positive vesicles, as no

PAS-positive vesicles were observed in 1NC calves. These data indicate that there may be some transfer of IgG in utero, as these calves were not fed IgG.

The intestine in the 24C treatment had the greatest quantity of PAS-positive vesicles in all segments of the intestine, except for the duodenum, which had no PAS-positive vesicles. The duodenum is not a major site of protein absorption. The distal jejunum had a 2.5-fold increase in the number of PAS-positive vesicles as compared to the proximal jejunum, and a 6.5-fold increase as compared to the ileum. This is in agreement with the findings of Jochims et al. (1994b), who observed large vesicles centrally located in the neonatal calf epithelial cells in the jejunum and ileum, in colostrum dosed calves at 1.5 hrs of life. To our knowledge, we are the first to count the vesicles in each intestinal segment, and the finding that the distal jejunum contains the greatest number of vesicles indicates the intestinal site for maximal IgG uptake in the neonatal calf. The 24NC calves also exhibited PAS-positive vesicles, although they had 72% fewer vesicles in the distal jejunum than the 24C calves.

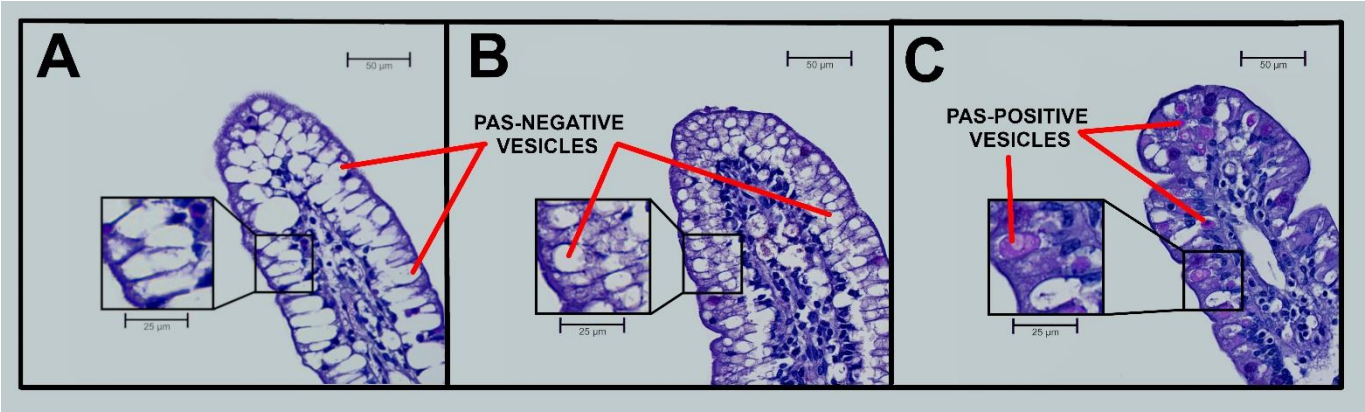


Figure 3.12 Vesicle morphology in distal jejunum in calves at 1 hr and 24 hrs, and in colostrum treated calves at 24 hrs of life. 1NC (A) calves have large, empty, PAS-negative vesicles indicated by the white space inside the intestinal epithelial cells while 24C (B) calves have some PAS-positive vesicles (pink) and some PAS-negative vesicles (white). 24NC (C) calves also have PAS-negative vesicles, though they appear smaller than those of the 1 hr old calves.

For PAS-negative vesicle count, which could indicate IgG absorption potential, vesicles are observed as central to the intestinal epithelial cells and are found in high abundance in 1NC and 24NC groups (Figure 3.11 and Figure 3.12). The intestine appears to be developmentally primed for IgG uptake, as indicated by the presence of PAS-negative vesicles at birth. The

proximal intestine (duodenum, 3.38 ± 1.42; proximal jejunum, 3.76 ± 1.20 vesicles per 20 μm^2) had fewer PAS-negative vesicles than the distal intestine, consistent with PAS-positive findings (distal jejunum, 11.6 ± 1.78; ileum 15.3 ± 1.20 vesicles per 20 μm^2, $P < 0.01$, Figure 3.13). The distal intestine appears to have a greater capacity for glycoprotein (IgG) uptake due to the overall number of vesicles present in the neonatal intestinal epithelial cells with the potential to be filled with IgG.

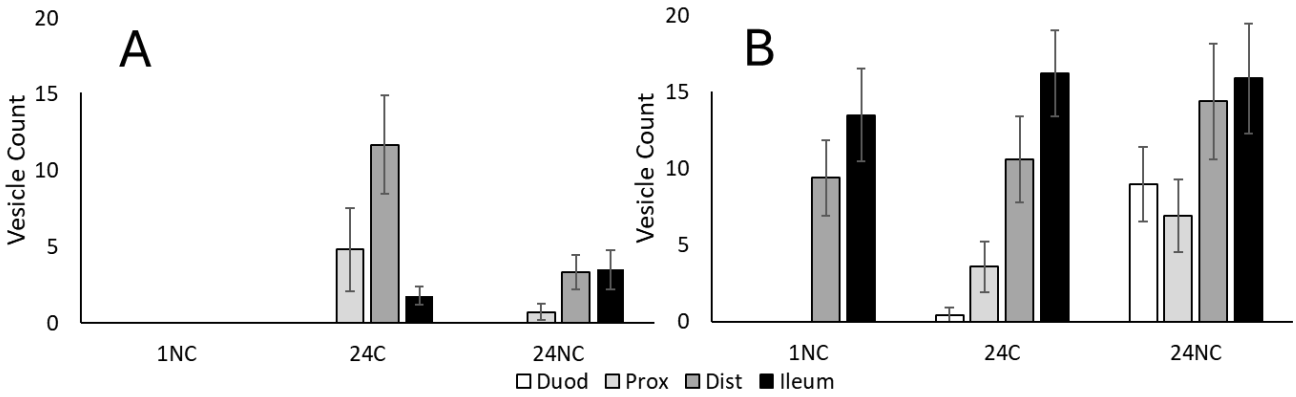


Figure 3.13 Count of PAS-positive and PAS-negative vesicles per 20μm^2 of villi tissue, in non-fed neonatal calves at 1 hr and 24 hr, and in colostrum fed calves at 24 hr. Data are presented as mean ± SEM, $P = 0.48$, n = 6/treatment., $P < 0.01$, n = 6 calves/treatment, 3 villi/calf. 1NC and 24NC calves were not fed colostrum and were euthanized at 1 or 24 hrs of life. 24C calves were fed one meal of colostrum within an hour of life and were euthanized at 24 hrs. (A) PAS-positive vesicles were present in 24C and in 24NC calves, (B) PAS-negative vesicles were present in all treatment groups though varied by tissue.

3.6 Discussion

In this study there were three main objectives – to 1) measure the timing of abomasal acidification over the first 24 hrs of life, 2) examine the effect of age colostrum feeding status on intestinal IgG transport and subsequent serum levels of IgG in four defined segments in the small intestine, 2) to examine the kinetics of IgG transport in four defined segments in the neonatal small intestine. The three treatment groups allowed for two comparisons – firstly, in non-fed neonates to observe the effect of age (1NC vs. 24NC), and secondly in colostrum fed neonates to observe the effect of feeding at 24 hrs of life (24C vs. 24NC).

3.6.1 Abomasal Acidification in the Neonatal Calf

Within the first 24 hrs of life, digesta pH numerically decreased with feeding in the abomasum, but not in other tissues. This study had a lower statistical power with only 6 animals per treatment, so it is possible that the numerical differences seen would become significant with a larger treatment group size. Acidification of the abomasum before 24 hrs of life appears to require nutritional stimulation in calves as the pH remained above 4 in non-fed calves (1NC and 24NC) but decreased in fed calves (24C). From these data, it is not clear when the pH would drop without nutritional stimulation. In a natural setting a calf would suckle at shorter intervals throughout the day as compared to commercial dairy practices where calves are fed a large amount twice a day. Therefore, in a natural setting, with colostrum and milk continuously entering the stomach, abomasal pH would likely remain higher due to the pH of the milk. In a production setting, the pH would increase after feeding but have time to drop between meals. This may be especially important in the neonate where colostral proteins, immune cells, and other nutrients could be damaged by the acid barrier of the stomach. Abomasal pH has been measured in calves that are two weeks old, in the context of feeding acidified milk replacer (Constable et al., 2006a), but to our knowledge has not been measured in calves less than 24 hrs old. Our data show that the first feeding does stimulate a decrease in abomasal pH, suggesting that normal stomach function in the neonate requires stimulation before 24 hrs of life. More investigation is needed to assess the effect of decreased pH on colostrum and milk components and their subsequent absorption profiles.

3.6.2 Effect of Colostrum Feeding on IgG Intestinal Morphology in the Neonatal Calf

The villi height:crypt depth ratio is an indicator of intestinal development, cellular proliferation and absorptive capacity, particularly in response to feeding, as crypts increase in depth and villi decrease in height or become broader with age, across different species (Hampson, 1986; Pluske et al., 1996). This morphological change is associated with type of diet, age of animal, and weaning age. The latter can occur at different ages depending on species, and in the calf it is usually age6-8 weeks of life in a production setting. Therefore, we expected no change in these morphological parameters between 1 hr and24 hrs of life. Our data showed that the villi height:crypt depth ratio did not differ with nutritional stimulation with colostrum compared to no-colostrum at 24 hrs (24C vs. 24NC), nor within 24 hrs in non-colostrum fed neonatal calves (1NC vs. 24NC). There was no change between treatment groups for villi heights

and crypt depths and thus no change in the villi height:crypt depth ratio, suggesting that epithelial maturation does not occur within the first 24 hrs of life

There were intestinal segment type differences in crypt depth, where, across all treatment groups, the duodenum had the deepest crypt depths (14% to 24% larger than other intestinal segments), and the distal jejunum had the shallowest crypt depths (66% to 74% smaller than other intestinal segments). These findings mirror those of Franco et al (2006) who examined the villi height and crypt depth in broiler chickens at different ages and found that the duodenum has larger crypt depths than the jejunum through 42 days of age. To our knowledge, these measurements have not been analyzed in calves within the first 24 hrs of life. In all, morphological analysis represented normal physiology of the neonate.

3.6.3 Effect of Colostrum Feeding on IgG Transport in the Neonatal Calf

Calves with less than 1000 mg/dL serum IgG at 24-48hrs of life are deemed to have FTPI (Lee et al., 2008). In this study, analysis of serum IgG confirmed that on average colostrum-fed calves had a serum IgG concentration of 1207 mg/dL at 24 hrs of life. Though current recommendations categorize our calves as having “fair” TPI (Lombard et al., 2020), the second lowest category, therefore the colostrum product (Premolac Plus, Zinpro, 135g IgG) we used provided adequate delivery and the correct formulation of IgG to the intestine for uptake. In this study, it was more important to feed the same amount of IgG to each calf than it was to ensure “good” or “excellent” TPI, and thus colostrum was not dosed by weight. Without consistent colostrum composition, comparisons could not be made between our treatment groups to evaluate age vs. feeding effects. We are the first to measure intestinal permeability-transport using Ussing chamber methods as a tool to evaluate ex-vivo uptake and transport kinetics of bovine IgG. At 24 hrs of life, 24C colostrum-fed calves had greater IgG transport, as indicated by total intestinal efflux, in all intestinal segments compared to 24 hr nonfed calves, as expected, demonstrating that colostrum feeding may further stimulate IgG absorption and transport. IgG transport was also observed in 1NC calves, showing that within 1 hr of birth, the distal jejunum and ileum have the capacity to transport IgG at the same level as a 24 hr old, colostrum fed calf. The capacity to transport IgG without colostrum stimulation appears to be lost by 24 hrs of life in unfed calves (24NC), emphasizing the importance of early colostrum feeding management practices within 24 hrs in the neonate. It appears that the first colostrum feeding is the most important to stimulate the intestine, after which, IgG can continue to be absorbed for at least 24

hrs of life. Without stimulation, however, the neonatal intestine loses the capacity to uptake immunoglobulins by 24 hrs of life.

FITC-dextran efflux was highest in 1NC, unfed calves, exhibiting over 50% greater flux than the 24C and 24NC groups over 2 hrs. All treatments increased with time, as expected in a cumulative Ussing chamber experiment, and there was an interaction of treatment × time, indicating that both the treatment group (fed vs. unfed effects or age effects) and time spent in the Ussing chamber were involved in FITC-dextran flux. It was expected that FITC-dextran flux would increase linearly with time (Thomson et al., 2019), which was the case in the 1NC calves for the first two hrs of the Ussing experiment, before flux was observed to decrease during the third hour. The peak of FITC-dextran flux at 2 hrs may indicate maximal paracellular transport in that timeframe.

PAS-positive vesicles indicate the presence of glycoproteins such as IgG (Abreu Velez et al., 2016). Our results are similar to those observed by Jochims et al (1994a). Jochims et al labeled some vesicles as “light type” others as “dark type” based on observed nanogold labeled IgG staining patterns, suggesting that the light and dark type vesicles indicated different stages of maturation of the intestinal enterocytes. They observed that in younger calves, large, centrally located vesicles were stained with IgG (light type) but in older calves the IgG staining was present throughout the cytosol (dark type). The light type staining indicates IgG contained within a vesicle in the neonatal epithelium, while the dark type indicate free IgG in the cells of the neonatal epithelium. Our data showed an abundance of PAS-positive vesicles in colostrum-fed calves 24 hrs verses no PAS-positive vesicles in the 1NC calves, as expected, but our imaging was not sensitive enough to be able to determine vesicle-bound vs. free IgG. The non-fed 24 hr calves displayed some PAS-positive vesicles, even though they were not fed colostrum. It is possible that endogenous IgG from the calf was stained by 24 hrs of life. Perhaps another metric to examine the PAS-positive vesicles in the 24NC calf would be to measure the size of the vesicles. Large, centrally located vesicles in the fed calves inform us of colostrum absorption, but smaller vesicles in the epithelium or lamina propria at 24 hrs in a non-fed calf would indicate potential endogenous IgG production.

It is important to note there was a large variation between animals for the presence of PAS-positive vesicles, a fact that was not noted by Jochims et al. and was observed in this study. The variation between animals in IgG uptake and transport is also reflected in serum IgG (Stott

et al., 1979b), where some animals seem to uptake and transport more IgG than others or, are capable of absorption for different lengths of time. For example, Stott et al. found that over 50% of calves who had delayed colostrum feeding until 24 hrs of life could not uptake any IgG to the serum; 30% were unable to uptake IgG to serum at 20hrs of life, 17% at 16 hrs of life, and 3% at 12 hrs of life (Stott et al., 1979b). In the current study, calves were able to transport IgG through the tissue at r24 hrs of life if fed colostrum within 1 hr of life – delayed colostrum feeding or no colostrum feeding appears to decrease the ability of the calf to absorb IgG, as observed with our 24NC group.

It is unclear why some calves, even when fed and treated the same, exhibit different histological features and absorptive capacity, but one factor that has not been investigated on this subject is the link between IgG uptake capacity and gestational length. The gestational length of the dairy cow differs by breed, parity, length of the previous lactation, and can even vary farm to farm (Norman et al., 2009). It is possible that a calf born at its expected birth date and a calf born a week past its expected birth date have differential absorption of IgG due to available developmental time in utero. Future studies should examine the effect of gestational length on IgG absorption in neonatal calves.

3.6.4 Effect of Intestinal Segment Type on IgG Transport in the Neonatal Calf

Our findings showed that at 24 hrs of life, efflux of IgG was not affected by the intestinal segment type. The 24NC group had low IgG efflux (under 15 mg/mL) in all intestinal segments at all timepoints, while the 24C group had significantly higher IgG efflux (above 16.4 mg/mL in hour1 and above 42 mg/mL in hr2) in all intestinal segments. Interestingly, in the 1NC group, IgG efflux was similar to the 24NC group in the proximal intestine (duodenum and proximal jejunum), but was similar to the 24C group in the distal intestine (distal jejunum and ileum). It appears that, at birth, IgG is absorbed preferentially in the distal intestine, but at 24 hrs of life (fed calves), IgG is absorbed throughout the entire small intestine. These findings match with early observations by Jochims et al. (1994a) and Staley et al. (1972) who both studied the structure of the neonatal intestine. Jochims used nanogold labeling to visualize IgG uptake in the neonatal intestine and noted that while IgG staining was visible in all sections of the small intestine, staining increased from the proximal to distal intestine. Though Jochims did not examine IgG kinetics, their histological findings complement our kinetic data. FITC-dextran paracellular efflux was 2-fold higher in the proximal intestine in newborn, unfed calves, than in

24 hr old calves, which was opposite of IgG transport, and showed us that small molecules and large molecules have preferential absorptive sites in the small intestine. Paracellular transport may play a greater role in the proximal intestine in neonates, where transcellular transport may play a greater role in the distal intestine.

It is also interesting to note that the 24C and 24NC groups demonstrated lower paracellular FITC-dextran efflux in the distal intestine compared to 1NC, and the 1NC had greater efflux in all intestinal segments. The neonatal calf intestine at birth has been called "leaky" and the kinetics of paracellular transport of molecules in neonatal calves has not been studied previously. Based on previous assumptions of neonatal absorption, it would be expected that the greatest amount of transport would occur in 1NC calves as they are the youngest group. Age has an effect on paracellular transport, with older calves able to perform more paracellular transport in the proximal intestine and less paracellular transport in the distal intestine.

The PAS-positive vesicles in the colostrum fed calves were found in the distal intestine, the same location as the maximum IgG transport observed in our flux experiments. The PAS-positive vesicles are therefore linked with IgG absorption, suggesting that the vesicles are required for IgG transport. Colostrum-fed calves retained the ability to transport IgG through 24 hrs of life, indicating that nutritional stimulation is required for maturation of the intestinal epithelial cells and that, prior to 24 hrs of life, nutrition is more important than age for stimulating maturation.

3.6.5 Effect of Feeding Colostrum on Gut Closure

Although it has been reported that absorption of macromolecules ceases around 24 hrs of life in the neonatal calf (Stott et al., 1979b), this study saw no difference between treatments for mucosal influx of IgG; therefore, the 1NC calves, 24C calves, and 24NC calves all have the same tissue-level capacity to uptake IgG into the intestinal epithelium, despite age and nutritional stimulation differences. This goes against what is normally talked about in the literature, where 24 hrs of life is said to be the point of gut closure and 12 hrs of life the point of declining IgG uptake capability (Jochims et al., 1994a; Le Dividich et al., 2005). It is possible that the acidification of the abomasum normally prevents intact IgG from reaching the small intestine at 24 hrs of life; thus, IgG absorption in the 24 hr old calf, though possible in an Ussing chamber environment, would not occur naturally. In opposition, it is possible that the intestine retains the

ability to absorb IgG but that the IgG does not end up in the serum and instead remains in the lumen, epithelium, or lymphatic vessels and structures.

Jochims et al. (1994a), suggest that gut closure may be “retrograde”, with the IgG efflux ceasing before IgG influx. This hypothesis is supported by our 24NC IgG efflux data, in which there is very little transport or permeability of IgG, compared to 1NC. These data, along with maximal IgG efflux by 24C colostrum-fed calves, suggests that nutritional stimulation by colostrum prolongs the time to gut closure and thus cessation of IgG trans-epithelial flux, and is a reflection of the available substrate for absorption in the fed calf. It is unclear why colostrum ingestion seems to prolong the ability to transport IgG in these calves, when previous literature suggests that a lack of nutritional stimulation would extend the time to gut closure (Stott et al., 1979b).

Stott et al. (1979a) also suggest that the volume of feeding may affect the time to gut closure as well, with calves fed 1 L of colostrum able to respond to a second feeding with increased serum IgG levels, but calves fed 2 L unable to respond. This suggests a maximum capacity of the intestinal vesicles to absorb IgG after a meal. In a natural setting where the calf would suckle many times and therefore have many small meals, this capacity is likely not an issue. In the current study we fed 104 g/L of colostrum replacer with 135 g total IgG; Stott’s study fed 1 and 2 L of an average 361 g IgG, and the increased amount of IgG may have contributed to their results. Either way, there seems to be an effect of maximal transport based on vesicle availability to transport IgG.

3.6.6 Limitations

We would like to acknowledge some limitations of our methods. The Ussing chamber, while a great ex-vivo tool for examining transport kinetics, must be recognized as an ex-vivo tool. The tissue has been removed from the animal and kept viable for several hrs, but it is not connected to a blood supply the same way that it is in-vivo. Since much of calf research discusses IgG transport into the bloodstream, we made the assumption that transport from the mucosal to the serosal side of the Ussing chamber indicates transport into circulation. However, the IgG could be transported into the lymph or the blood.

While 24 hrs of life is the typical age defined as “gut closure”, it is possible that some of our age-effects would change if calves were euthanized even a couple hrs later. To really understand the timing of gut closure, experiments should extend beyond 24 hrs of life. In this

study, colostrum feeding amount was kept the same for all animals to investigate the effects of transport when colostrum amount did not change. However, in a production setting, animals are often fed based on their bodyweight at birth. It would be beneficial to investigate the effect of colostrum feeding on transport kinetics at differing colostrum inclusions.

3.7 Conclusion

In this study, we evaluated the impact of the first 24 hrs of life and colostrum feeding status on IgG transport kinetics and histological features in the neonatal calf. Though acidification of the abomasum may play a role in the cessation of IgG uptake, the capacity for IgG uptake and transport is the same at 1 hr of life as it is at 24 hrs of life if the calf is fed. IgG is preferentially absorbed in the distal intestine, while smaller molecules undergo greater paracellular transport in the proximal intestine. Histologically, PAS positive vesicle counts coincide with colostrum-IgG feeding status and location of maximum transport was in the distal intestine. These data may not facilitate direct changes to colostrum farming feeding practices, but the results inform on the effect of colostrum feeding vs no-colostrum feeding at 24 hrs in the neonate calf, and do pave the way for continued research to understand mechanisms involved in IgG transport. The advice for producers remains the same – feed your calves an adequate amount of IgG within the first 4-6 hrs of life as timing is crucial for the proper absorption of IgG.

4.0 Influence of the First 24 hrs of Life on IgG Transport Kinetics, Paracellular Transport, and IgG Passage Through the Neonatal Bovine Small Intestine

4.1 Abstract

Transfer of passive immunity (TPI) and intestinal immunoglobulin uptake are important for neonate calf immune function, prevention of disease and survival in the dairy industry (Godden et al., 2019; Lombard et al., 2020). Maturation of the gut progresses with age in the neonatal calf, but how age affects neonatal absorption of IgG during the first day of life is unclear. The aim of this study was to examine IgG transport kinetics and histological features of different sections of the small intestine overtime in the first 24 hrs in neonatal calves. Holstein × Angus bull calves (n = 30) were assigned to one of five treatment groups in a randomized complete block design: one group not fed colostrum and euthanized at 1 hr of life (1NC), and four groups fed colostrum within an hour of life and euthanized at 6 (6C), 12 (12C), 18 (18C) or 24 hrs of life (24C). At euthanasia, intestinal tissue samples were collected for Ussing chamber IgG kinetics, histology, and IgG concentration in tissue, and blood and digesta samples were collected to examine IgG concentration in digesta and digesta pH. A Periodic-Acid Schiff's stain was utilized to investigate intracellular vesicle quantity, while a 4KDa FITC-dextran in the Ussing chambers was used to determine intestinal tissue permeability and to examine paracellular transport. Abomasal pH initially increased with colostrum feeding and then dropped steadily at each time point, as expected, until 24 hrs of life; other intestinal segments did not have a change in pH with feeding or with age. Digesta IgG peaked in the small intestine (duodenum, proximal and distal jejunum, and ileum) at 6 hrs and decreased by 12 hrs in the distal intestine, indicating absorption and passage of digesta through the intestinal tract. The % total mucosal uptake of IgG into the intestine did not differ across time in the first 24 hrs of life in the neonate calf (approximately 55% influx, or 373 mg of IgG into 1 cm^2, indicating that absorption capacity for IgG does not change within the first 24 hrs of life. Total % Serosal efflux of IgG differed by treatment but not by intestinal segment, with 18C and 24C calves having the largest capacity for transepithelial flux of IgG. Overall, colostrum-fed calves have the capacity to absorb IgG for up to 24 hrs which is longer than previously reported.

4.2 Introduction

It is reported in agricultural studies that the quicker colostrum is fed to a calf, ideally within 4-6 hrs of life, the better chance the calf has at absorbing IgG and increasing serum levels to support immune function and prevention of disease (Geiger, 2020). Serum IgG levels at 1 day of life are predictive of serum IgG levels for the first 16 days of life (Correa et al., 2022), making colostrum absorption of utmost importance in the first 24 hrs for the first weeks of life. One study delayed feeding colostrum to calves by 6 hrs after birth and found that apparent efficiency of absorption – a measure of the percentage of IgG fed that is transferred to the bloodstream - decreases from 51.8% to 35.6% (Fischer et al., 2018). Recent literature suggests measuring serum IgG levels at 24 hrs of life to 48hrs of life to determine if transfer of passive immunity (TPI) has been achieved, which is a serum IgG level above 1000 mg/dL (Lima et al., 2024; Murayama et al., 2024). Serum IgG levels within the first 24 hrs of life have been shown to increase steadily with colostrum feeding, (Lima et al., 2024; Murayama et al., 2024), but IgG absorption over time and the process of gut closure on IgG uptake and mechanisms of transport throughout the first 24 hrs of life has not been reported.

Immunoglobulin absorption in the intestine and transport to the bloodstream is critical for immune protection of the neonatal dairy calf (Weaver et al., 2000; Lombard et al., 2020) and cessation of that absorption is termed “gut closure” (Lecce, 1965). Gut closure is stimulated by colostrum feeding but delaying colostrum feeding by 6 or 12 hrs does not linearly extend the time to gut closure by 6 or 12 hrs (Stott et al., 1979b), likely due to an effect of age without nutritional stimulation causing a natural progression of maturation. Due to this age-related maturation progression, our current knowledge of immunoglobulin-IgG absorption is from studies that have fed colostrum to dairy calves, lambs, or kids in different study designs, with different age at feeding colostrum, amount of colostrum and number of colostrum feedings, (Stott et al., 1979a; b; Jaster, 2005) and subsequent effects on serum IgG. It has been reported that spontaneous gut closure, occurring without nutritional stimulation, occurs around 24 hrs of life if calves are not fed, and that nutritionally stimulated gut closure occurs around the same time(Stott et al., 1979b), but this has not been defined at the tissue or transport kinetics level.

At the tissue level, histological investigations have reported the presence of large vesicles centrally in the neonatal intestinal epithelial cells, upon absorption of following colostrum feeding(Staley et al., 1972; Jochims et al., 1994a). These vesicles have been confirmed

to contain IgG, using a gold-labelling technique, up until 24 hrs of life. These vesicles have been identified as pinocytotic transport vesicles, and it has been determined that their purpose is to transport and store IgG during the period of gut closure, up until 24 hrs of life. The link between the number of IgG-positive vesicles and the rate of IgG transport is unclear, though these vesicles have been reported to transport immunoglobulins into the lymphatics (Dobbins and Rollins, 1970).

The overall objective of this study was to examine the effect of time following colostrum feeding within the first 24 hrs of life in the neonate calf on IgG absorption kinetics and the relationship to vesicle transport in the small intestine. In addition, we examined the effect of feeding on abomasal acidification in the first 24 hrs of life. We hypothesized that 1) IgG influx and efflux in the intestine would decrease over time, and that 2) the IgG-containing vesicles would decrease in number over time within the first 24 hr in the neonate calf and 3) the abomasum pH would decrease with time, reaching adult cow levels by 24 hrs of life.

4.3 Materials and Methods

4.3.1 Calves and Study Design

Between October 2021 and March 2023, Holstein × Angus bull calves (n = 30) were born on a commercial farm. At birth, calves were weighed and assigned to one of five treatment groups (n = 6); 1. not fed colostrum and euthanized at 1 hour of life (1NC), and 2-5. fed colostrum within 1 hr of life and euthanized at 6 (6C), 12 (12C), 18 (18C) or 24 hrs of life (24C). The 1NC group was used as a baseline and all other euthanasia times were selected to target critical points during the first 24 hrs of the calf's life; 6 hrs of life has been cited as the point at which absorption potential in the calf starts to decline (Fischer et al., 2018), 12 hrs of life has been cited as the point at which gut closure can begin to occur spontaneously (Stott et al., 1979b), 18hrs of life was the halfway point between the "start" of gut closure (12hrs) the mean time of gut closure (24 hr24 hrs), as measured by Stott et al (1979b). Animals were matched for birth weight in each group. All calves were cleaned by their dams but were removed from the dam before the calf could stand and suckle. After removal from their dam, calves were placed in an individual hutch bedded with straw until euthanasia. All calves were euthanized via captive bolt and exsanguination. Blood was collected during exsanguination into a vacutainer with a clot

activator (BD Vacutainer, silicone-coated interior, Fisher Scientific). All animal procedures were approved by the University of Alberta (AUP #3673).

4.3.2 Colostrum Treatment

A commercial colostrum replacer containing 135 g IgG in 1.3 L of warm water was provided via an esophogeal feeding tube (Premolac, Zinpro Inc.). The colostrum replacer in question only contains the whey fraction of colostrum and is devoid of fat and casein, which would naturally be removed during the clotting process in the abomasum of the calf (Hocquette and Bauchart, 1999).

4.3.3 Intestinal Dissection and Digesta pH

After euthanasia, digesta (stomach and intestine fluids) were collected from the abomasum, duodenum, proximal jejunum, distal jejunum, ileum, and cecum. Intestinal tissue samples from the same intestinal segments were rinsed with phosphate buffered saline (PBS; Fisher Scientific) and placed in ice-cold Krebs buffer; additional small intestinal tissue samples were washed with PBS and then one sample was preserved in 10% buffered formalin while the other sample was snap frozen and stored at -80°C until later analysis of IgG concentration and histological staining. Digesta pH was also measured from the abomasum, each section of the small intestine, and the cecum.

4.3.4 Intestinal and Vesicle Histology

Periodic Acid-Schiff's staining method was used to identify glycoproteins in intestinal segments (Pluta et al., 2011). After 24 hrs in 10% buffered formalin for fixation, samples were transferred to a 70% ethanol solution until they were processed, encased in paraffin, and 5-µm thick sections were taken and mounted on slides (Al-Sabawy et al., 2021). The slides were then stained with Periodic Acid-Schiff's stain, as follows - slides were first de-waxed in two rounds of toluene and then rehydrated with a progression of ethanol dilutions (100%, 90%, 70%, 50%, and tap water). After rehydration, slides were added to a solution of Periodic Acid (Fisher Sci, reconstituted in nitric acid) for 5 mins, placed in tap water for 1 min, and then added to fresh Schiff's reagent (Thermofisher) for 10 mins. After the Schiff's reagent, slides were rinsed with running tap water for 5 mins, at which point slides were counterstained with Hematoxylin Gill III (Leica) for 50 seconds and then were rinsed with running tap water for 12 mins. Finally,

coverslips were applied with DPX mountant (Sigma Aldrich) and cured overnight in a 37° incubator.PAS staining revealed the presence of PAS-positive vesicles in the small intestinal tissues; one image was taken of three separate villi per slide, and the number of vesicles was counted. Counting was performed using an assistive software for manually counting objects in an image (DotDotGoose, American Museum Of Natural History). Briefly, DotDotGoose displayed a 200 x 200 pixel grid over the selected image. A 3 x 3 section of the grid was selected and the PAS positive or "empty" vesicles inside were counted. As each vesicle was clicked on, DotDotGoose recorded the number of clicks, and added a dot so that vesicles were not counted twice (Figure 3.1).

4.3.5 Bioavailability of IgG

Whole blood samples were allowed to clot at room temperature before being centrifuged at 2000 × g for 20 mins at 4°C, after which the serum was removed, aliquoted, and frozen at -80°C. For analysis, each serum sample was plated on a radial immunodiffusion plate with antigens against bovine IgG (RID; Triple J Farms Bovine IgG RID Plates) according to manufacturer's instructions. Plates were incubated for 18 hrs at room temperature before being read with a jeweler's loupe capable of measuring 1/10 of a millimeter. Apparent efficiency of absorption was calculated according to (Halleran et al., 2017a).

The equation for AEA is as follows:

$$AEA\ \% = \left\{ \frac{serum\ IgG\ concentration\ \left(\frac{g}{L}\right) \times\ body\ weight\ (kg) \times [0.7(estimated\ \%\ blood\ volume)]}{colostral\ IgG\ concentration\ \left(\frac{g}{L}\right) \times volume\ colostrum\ administered\ (L)} \right\} \times 100$$

4.3.6 Intestinal Permeability

Intestinal tissue samples were mounted in Ussing chambers (NaviCyte System, Harvard Apparatus Inc.), approximately 2.5 hrs after euthanasia. Each intestinal segment (duodenum, proximal jejunum, distal jejunum, ileum) had three replicate chambers for a total of 12 chambers. The outer serosal and tunica muscularis layers were not peeled, as neonatal tissue is very thin, and there was risk of damaging the epithelium before mounting. The mucosal side of the chamber was filled with carbogenated Krebs' Buffer supplemented with 130 mg/mL bovine IgG and 1 mg/mL FITC-dextran (4 KDa; used as a permeability marker and indicator of paracellular transport), at a pH of 7.4; the serosal side of the chamber was filled with unsupplemented,

carbogenated Krebs' buffer at a pH of 7.4. Intestinal samples were given 20 mins to equilibrate to 37°C before initial samples were taken. Samples (600 µL) were taken from the mucosal chamber every two hrs for four hrs, and from the serosal chamber every hour for four hrs. After a sample was taken, it was replaced with the supplemented Krebs buffer (mucosal chamber) or non-supplemented Krebs buffer (serosal chamber). Samples from the Ussing chamber experiment were analyzed for FITC-dextran and bovine IgG.

4.3.6.1 FITC-dextran Flux as a Permeability and Paracellular Transport Marker

FITC-dextran mucosal influx and serosal efflux values were used to assess tissue integrity as a quality control measure, and to measure paracellular transport across the neonatal calf small intestine. To measure FITC-dextran concentration, mucosal and serosal samples were read at an excitation of 485 nm and an emission of 520 nm on a plate reader (BioTek Synergy H1, Aligent). The concentration of FITC-dextran was determined using a standard curve prepared at the same time as the mucosal buffer to minimize differences from FITC degradation. For FITC-dextran trans-epithelial flux, serosal concentration thresholds were set based on break points in the data, where an unexpectedly high concentration of FITC-dextran identified damaged tissues (Figure 4.1, Aguanno et al., 2021). These thresholds were used to determine which tissues were intact during the experiment and which had degradation or other permeability issues.

We acknowledge that there are limitations to eliminating samples in this way, but in this experiment voltage measurements and histological samples were not taken throughout the Ussing experiment, and thus FITC-dextran efflux is the best quality control measurement of integrity for these intestinal tissue samples. Integrity of the tissue was considered to be low when the initial serosal concentration of FITC-dextran was above 0.005 mg/mL. The break-point of 0.005 mg/mL was chosen based on analysis of the entire sample set – there was a natural break-point in the data between 0.005 mg/mL and 0.01 mg/mL FITC-dextran for initial (0hr) serosal efflux samples. In addition, integrity of the tissue was considered to be low when the difference between sequential samplings (ex. corrected concentration of FITC-dextran at hour 2 – corrected concentration of FITC-dextran at hour 1) was above 0.005 mg/mL. The break-point of 0.005 mg/mL was again chosen based on analysis of the entire sample set – there was a natural break-point in the data between 0.005 mg/mL and 0.01 mg/mL FITC-dextran for the difference between sequential samples (ex. 2 hr – 1 hr). The difference between sequential samples

indicated that, for example, FITC-dextran concentration spiked unexpectedly high between hrs 1 and 2, and thus the tissue at hour 2, 3, and 4 was degraded or otherwise compromised. Samples were eliminated from the overall dataset (for both FITC-dextran and IgG data points) based on the FITC-dextran thresholding, resulting in the following n per treatments (Table 4.1). FITC-dextran efflux data was unavailable for 5 calves due to a plate reader malfunction; those samples were removed from IgG and FITC-dextran flux analysis due to inability to assess the tissue integrity.

Table 4.1 Number of samples removed from analysis due to FITC-dextran thresholding or due to lack of FITC-dextran data, by treatment group.

Treatment Group	n samples removed due to FITC thresholding	n samples removed due to unavailable FITC-dextran data	Total n removed	Total n used in analysis
1NC	81	0	81	279
6C	103	0	103	257
12C	55	48	103	257
18C	35	96	131	229
24C	55	48	103	257

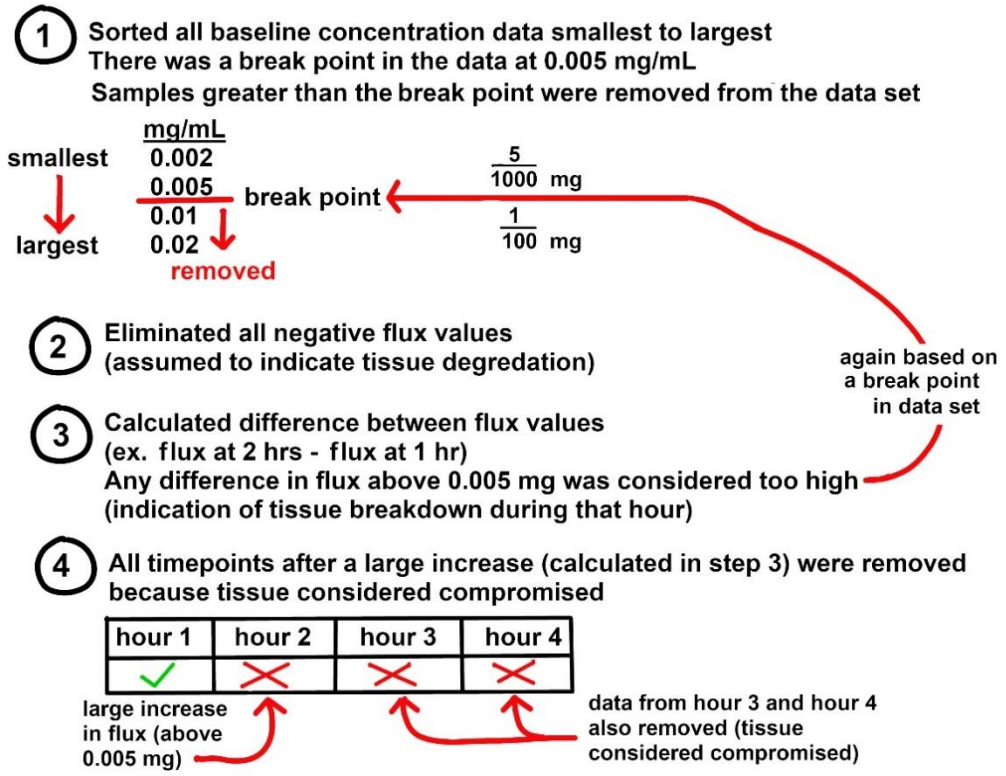


Figure 4.1 A diagram of how FITC-dextran samples were eliminated from the data set. 1) Baseline concentrations were used to determine which tissues were compromised from the beginning of the experiment. Break points in the data were used to determine thresholds for intact vs. compromised tissue 2) negative flux values were also removed, mostly from data in the last hour of the experiment 3) a large increase in flux (as determined by breakpoints) indicated tissue breakdown 4) all timepoints after the tissue was compromised were removed from the dataset.

4.3.6.2 IgG Flux in Neonatal Intestinal Segments

IgG influx and efflux from intestinal tissue segments allowed us to assess IgG uptake during the first 24 hrs of life in the neonatal calf. Gut closure timing has been historically defined by the circulating serum IgG peak concentration, (Stott et al., 1979b), but to our knowledge has not been assessed at the intestinal tissue level.

IgG concentrations in mucosal and serosal samples were measured using a commercial kit for bovine IgG quantification according to manufacturer's instructions (Mabtech Bovine IgG ELISAflex, HRP conjugated) with in-house standards of $1x10^{-1}$ mg/mL, $1x10^{-2}$ mg/mL, $1x10^{-3}$ mg/mL, $1x10^{-4}$mg/mL, and $1x10^{-5}$ mg/mL IgG. Briefly, a standard sandwich ELISA utilized a matched antibody pair specific to bovine IgG. The detection antibody was conjugated to horseradish peroxidase and was able to be quantified using the substrate tetramethylbenzidine (TMB) when samples were read on a plate reader at 450 nm.

4.3.7 Protein Extraction

Extracted protein was analyzed for bovine IgG content to track the location of IgG throughout the first 24 hrs of life within the intestines, and to assess the degree of IgG that is stored in the tissues throughout that timeframe. Protein was extracted from snap-frozen small intestinal tissue (duodenum, proximal jejunum, distal jejunum, and ileum) and from digesta collected from the same segments plus the abomasum and the cecum. For intestinal tissue protein extraction, 600 µL RIPA lysis buffer (1 M tris pH 8, 150 mM NaCl, 0.1% SDS, 0.5% Sodium deoxycholate, 1% Triton X-10, 1mM PMSF, (Mishra et al., 2017)) was added to 5 mg sample and vortexed for 30 seconds to homogenize. Samples were then agitated at 4°C for 2 hrs, centrifuged at 16,000 x g for 20 mins, and the supernatant was collected and stored at -80°C. For digesta protein extraction, PBS + 0.1% Tween20 was used as an extraction buffer at 1 mL/100 g digesta sample (Gumber et al., 2007). After addition of buffer, samples were vortexed to form a slurry, rested for 5 mins on ice, and then centrifuged for 10 mins at 2000 x g. The supernatant was removed and stored at 80°C. Extracted protein from intestinal tissue and from digesta were then run on a commercial kit for bovine IgG quantification (Mabtech Bovine IgG ELISAflex, HRP conjugated).

4.4 Statistical Analysis

Using six animals per treatment allows us to detect, with 80% power, a difference of 20% between treatment means for all variables when the coefficient of variation within treatments is 10% (Berndtson, 1991). Data were analyzed using a one-way ANOVA to look at differences between treatments (digesta pH, serum IgG, AEA) or two way ANOVA to look at differences between treatments, intestinal tissue segments, and the interaction of treatment × intestinal tissue segment (PAS-positive and PAS-negative vesicle count, villi height, crypt depth, villi

height:crypt depth ratio, tissue and digesta IgG). All statistical analysis were run using R (RStudio 2023.03.1+446 "Cherry Blossom").

Ussing parameters were analyzed by repeated measures over the course of the 4hr experiment to account for the same chamber being samples at multiple timepoints (FITC-dextran influx and efflux, IgG influx and efflux), There were no three-way interactions of treatment x tissue x time in the repeated measures analyses. Outliers were removed from analysis by using a 1.5 interquartile range (25^{th} – 75^{th} percentile).

Data were analyzed according to the following model:

$$Y = \mu + T_i + \varepsilon_{ij}$$

where Y is the response, μ is the overall mean, T_i is the treatment effect, and ε_{ij} is the residual error. Significance was declared at $P \leq 0.05$ and tendencies were declared at $0.05 < P \leq 0.10$. Numbers displayed are least squares means ± standard error of the mean, unless otherwise indicated as is standard in agricultural research.

4.5 Results

4.5.1 Digesta pH

The pH of the stomach and intestinal lumen digesta was assessed to identify the time-course of abomasal acidification and to establish normal intestinal pH values over the first 24 hrs of life in the colostrum fed calf. The abomasal pH increased with colostrum feeding at 6 hrs, although this was not significant, and then decreased over time, reaching a low of 2.7 at 24 hr24 hrs of life in fed calves. (1NC, 4.19 ± 0.47; 6C, 5.09 ± 0.43; 12C, 3.36 ± 0.43; 18C, 3.11 ± 0.53; 24C, 2.7 ± 0.61 pH, P = 0.01, Figure 4.2). The pH of the abomasum at 24 hrs was 28% higher than that of an adult cow, where the normal pH is 2.1 to 2.2 (Constable et al., 2006b); pH of the neonatal calf stomach and intestine at 24 hrs of life has not been published. Digesta pH was did not differ across time from 0 to 24 hrs in the duodenum ($P = 0.34$, average pH = 6.08 ± 0.26), proximal jejunum ($P = 0.24$, average pH = 6.17 ± 0.19), distal jejunum ($P = 0.14$, average pH = 7.08 ± 0.22), ileum ($P = 0.15$, average pH = 7.35 ± 0.25), and cecum ($P = 0.33$, average pH = 6.91 ± 0.32), as expected.

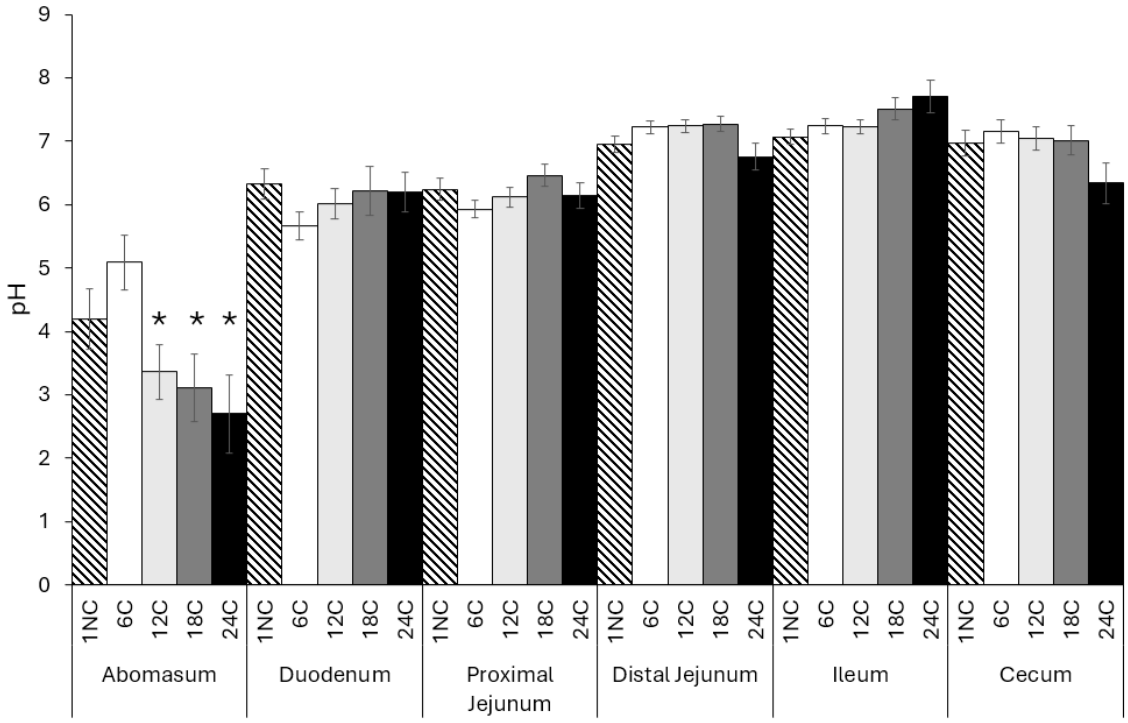


Figure 4.2 Digesta pH of intestinal segments in non-fed neonatal calves at 1 hr and colostrum-fed calves at 6, 12, 18, and 24 hrs. Data are presented as mean ± SEM. n = 6/treatment. Most tissues had consistent pH regardless of age (duodenum, $P = 0.31$; proximal jejunum, $P = 0.24$; distal jejunum, $P = 0.14$; ileum, $P = 0.15$; cecum, $P = 0.33$). The digesta pH generally increased from the proximal to distal sections of the intestine. The 6 hr treatment in the abomasum tended to differ from the 12 hr ($P = 0.07$) and 18 hr treatments ($P = 0.06$), and did differ from the 24 hr treatment ($P = 0.03$).

4.5.2 Bioavailability and Efficiency of Absorption of IgG

Serum IgG was used to determine the bioavailability of IgG from colostrum. Serum IgG increased from 0 to 6hrs as expected and then increased by 30% from 6-12 hrs, and then plateaued to 24 hrs. These data were matched by the apparent efficiency of absorption (Figure 4.3). Transfer of passive immunity, as defined by a serum IgG level >1000 mg/dL, was achieved by 12 hrs of life (1NC, 000 ± 000; 6C 590 ± 49.9; 12C, 1195 ± 230; 18C, 1110 ± 82.1; 24C, 1207 ± 171 mg/dL, $P < 0.01$, Figure 4.3). Apparent efficiency of absorption (AEA), a measure of the percent of IgG that was absorbed and transported into serum, increased to a peak at 12 hrs of life and then stabilized, with a slight decline of 11% and 14% 18 and 24 hrs of life (AEA: 1NC,

0.00 ± 0.00; 6C, 17.2 ± 1.92; 12C, 34.6 ± 7.16; 18C, 30.8 ± 2.06; 24C, 30.1 ± 3.32 %, $P < 0.01$, Figure 4.3), indicating that calves at 12 hrs of life had absorbed the largest percentage of IgG. Clearance of IgG from the blood, or reuptake into the tissue or lymphatics may explain why 18 hr and 24 hr calves had 11% and 14% decreased apparent efficiency of absorption as compared to 12C, respectively. One calf in the 1NC group had 82 mg/dL serum IgG at birth but was not fed colostrum; these data were removed from serum IgG and apparent efficiency of absorption analysis because while some calves do have circulating IgG at birth (Hiltz and Laarman, 2019), in this study it was an outlier. By 24 hrs of life, calves had a "normal" apparent efficiency of absorption percentage (approx. 30-35%Halleran et al., 2017a).

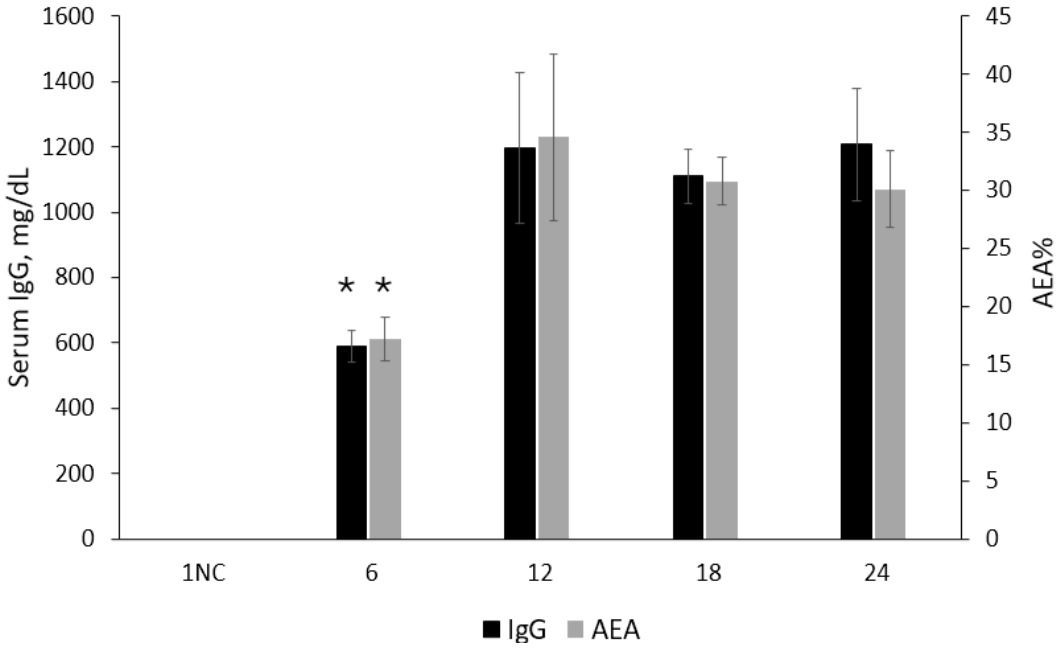


Figure 4.3 Serum and apparent efficiency of absorption of IgG in non-fed neonatal calves at 1 hr and in calves fed colostrum at 6, 12, 18, and 24 hrs, n = 6/treatment. Values for both serum IgG and AEA at 6 hrs were significantly different from those at 12 hrs of life ($P < 0.01$). One calf in the 1NC group had 82 mg/mL circulating IgG at birth but was not fed colostrum; these data were removed due to being an outlier. On average calves had >1000 mg/mL serum IgG in the 24C group, indicating transfer of passive immunity was achieved.

4.5.3 Tissue Integrity and Paracellular Permeability

FITC-dextran is used as a permeability marker for quality control to indicate tissue integrity and as a marker of paracellular transport (Hubbard et al., 2014; Bzik and Brayden, 2016). Our data showed linearity of FITC-dextran over time in a constant manner with no co-efficient variance overtime and thus concentration values were assessed at each time point instead of regression analyses. Mucosal concentration of FITC-dextran did not differ by intestinal tissue segment ($P = 0.92$, Figure 4.4).

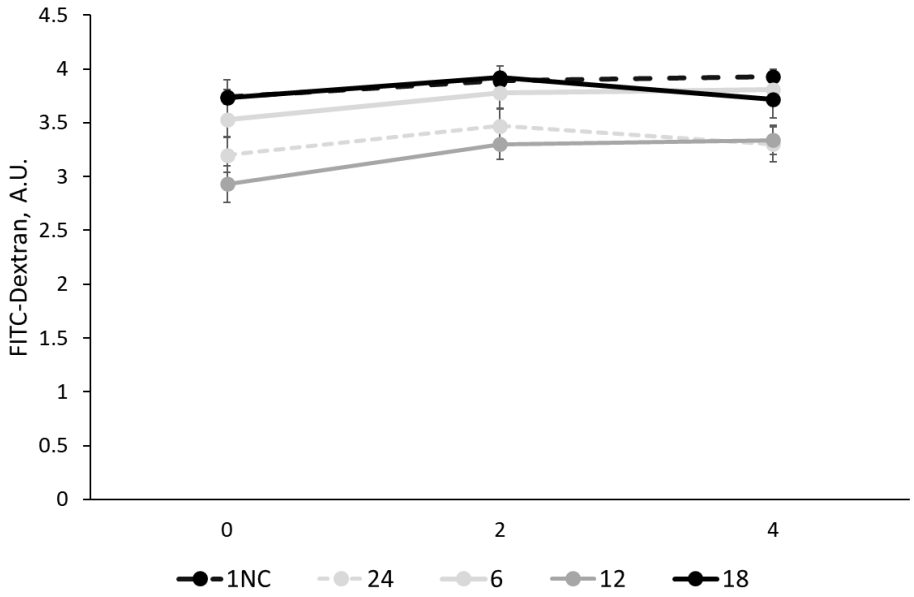


Figure 4.4 Total mucosal chamber FITC-dextran concentration in non-fed neonatal calves at 1 hr and in calves fed colostrum at 6, 12, 18, and 24 hrs, n = 6/treatment. FITC-dextran was in excess for the duration of the experiment, as planned.

FITC-dextran efflux, as indicated by serosal levels, was 66% higher in the youngest calves (1NC and 6C) than at all other time points (1NC, 0.03 ± 0.01; 6C, 0.03 ± 0.01; 12C, 0.01 ± 0.00; 18C, 0.01 ± 0.00; 24C, 0.01 ± 0.00 A.U., $P = 0.01$, Figure 4.5). FITC-dextran serosal efflux differed by intestinal tissue segment where the duodenum had 75% higher efflux than the distal intestine segments and the proximal jejunum had 66% higher efflux than the distal intestine segments (duodenum, 0.04 ± 0.01; proximal jejunum, 0.03 ± 0.01; distal jejunum, 0.01

± 0.00; ileum, 0.01 ± 0.00 A.U., $P = 0.01$). These data suggest the proximal intestine segments have greater paracellular transport of smaller molecules as indicated by FITC-dextran flux.

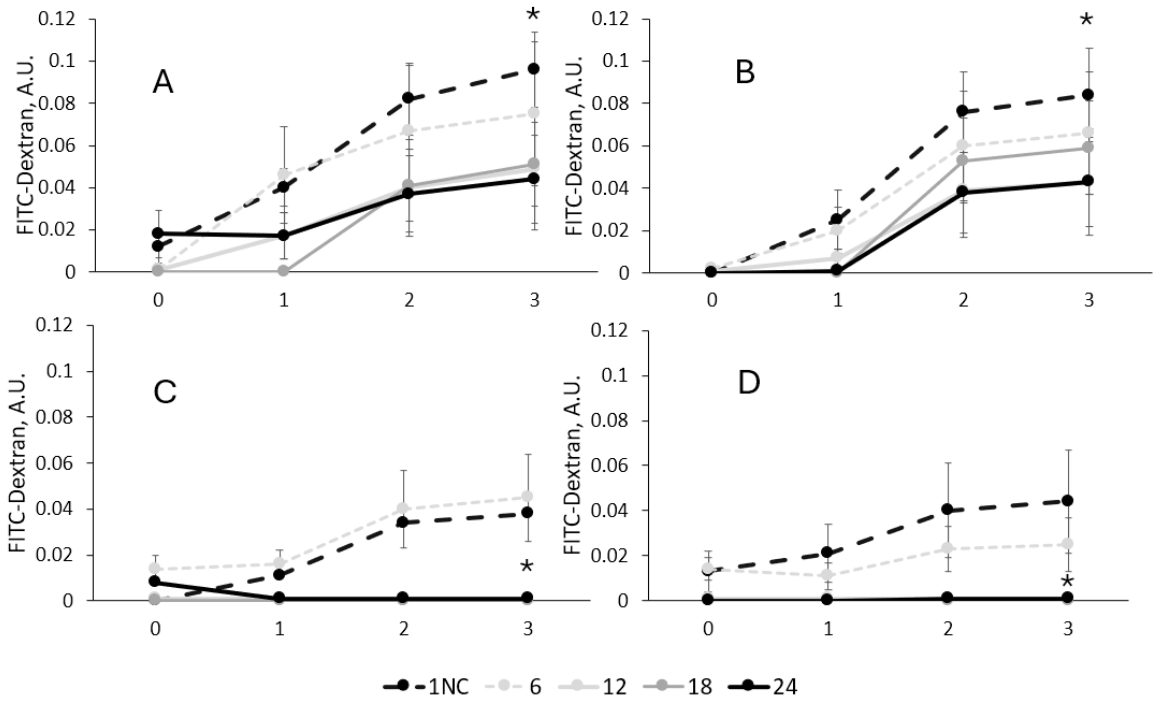


Figure 4.5 Serosal FITC-dextran efflux for non-fed neonatal calves at 1 hr and colostrum treated calves at 6, 12, 18, and 24 hrs, n = 6/treatment. There is an effect of treatment ($P = 0.01$), intestinal tissue segment ($P = 0.01$), and time ($P < 0.01$), plus an interaction of intestinal tissue segment × time ($P < 0.01$). A) duodenum, B) proximal jejunum, C) distal jejunum, and D) ileum all exhibit a similar pattern of absorption. There is more FITC-dextran transport in the 1NC and 6C groups than in other groups, and there is more transport in the proximal intestine than the distal intestine.

4.5.4 Digesta and Intestinal Tissue IgG Concentrations

Digesta IgG was measured to track the passage of IgG through the intestinal tract for the first 24 hrs of life, to better understand IgG clearance rate. Digesta IgG concentration was greatest in all tissue segments at 6 hrs post-colostrum feeding demonstrating that digestion and absorption occurred within 6 hrs and IgG was then cleared from the lumen at 6-12 hrs, likely into circulation and lymphatics after this time (reflecting normal physiology) (abomasum, 19.9 ± 2.92; duodenum, 10.9 ± 3.36; proximal jejunum, 12.6 ± 7.42; distal jejunum, 12.0 ± 5.49; ileum,

9.44 ± 3.71; cecum, 5.52 ± 3.14 mg/mL; $P < 0.01$, Figure 4.6). After 6 hrs of life, digesta IgG declined to close to 0 mg/mL in the abomasum and all segments of the small intestine but remained around a concentration of 5 mg/mL in the cecum until 24 hrs of life. These data demonstrate that IgG clears from the proximal small intestine tissue by 12 hrs, and from the distal small intestine contents by 18 hrs of life in calves fed at 1 hr of life. From these data it is not clear how long IgG will remain in the cecum, or how long it will take for the non-absorbed colostral IgG to leave the body. We did not measure IgG in feces – a limitation of this study – and so cannot confirm how much IgG was excreted and how much was retained in the tissues and lymphatics instead of passing into blood circulation.

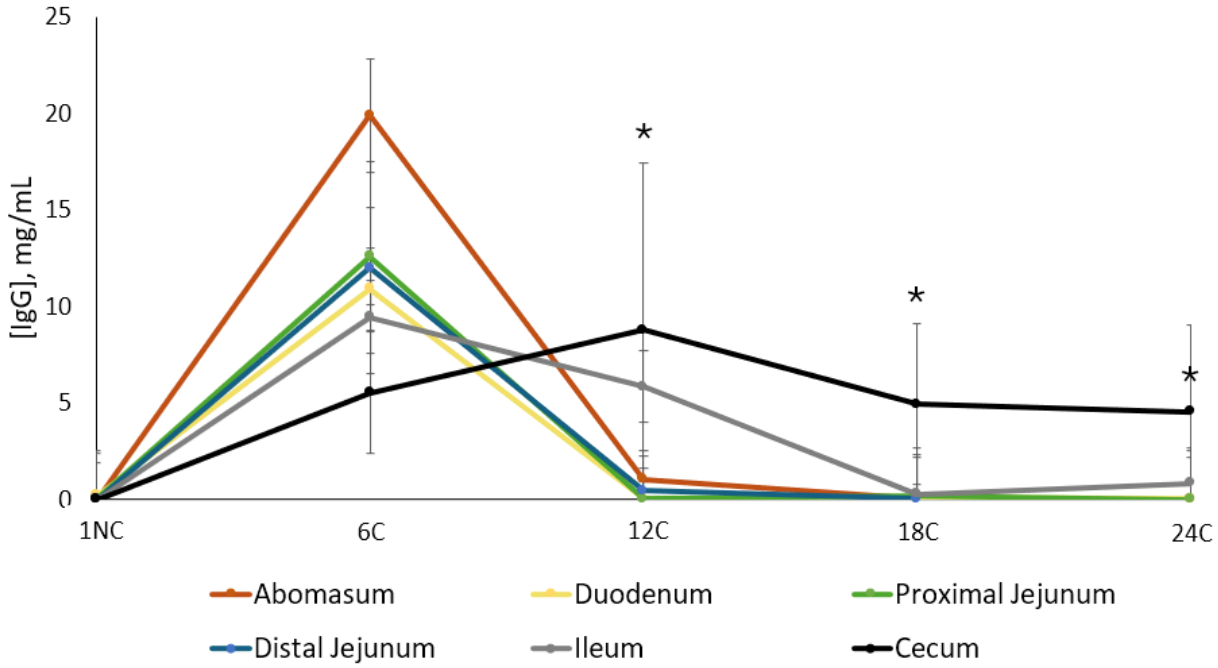


Figure 4.6 Digesta IgG concentration in non-fed calves at 1 hr and colostrum treated calves at 6, 12, 18, and 24 hrs. Data are presented as mean ± SEM, n = 6/treatment. Digesta IgG peaked in 6C calves before declining in every sampling location except cecum at 12 hrs of life. Digesta IgG concentrations remained constant in the cecum from 18 to 24 hrs of life. And declined to near 0 mg/mL in all other sampling locations.

Intestinal tissue IgG concentration was analyzed to better understand the amount of intestinal tissue uptake and storage of IgG during the first 24 hrs of life in the neonatal calf.

Intestinal tissue IgG concentrations were highest in the proximal and distal jejunum, especially in 6C and 12C calves (Proximal Jejunum – 1NC, 0.00 ± 0.00; 6C, 335 ± 169; 12C, 120 ± 46.8; 18C, 88.3 ± 21.7; 24C, 38.4 ± 12.1 mg IgG / g tissue | Distal Jejunum – 1NC, 0.00 ± 0.00; 6C, 72.6 ± 3.22; 12C, 161 ± 53.3; 18C, 5.58 ± 1.90; 24C, 7.23 ± 2.07 mg IgG/g tissue, $P < 0.01$, Figure 4.7), suggesting that in these locations, IgG is being absorbed and transported, but also could be remaining in the tissue at those locations. Duodenum intestinal tissue IgG concentrations were 98%, 95%, 96%, and 82% smaller than their proximal jejunum counterparts for 6C, 12C, 18C, and 24C calves, respectively.

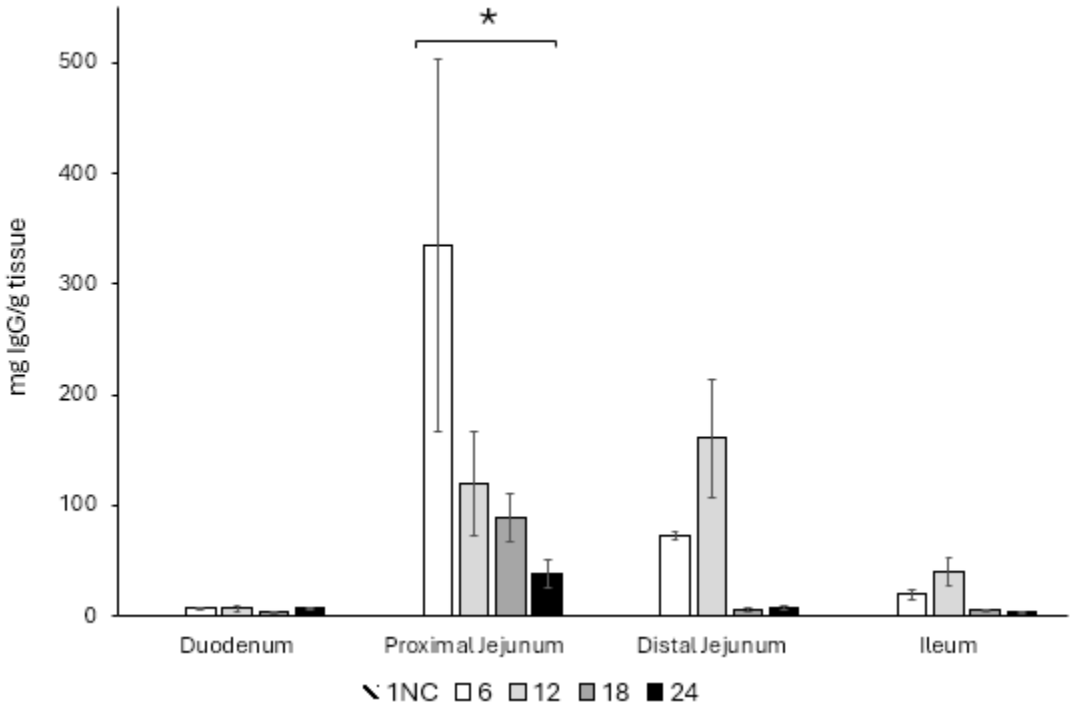


Figure 4.7 Intestinal tissue IgG concentration in non-fed calves at 1 hr and colostrum-fed neonatal calves at 6, 12, 18, and 24 hrs. Data presented as mean ± SEM, n = 6/treatment. 1NC calves were not fed colostrum and were euthanized at 1 hr of life. 6C, 12C, 18C, and 24C calves were fed one meal of colostrum within an hour of life and were euthanized at 6, 12, 18, or 24 hrs of life, respectively. Tissue IgG was highest in the proximal and distal jejunum, especially in the 6C and 12C groups ($P = 0.02$).

4.5.5 IgG Flux Across the Neonatal Intestinal Epithelium

IgG influx and efflux from small intestinal tissue segments was measured to assess IgG transport capacity throughout the first 24 hrs of life in neonatal calves. There was no difference between treatments for % of total mucosal IgG influx (1NC, 55.1 ± 3.06; 6C, 60.2 ± 2.57; 12C, 58.4 ± 4.76; 18C, 55.7 ± 5.35; 24C, 59.3 ± 5.84 %, $P = 0.90$, Figure 4.8 and Figure 4.9), indicating that IgG influx does not change with age in the first 24 hrs of life, a novel finding that goes against the concept of "gut closure". Since 130 mg/mL IgG (780 mg total) was loaded into the mucosal donor chamber, this represents an uptake of 373 mg/ cm^2 of tissue for 1NC, 408 mg/cm^2 of tissue for 6C, 393 mg/cm^2 of tissue for 12C, 377 mg/cm^2 of tissue for 18C, and 401 mg/cm^2 of tissue for 24C over a 2 hr period, which is equivalent to approximately 55-60% total uptake. This appears to be a large capacity for uptake into the intestinal tissue however it should be noted that this could also represent adhesion to the chamber plastic and mucosa brush border, as well as uptake into the epithelium and tissue. There were also no differences between intestinal tissue segments ($P = 0.57$), suggesting that IgG influx is not location dependent in the small intestine.

Serosal IgG efflux was highest in colostrum-fed groups at 0-1 hr and increased at 1-2 hrs in 1NC, 18C and 24 C groups. ($P < 0.01$). It was not until 2 hrs that there was an increase in 1NC efflux, indicating that, in all other treatment groups, IgG was fluxed through the tissue in the first hour, likely due to a higher concentration of IgG with colostrum feeding. In 1NC calves, efflux of IgG into the serosal chamber was likely below the limit of detection in the first hour, rather than no IgG being fluxed at all. The IgG could have been gathering in the epithelial enterocytes, intercellular vesicles, lamina propria, and vasculature and lymphatic structures instead of being transported out of the tissue as efflux. These data indicate a lag effect on IgG transport, and perhaps are an indication of the timing of vesicular transport.

The 18C group had the largest amount of efflux at hr 2 in the Ussing chambers, 26% and 32% more than both the 1NC and 12C groups respectively (1NC, 28.5 ± 7.71; 6C, 47.6 ± 10.9; 12C, 23.2 ± 8.73; 18C, 82. 3 ± 22.3; 24C, 46.8 ± 10.3 mg/mL, $P < 0.01$, Figure 4.10), indicating that at 18hrs of life, either an accumulation of fed colostral IgG was able to efflux, or that the capacity for IgG trans-tissue flux is increased. It is more likely that, during the first 18hrs of life, IgG was transported into the intestinal epithelium and remained in vesicles, the lamina propria, lymph vessels and blood vessels of the epithelium. Increased efflux at 18 hrs could represent

increased accumulation in the tissue prior to 18 hrs, as compared to a fed calf at 6 or 12 hrs of life. A younger calf would still be absorbing and accumulating IgG, whereas at 18 hrs old there may be a threshold reached for IgG absorption potential of the tissue.

During hour 3 of the experiment IgG efflux decreased in all treatment groups (1NC, 3.16 ± 1.22; 6C, 13.2 ± 0.97; 12C, 15.5 ± 2.5; 18C, 10.3 ± 1.86; 24C, 8.72 ± 1.64 mg/mL), indicating that vesicle transport/flux of IgG was decreased and may reflect a mechanism for IgG reuptake into the tissue, or that the tissue was simply dying by this point in the experiment Total protein analysis of the hour 3 samples had similar results with IgG levels decreasing, indicating that the IgG may have returned to the tissue rather than become denatured (data not shown). It could also be that IgG was retained in the tissue and efflux and vesicle emptying were slowed or stopped at hr3 of the experiment, or that the tissue was simply dying due to time outside of the calf However, confirmation of a lack of protein in the serosal chamber, and accounting for the dilution effect, leads to the conclusion that the IgG is somewhere other than in the serosal fluid. Proteins can become stuck to the chamber walls, or to the tissue surface, which is a limitation of our study, but for all treatments and chambers to experience a decrease in serosal concentration of approximately 90% suggests some of the IgG may be transported back into the tissue. The FcRn receptor, to be discussed in the next chapter, is involved in the transport of IgG into circulation and for salvage and extension of the IgG half-life. In the ex-vivo model of the Ussing chamber, it is not clear if FcRn could reuptake IgG into the tissue in this way. Adding TER measurements to this experimental model would have indicated tissue viability and integrity, and should be considered for future experiments.

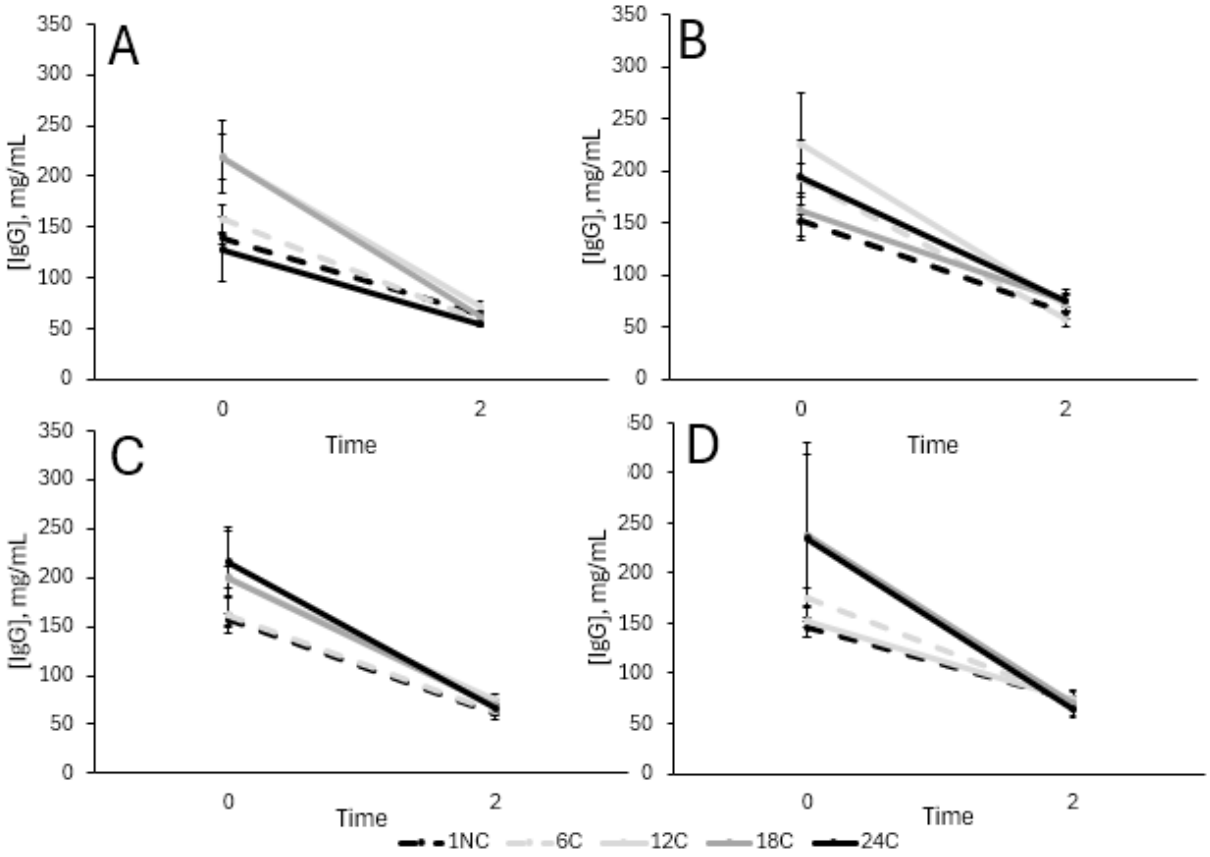


Figure 4.8 Mucosal influx of IgG into the intestine in non-fed at 1 hr and in colostrum-fed neonatal calves at 6, 12, 18, and 24 hrs of life. Data are presented as mean ± SEM, n = 6/treatment, $P = 0.90$. IgG concentration in the mucosal chamber decreased over time, with up to 60% in 2 hrs entering the tissue or remaining trapped in the brush border. There were no significant differences for A) duodenum, B) proximal jejunum, C) distal jejunum, or D) ileum.

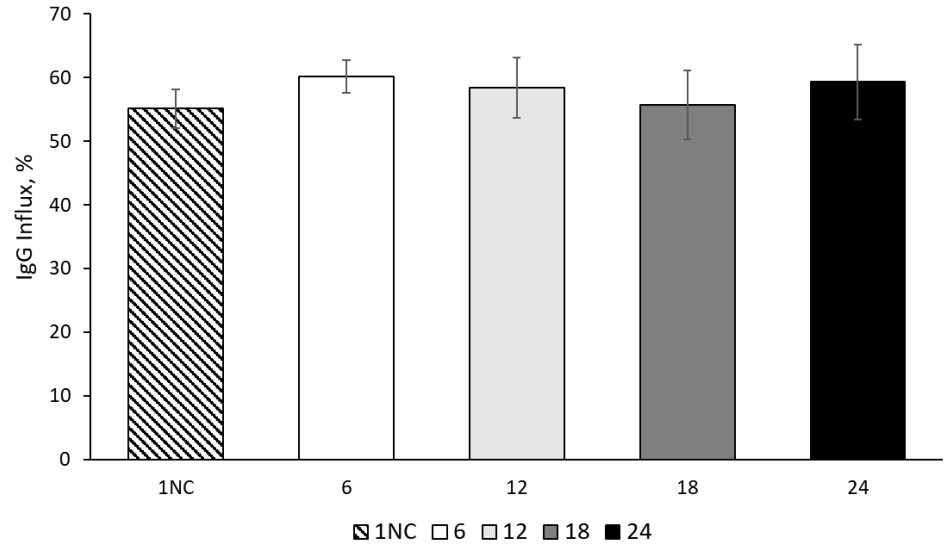


Figure 4.9 Total % mucosal influx of IgG into the intestine in non-fed at 1 hr and in colostrum-fed neonatal calves at 6, 12, 18, and 24 hrs of life. Data are presented as mean ± SEM, n = 6/treatment, $P = 0.90$. The initial measurement of 780 mg IgG loaded into the mucosal chamber (0hr) was considered 100%, and the measurement at 2 hrs was subtracted from 100% to obtain the % influx. There were no differences between treatments for mucosal IgG influx ($P = 0.90$).

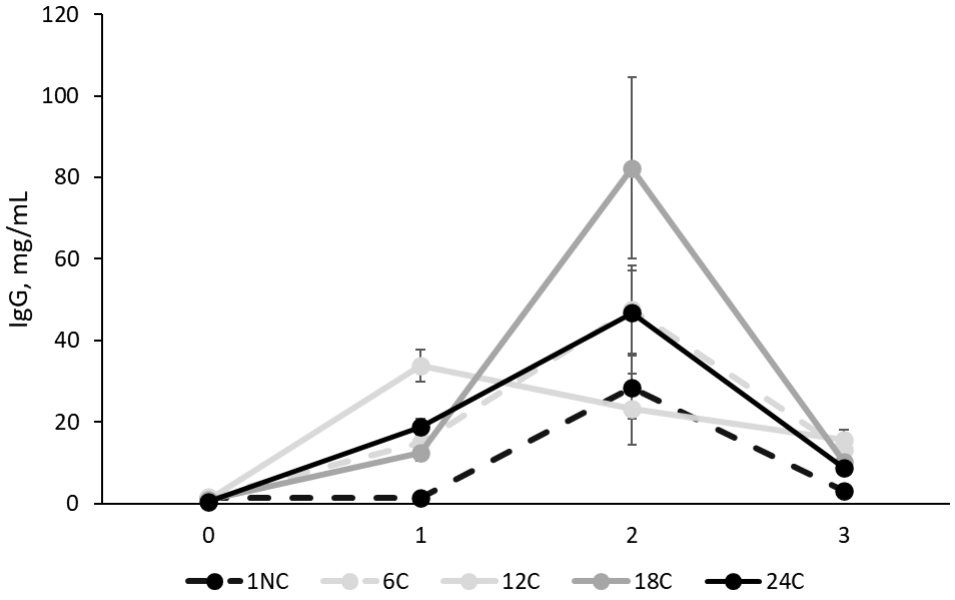


Figure 4.10 Serosal IgG efflux in the Ussing chamber in non-fed calves at 1 hr and colostrum treated calves at 6, 12, 18, and 24 hrs. Data are presented as mean ± SEM, n = 6/treatment. There

was no effect of treatment ($P = 0.11$) or tissue ($P = 0.51$) but there was an effect of time ($P < 0.01$). The 18C calves have the greatest serosal efflux of IgG at hour 2 of the experiment, and all treatments have a decrease in IgG concentration at hour 3.

4.5.6 Intestinal Morphology and IgG-containing Vesicles in the Small Intestine

A Periodic Acid-Schiff's stain was used to identify glycoproteins (IgG) within the intestinal villus epithelium.PAS-positive vesicles were noted in the intestinal epithelium and were counted to identify differences between treatment groups within the first 24 hrs of life. PAS-negative vesicles, suggesting IgG uptake capacity, were also noted and counted in the intestinal villus epithelium. The 12C calves had the greatest number of PAS-positive vesicles, especially in the distal jejunum (duodenum, 3.56 ± 2.59; proximal jejunum, 11.7 ± 2.89; distal jejunum, 42.1 ± 12.0; ileum, 27.6 ± 9.69 PAS-positive vesicles / 20 μm^2, $P < 0.01$, Figure 4.11, Figure 4.12). The number of PAS-positive vesicles increased until 12 hrs of life, and then decreased until 24 hrs of life (1NC, 0.05 ± 0.05; 6C, 15.4 ± 3.82; 12C, 21.2 ± 4.82; 18C, 11.9 ± 3.22; 24C, 4.94 ±1.46 PAS-positive vesicles / 20 μm^2 , $P < 0.01$, Figure 4.13), indicating an increase in absorption of IgG until 12 hrs of life and a decrease from 12 to 24 hrs of life.. For PAS-negative vesicle count, the beginning of the intestine (duodenum, proximal jejunum) differed from the end of the intestine (distal jejunum, ileum), where the beginning of the intestine had fewer PAS-negative vesicles (duodenum, 3.38 ± 1.42; proximal jejunum, 3.76 ± 1.20; distal jejunum, 11.6 ± 1.78; ileum 15.3 ± 1.20 PAS-negative vesicles/20 μm^2, $P < 0.01$). This suggests that IgG uptake capacity was greatest in the distal intestine, as has been previously reported (Jochims et al., 1994c).

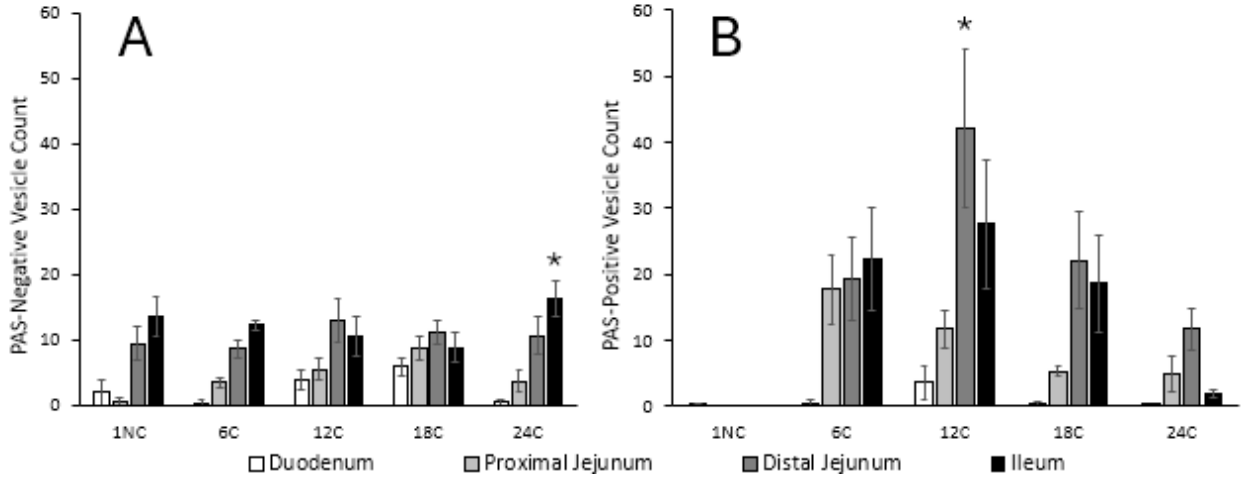


Figure 4.11 PAS-negative and PAS-positive vesicle count in non-fed calves at 1 hr and colostrum treated calves at 6, 12, 18, and 24 hrs. Data are presented as mean ± SEM, n= 6/treatment. (A) PAS-negative vesicles were present in all treatment groups though varied by tissue ($P < 0.01$), with the greatest number in the distal jejunum and ileum and (B) PAS-positive vesicles were present in all groups except 1NC, spiking in the distal jejunum in the 12C group ($P < 0.01$)**.**

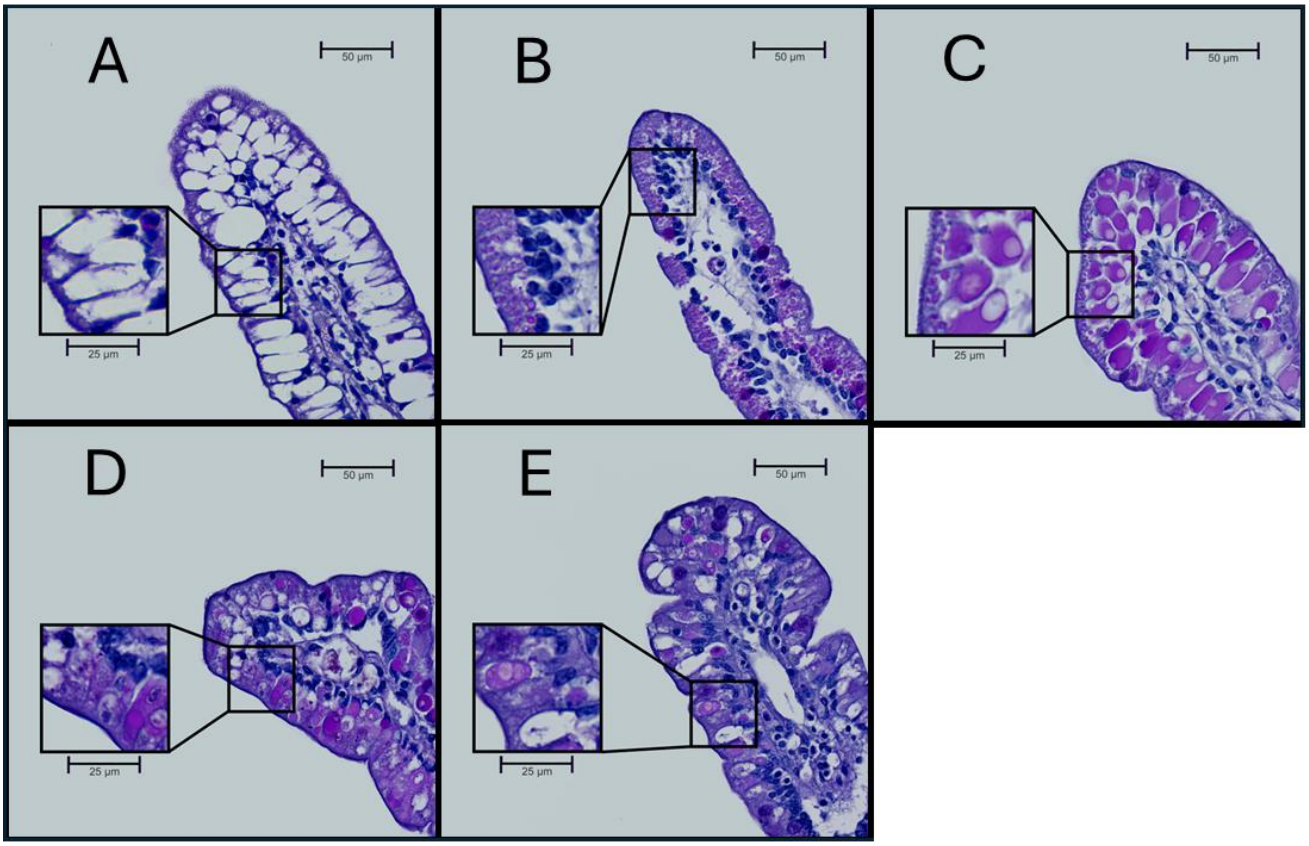


Figure 4.12 Histological comparison of distal jejunum tissue for non-fed neonatal calves at 1 hr and colostrum treated calves at 6, 12, 18, and 24 hrs, n = 6/treatment. A) 1NC calves have large, empty vesicles while B) 6C calves have numerous small pink PAS-positive vesicles. C) 12C calves have large PAS-positive vesicles and D) 18C calves have a variety of sizes of vesicles. E) By 24 hrs of life, 24C calves have some full and some empty vesicles.

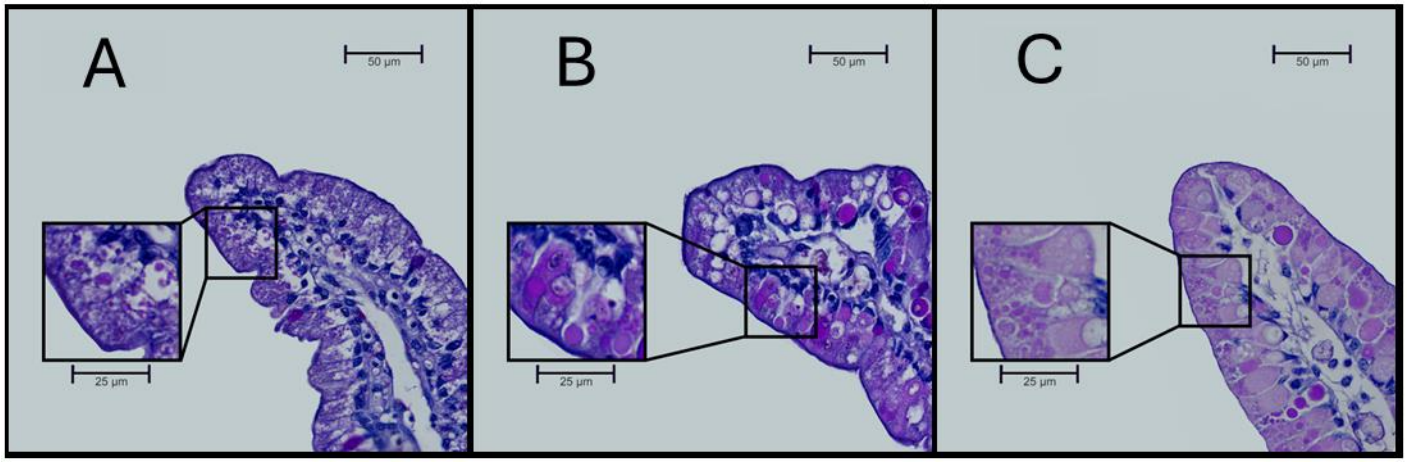


Figure 4.13 Histological comparison of three distal jejunum villi from 18C calves, demonstrating the variation found even within calves treated, fed, and euthanized at the same time. 18C calves were fed one meal of colostrum within an hour of life and were euthanized at 18hrs of life. A) Small sparse vesicles are seen, B) large full vesicles are seen with empty vesicles, and C) large and small full vesicles are seen with few empty vesicles

Basic morphometric measurements were taken to assess if there were differences in intestinal surface area over time in the neonate within the first 24 hrs of life, following colostrum-feeding, as these measurement reflect GIT development and absorptive area-capacity (Paulsen et al., 2003). Villi height and crypt depth were not significantly different, however VH and CD both had a tendency to increase over time from 6 to 24 hrs following colostrum feeding(1NC, 525 ± 21.7; 6C, 422 ± 35.6; 12C, 459 ± 21.4; 18C, 449 ± 21.5; 24C, 466 ± 19.0; µm, $P = 0.054$, Table 4.2), and with 1NC having the greatest VH and CD although not significantly different from other groups. There were no differences between villi height:crypt depth ratio over time from 6-24 hrs following colostrum feeding ($P = 0.89$).

Villi height did not differ by intestinal tissue segment ($P = 0.44$) but crypt depth did differ by intestinal tissue segment (duodenum, 165 ± 10.2; proximal jejunum, 140 ± 5.72; distal jejunum, 118 ± 4.53; ileum, 131 ± 6.62, $P < 0.01$), with deeper crypts in the duodenum and proximal jejunum than in the distal jejunum and ileum. These data are similar to those that have been previously reported in neonatal Holstein bull calves (Yang et al., 2015), suggesting that our calves had normal physiology at birth. Crypt depth also tended to differ by treatment (1NC, 149 ± 7.43; 6C, 122 ± 5.93; 12C, 141 ± 8.00; 18C, 143 ± 8.95; 24C, 132 ± 7.89 µm, $P = 0.06$).

The villi height:crypt depth ratio was significantly higher in the distal jejunum overall as expected as this is the absorptive segments of the GIT (duodenum, 3.36 ± 0.27; proximal jejunum, 3.39 ± 0.21; distal jejunum, 4.40 ± 0.28; ileum, 3.88 ± 0.23, $P = 0.01$).

Table 4.2 Villi height, crypt depth, and villi height:crypt depth ratio (V:C ratio) in non-fed neonatal calves at 1 hr and colostrum treated calves at 6, 12, 18, and 24 hrs. Data are presented as mean ± SEM, n = 6/treatment. There was a tendency for treatments to differ from each other for villi height ($P = 0.054$) and crypt depth ($P = 0.06$), with 1NC having the longest villi and longest crypt depths.

	1NC	6C	12C	18C	24C	*P*-Value
Villi height (µm)	525 ± 21.7	422 ± 35.6	459 ± 21.4	449 ± 21.5	466 ± 19.0	0.054
Crypt depth (µm)	149 ± 7.43	122 ± 5.93	141 ± 8.00	143 ± 8.95	132 ± 7.89	0.06
V:C ratio	3.72 ± 0.27	3.53 ± 0.31	3.51 ± 0.25	3.40 ± 0.31	3.72 ± 0.22	ns
		Duodenum	Proximal Jejunum	Distal Jejunum	Ileum	*P* -Value
Villi height (µm)		492 ± 21.0	458 ± 19.0	471 ± 21.3	441 ± 26.8	ns
Crypt depth (µm)		162 ± 7.09	144 ± 6.32	121 ± 6.21	121 ± 4.80	< 0.01
V:C ratio		3.18 ± 0.19	3.29 ± 0.17	4.16 ± 0.27	3.72 ± 0.27	0.01

4.6 Discussion

4.6.1 Effect of the First 24 hrs of Life on Intestinal Morphology in the Neonatal Calf

Villi height and crypt depth are indicators of intestinal growth and development in livestock species and humans, and the ratio of villi height:crypt depth can indicate absorptive capacity (Keating et al., 1995), can be altered in injury and disease (Hampson, 1986). In piglets, the villi height:crypt depth ratio decreased in weaned animals, as soon as 24 hrs after the completion of the weaning transition (Hampson, 1986), providing evidence of morphological changes within a 24 hr time period. In this study, villi height and crypt depth tended to decrease between 1NC and 6C calves; we cannot find other evidence in the literature of morphological changes occurring within 5hrs. It is possible that 1NC calves had greater villi heights and deeper crypt depths to begin with; one theory that may explain the difference in morphological features is that gestational age may have differed between treatments in this study. We do not have the data to confirm this, but cows can give birth weeks before or weeks after their due date (Fitzgerald et al., 2015). A calf born early and a calf born late may have morphological differences at birth, due to the length of gestation, and we do not know the gestational ages of the calves in this study. While it is expected that the villi height decrease and crypt depth increase in size with age, it is not expected to occur within the timeframe of this study.

4.6.2 Effect of the First 24 hrs of Life on IgG Transport in the Neonatal Calf

To assess transfer of passive immunity (TPI), most farms take a blood sample at day 1 of life and analyze the sample for total protein concentration, indicative of serum IgG concentration (Correa et al., 2022). In this study, TPI was achieved by 12hrs of life and serum IgG concentrations remained above the failure of transfer of passive immunity threshold of 1000 mg/dL (Lee et al., 2008) through to 24 hrs of life. IgG uptake in the neonatal calf is rapid and efficient for the first hrs of life – IgG concentrations were halfway to successful TPI (500 mg/dL serum IgG) by 6 hrs of life, or only 5 hrs post-feeding. In addition, IgG was cleared from the small intestine digesta by 12 hrs of life, though whether this was normal passage rate or an effect of IgG influx is not clear. IgG concentrations in the tissue were highest in the proximal jejunum at 6 hrs of life, reaching upwards of 50% IgG content in total protein of the tissue sample. These data show the incredible ability of the neonatal calf to intake large amounts of IgG in a relatively

short amount of time (5hrs post feeding). Interestingly, efflux of IgG at 6 hrs of life followed a similar pattern to efflux of IgG at 24 hrs of life, increasing at the same rate, despite age differences and supposed "gut closure" at 24 hrs of life. From these data, gut closure does not appear to occur at 24 hrs of life. While the intestinal epithelium and lamina propria have a large capacity to intake and store IgG, it does not hold onto all of the IgG and instead steadily shuttles it either back into the lumen, or forward into circulation (He et al., 2008) .

There was no effect of age of the calf on mucosal influx of IgG in the first 24 hrs following colostrum feeding. Unfed calves in the 1NC group had the same capacity to uptake IgG into the tissue as 24C fed calves, even though the latter had not been fed for 23 hrs. The absorption-permeability of IgG through tissue remains intact at 24 hrs of life, despite considerations of "gut closure" based on serum IgG levels.

IgG efflux also did not differ by age. As described in chapter 3, fed calves had efflux of IgG during the first hour of the Ussing experiment, while unfed calves did not have measurable efflux of IgG until the second hour. It appears that fed calves have a store of IgG in the tissue from colostrum uptake which is released within one hour, while full transcytosis takes up to two hrs to occur. The 18C group had the greatest amount of IgG transport at two hrs in the Ussing chamber – 26% and 32% more efflux than both the 1NC and 12C groups respectively. It is not clear why there was a spike in IgG efflux at 18 hrs of life, as this has historically been thought to be a time point of declining IgG absorption potential (Stott et al., 1979b). However, the data in this study show that IgG uptake potential is intact at 24 hrs of life, so it is possible that our historical assumptions about IgG absorption in neonatal calves are incorrect.

We assessed PAS-positive vesicle counts in neonatal calf intestinal tissue to estimate colostrum-IgG uptake at each time point following feeding. The PAS stain reveals the presence of glycoproteins (IgG), and of PAS-negative, or "empty" vesicles indicating IgG uptake potential. The number of PAS-positive vesicles peaked at 12 hrs of life, coinciding with the maximal tissue IgG content in the same intestinal tissue segment, preceding the increased IgG efflux at 18 hrs of life. An increase in PAS-positive vesicles does not correspond directly to an increase in IgG efflux but may precede IgG efflux. These data provide evidence that vesicle transport is occurring before IgG efflux, as is normal physiology.

4.6.3 Effect of Intestinal Segment Type on IgG Transport

Intestinal tissue segment type did not have an effect on the influx or efflux of IgG. In chapter 3 we examined the effect of colostrum feeding on IgG flux and found that IgG was preferentially absorbed in the distal intestine. When examining the time course of the first 24 hrs as a whole in chapter 4, we do not have any difference in influx or efflux between tissue segments, suggesting that colostrum feeding has a greater effect on IgG transport location than the age of the calf over the first 24 hrs of life.

The greatest number of PAS-positive vesicles were present in the distal jejunum, in contrast to data from Jochims et al (1994c) who noted the vesicles mostly in the ileum. Jochims, however, did not note exactly where their distinction between jejunum and ileum lies, and we took a distal jejunum sample at 100 cm proximal of the ileal-cecal junction. It is possible that our "distal jejunum" and Jochims' "ileum" are one and the same. Our data show that Thus far, there is no specific link between the PAS-positive vesicles and IgG absorptive capacity, as even those who have investigated IgG uptake on a tissue level have not measured the kinetics of transport (Stott et al., 1979a; Jochims et al., 1994d; Kaup et al., 1996).

4.6.4 Paracellular Transport in the Neonatal Calf

12C and 24C groups had increased transport of FITC-dextran into the intestinal epithelium, which may indicate a decrease in tissue integrity in mature tissues (Thomson et al., 2019), though the kinetics of paracellular transport in neonates is largely unstudied (Rouwet et al., 2002). Efflux of FITC-dextran differed by hour following colostrum feeding and by intestinal tissue segment, with approximately 61% more transport occurring in proximal intestine than in the distal intestine. In addition, the younger time points (1NC and 6C) had 47% more FITC-dextran efflux demonstrating permeability than the older timepoints (12C, 18C, and 24C), indicating an age effect on paracellular transport.

4.6.5 Gut Closure Timing

There was very little IgG measured in the intestinal tissue or in the lumen contents (digesta) at 24 hrs of life (0.7 to 3.8% IgG in tissue and 4.54 mg/mL in digesta in the cecum only), as compared to 6hrs of life (6% to 33.5% IgG in tissue and up to 19.9 mg/mL in abomasal digesta). Despite the intestinal tissue IgG content, IgG transport capacity into the tissue remained intact at 24 hrs of life. It has long been assumed that IgG transport capacity is extremely low at

24 hrs of life, since age of the calf is known to affect the amount of IgG that is transported into serum (Hopkins and Quigley, 1997). With serum IgG levels of utmost importance for calf health and survival (Windeyer et al., 2014), the findings of the current study bring into question when the gut actually "closes". Our data shows serum levels peaked at 12 hrs of life, but IgG transport did not cease at 12hrs of life. From our data the first colostrum meal passes by the absorptive sites in the small intestine by 12-18 hrs of life, ending up in the cecum and more distal sections of the intestine. Data from the current study suggest that it is not the capacity of the tissue to uptake IgG that limits absorption - if the transport capacity for IgG uptake is intact, a second meal of colostrum could be beneficial to calves, and indeed other studies have found a second feeding to improve serum IgG levels (Hopkins and Quigley, 1997; Jaster, 2005; Hare et al., 2020). In the abomasum, feeding increased the pH at 6 hrs of life but the pH steadily dropped thereafter, reaching a low of 2.7 at 24 hrs of life. Acidification of the abomasum occurs relatively quickly after the first feeding which is similar to humans where gastric acid is secreted within 3-6 hrs post birth (Ames, 1959). To our knowledge, abomasal pH has not been measured in neonates of other species.

4.6.6 Limitations

The colostrum replacer product used in this study (Premolac Plus, Zinpro, 135g IgG) provided a consistent amount of IgG to each calf, allowing us to analyze the effect of age on IgG uptake and transport. Without consistent colostrum composition, comparisons could not be made between our treatment groups. It is a limitation to the study, however, that we could not both keep the amount of IgG administered the same, and account for differences in calf body weights at birth. Feeding IgG by bodyweight may have reduced some of the variation seen in tissue IgG concentrations, because the inclusion of IgG per kg would have been constant. PAS-positive vesicles most likely contain IgG, as seen by Jochims et al (1994c), though in this study we did not confirm the presence of IgG within the vesicles. PAS staining stains for glycoproteins, of which IgG is in the greatest quantity in colostrum, but there are also other immunoglobulins (IgA and IgM) that could be occupying the vesicles.

4.7 Conclusion

In this study, we evaluated the impact of the first 24 hrs of life on IgG transport, paracellular transport, and unique PAS-positive or PAS-negative vesicles in the neonatal calf.

Despite previous claims, the 24 hr old nutritionally stimulated intestine is capable of the same amount of IgG transport as the 1 hr old unfed intestine. This phenomenon, in combination with our observations of PAS-positive vesicles and digesta passage through the intestinal tract, indicate that a second feeding of colostrum could be readily absorbed by the calf. Paracellular transport in the calf occurs preferentially in the proximal small intestine, unlike IgG transport, which was equal across all intestinal tissue segments. Though we do not completely understand the mechanisms of absorption, it is clear that the neonatal calf small intestinal tissue is not losing the capacity to uptake macromolecules as quickly as previously believed

5.0 FcRn and IgG Within the Neonatal Calf Intestine During the First 24 hrs of Life

5.1 Abstract

The Fc receptor of the neonate (FcRn) is involved in the transport of immunoglobulin G (IgG) across the intestinal epithelium in most mammals. Despite this, absorption of IgG in the bovine calf has been called "non-selective" for the first 24 hrs of life, suggesting that absorption occurs without the aid of a receptor. This study examined the colocalization between IgG and FcRn along with markers of lymphatic and endothelial vasculature to further our understanding of the mechanism of IgG transport in the small intestine of the neonatal calf. Holstein × Angus bull calves (n = 30) were assigned to one of five treatment groups in a randomized complete block design: one group not fed colostrum and euthanized at 1 hr of life (1NC), serving as a baseline control group, and four groups fed colostrum within an hour of life and euthanized at 6 (6C), 12 (12C), 18 (18C) or 24 hrs of life (24C), serving as a time-course throughout the first day of life. At euthanasia, intestinal tissue samples were collected for histological analysis and IgG content, and intestinal luminal contents were collected for IgG content. Intestinal tissue IgG abundance as measured by immunofluorescence did not differ by time or colostrum treatment in the first 24 hrs of life but did differ by intestinal tissue segment, with the greatest amount of IgG present in the distal jejunum and ileum villi, as expected. IgG was observed in all small intestinal segments at birth with and without colostrum feeding and this is a novel finding. FcRn was colocalized with the lymph vessel marker PROX1, and weakly colocalized with the blood vessel marker VEGFR2, indicating that FcRn may have greater transport activity in lymphatic vessels compared to blood vessels in the villus and lamina propria. FcRn abundance did not differ by intestinal tissue segment but did differ by age at euthanasia, where unfed calves at 1 hr post-partum had the greatest abundance of FcRn within intestinal villi. A greater amount of FcRn at 1 hr post-partum suggests that the neonatal intestine is primed ready to transport IgG.

FcRn was strongly colocalized with IgG in all sections of the small intestine throughout the first 24 hrs of life. These data show that FcRn is most abundant at 1 hr in the neonatal calf and is colocalized to IgG within the intestinal tissue in the first 24 hrs of life, suggesting that, IgG transport in the calf is at least partially transporter dependent rather than transported entirely by non-selective permeability of the intestinal epithelium.

5.2 Introduction

Colostrum feeding is essential for the bovine neonate to obtain passive immunoglobulin immunity (Lombard et al., 2020), for protection against intestinal pathogens, establishment of the intestinal microbiome, and for development of neonatal mucosal and systemic immunity (Ma et al., 2019). The protein fraction of bovine colostrum contains 65 - 90% immunoglobulin G (IgG) (Puppel et al., 2019), making IgG one of the most abundant components of colostrum and the dominant immunoglobulin for immune protection of the neonatal gut shortly after birth. Immunoglobulins A and M are also present in bovine colostrum, constituting each 7% of the total immunoglobulin content (Stelwagen et al., 2009). IgA primarily remains in the lumen of the intestine on the epithelial surface, and IgM appears to be transported into circulation at a similar rate to IgG (Butler, 1969), and they both utilize a different receptor to IgG (Rojas and Apodaca, 2002). Factors influencing the transfer of passive immunity, typically defined in bovines by serum IgG concentrations, include dam factors (colostrum quantity and quality), producer factors (administration of colostrum), and calf factors (absorptive capacity; McGuirk and Collins, 2004). IgG absorption in the neonatal calf has primarily been investigated on a systemic level, with a focus on serum IgG levels to indicate bioavailability, however there is limited data on IgG absorption at the intestinal tissue level (Jaster, 2005; Windeyer et al., 2014), There is extensive knowledge of ideal colostrum collection and storage conditions, and IgG content requirement, (Argüello et al., 2003; Cummins et al., 2017) as well as best practices for administration of colostrum (Lombard et al., 2020), however these are based on a lack of information on intestinal absorption and passive transfer of IgG.

In the calf, bioavailability of IgG as determined by serum IgG concentrations has been shown to occur within the first 24 hrs of life (Stott et al., 1979b) and the uptake is generally considered to be “non-selective” or occurring without the aid of a receptor (Besser et al., 1985; Geiger, 2020). However, the neonatal Fc receptor (FcRn) has recently been identified in the bovine small intestine (Zhu et al., 2020) and has been previously identified in the bovine mammary gland (Kacskovics, 2004). The FcRn receptor functions to transport IgG throughout many tissues (Pyzik et al., 2019), although it is not yet known if FcRn functions in the intestine to transport IgG into the bloodstream or lymphatics in neonatal calves.

FcRn has been shown to transport IgG from the lumen of the gut into systemic circulation in the rat (Rodewald and Kraehenbuhl, 1984), human, and rabbit (Pyzik et al., 2015). In addition,

FcRn is responsible for transcytosis of IgG across the placental barrier in neonates (Latvala et al., 2017). Though previous chapters of this thesis have described IgG inside of intracellular vesicles, those vesicles will also fuse with lysosomes for degradation, though the extent to which this occurs in the neonatal intestinal epithelium is unknown. In the adult animal, FcRn salvages IgG from lysosomes that would normally degrade the IgG by binding to IgG in the acidic pH of the lysosome and returning it to the intestinal lumen or passing it into circulation (Lencer and Blumberg, 2005), thereby extending the half-life of IgG (Rath et al., 2013). By returning IgG to the lumen of the intestine or transporting it into systemic circulation, FcRn contributes to both immune surveillance and host defense depending on the direction of IgG transport (Tzaban et al., 2009). Specific maternal IgGs that are recycled to the lumen of the gut by FcRn can manipulate the neonatal microbiome by contributing to specific immune responses against pathogens, helping to promote commensal populations and regulate pathogenic populations (Sanidad et al., 2022). The efflux of IgG back out into the intestinal lumen is therefore just as important to host defense as is the transport of IgG into the mesenteric lymph and circulatory system, and the systemic lymphatic and circulation. IgG and FcRn binding is highly pH dependent (He et al., 2008); with binding optimized at pH of 6 to 6.5, and unbinding at pH of 7, therefore the pH of the brush border membrane, basement membrane, or cytosol microenvironment important for the binding, transport, and release of IgG (He et al., 2008). It is not known how the pH of the intestinal digesta, which can be at pH 7 or above, impacts IgG-FcRn binding in the neonatal calf.

IgG is of utmost importance to the neonatal calf for passive immunity and protection of the intestinal epithelium, but the transport of IgG through the neonatal intestine in the bovine calf is understudied and the involvement of FcRn in IgG transport to systemic circulation unknown. This study examined the abundance as well as physical localization of IgG and FcRn within the neonatal small intestinal epithelium. It also examined the physical location of FcRn in relation to the lymphatic and blood vessels within the intestinal villi. We hypothesized that FcRn and IgG would be closely colocalized and that age would decrease the abundance of both IgG and receptor in the neonate intestine in the first 24 hrs post-partum. In addition, we hypothesized that FcRn would be closely colocalized to both the lymphatic and blood vessels, indicating selective receptor uptake of IgG in the neonatal calf.

5.3 Materials and Methods

5.3.1 Calves and Study Design

Between October 2021 and March 2023, Holstein × Angus bull calves (n = 30) were born on a commercial farm. At birth, calves were 1. removed from their dam before they could stand and suckle 2. weighed and assigned to a treatment group balanced by birthweight and 3. placed in an individual hutch bedded with straw until the time of euthanasia.

Calves were assigned to one of five treatment groups (n = 6). One of the treatment groups was not fed colostrum and was euthanized at 1 hr of life (1NC); this group served as a baseline group. The remaining groups were euthanized at 6 (6C), 12 (12C), 18 (18C) or 24 hr24 hrs of life (24C); these groups represent a time-course to observe the effect of age throughout the first 24 hrs of life. All calves were euthanized using a captive bolt gun and exsanguination. All animal procedures were approved by the University of Alberta (AUP #3673).

5.3.2 Colostrum Treatment

A commercial colostrum replacer containing 135 g IgG in 1.3 L of warm water was provided via an esophogeal feeding tube (Premolac, Zinpro Inc.). The colostrum replacer in question only contains the whey fraction of colostrum and is devoid of fat and casein, which would naturally be removed during the clotting process in the abomasum of the calf (Hocquette and Bauchart, 1999).

5.3.3 Intestinal Dissection and Digesta pH

After euthanasia, intestinal tissue samples were taken from the duodenum, proximal jejunum, distal jejunum, and ileum, were washed in PBS, and then preserved in 10% buffered formalin for histological analysis. Luminal contents (digesta) were taken from the same locations and snap-frozen in liquid nitrogen. Digesta pH was measured at the time of sampling.

5.3.4 Intestinal Histology

Immunofluorescence techniques were used to localize and quantify FcRn, IgG, PROX1 (lymphatic vessel marker), and VEGFR2 (blood vessel marker). After 24 hrs in 10% buffered formalin for fixation, samples were transferred to a 70% ethanol solution until they were processed, encased in paraffin, and 5-µm thick sections were mounted on slides (Al-Sabawy et al., 2021). Slides were then stained using an immunofluorescence technique from Laarman et al

(2013). Briefly, slides were dewaxed using 100% tolune and were then rehydrated via a decreasing ethanol gradient (100%, 90%, 70%, 50%, tap water). Antigen retrieval was performed by boiling slides in a 10mM sodium citrate buffer for 15 mins (95°C). Primary antibodies were incubated on slides in a humidity chamber for 90 mins. Primary antibodies used were anti-bovine IgG (1:150 dilution, monoclonal, rat host, Mabtech MT134), anti-bovine FcRn (1:200 dilution, polyclonal, rabbit host, Biomatik custom antibody), anti-mouse PROX-1 (1:100 dilution, monoclonal, Novus Biologicals), anti-rat VEGFR2 (1:200 dilution, monoclonal, Abcam). PROX1 was used as a lymph vessel marker (Kong et al., 2017) and VEGFR2 was used as a blood vessel marker (Meissner et al., 2011). A positive control of non-modified IgG was used to assess the validity of the anti-bovine IgG antibody as it has not been previously used in IF staining procedures. After primary incubation, slides were rinsed 7 times with PBS and then incubated with secondary antibodies, in the dark in a humidity chamber, for 30 mins. Secondary antibodies (Goat ant-Rabbit IgG, Alexa 488, Invitrogen; Goat anti-mouse IgG, Alexa 568, Abcam Biochemicals; Goat anti-rat IgG, Dylight 680; Novus Biologicals) were selected so that the excitation and emission of the fluorophores were at least 100 nm different from each other to avoid spectral overlap. Slides were rinsed 7 times after the secondary antibody incubation, and coverslips were mounted with Prolong Anti-fade Gold with DAPI nuclear stain.

Slides were stained with two targets to obtain colocalization results. Staining pairs were as follows: FcRn:IgG, FcRn:PROX1, FcRn:VEGFR2. Intensity of fluorescent signal of an entire villi per image was measured for FcRn and IgG, and that intensity was divided by the number of nuclei in the villi to obtain intensity per cell measurements. Nuclei were counted using the DAPI nuclear stain. Localization within sections of the villi was also calculated. Localization was achieved by measuring the intensity in the bottom 1/3, middle 1/3 and top 1/3 of intestinal epithelial villi. Colocalization correlations were calculated using CellProfiler, a program which can compare two channels of a single fluorescent image and analyze physical colocalization.

5.4 Statistical Analysis

Using six animals per treatment allows us to detect, with 80% power, a difference of 20% between treatment means for all variables when the coefficient of variation within treatments is 10% (Berndtson, 1991). Data were analyzed using a two-way ANOVA (colocalization, localization, intensity) in R (RStudio 2023.03.1+446 "Cherry Blossom") to look at the effect of

intestinal tissue segment and treatment. A students T-test was run to identify if PROX1:FcRn colocalization differed from VEGFR2:FcRn colocalization. Outliers were removed from analysis by using a 1.5 interquartile range (25^{th} – 75^{th} percentile).

Data were analyzed according to the following model:

$$Y = \mu + T_i + \varepsilon_{ij}$$

where Y is the response, μ is the overall mean, T_i is the treatment effect, and ε_{ij} is the residual error. Significance was declared at $P < 0.05$ and tendencies were declared at $0.05 \leq P < 0.10$. Numbers displayed are least squares means ± standard error of the mean, unless otherwise indicated.

5.5 Results

5.5.1 IgG and FcRn Abundance

Immunofluorescence staining was utilized to evaluate the location and abundance of the FcRn receptor in the neonatal calf in relation to bovine IgG. Strong positive colocalization of FcRn and IgG would suggest binding interaction, however this technique does not definitively prove an interaction between the two proteins. While one form of FcRn has been identified in the bovine small intestine epithelium, and three other forms identified in neonatal immune cells via mRNA gene expression analysis (Zhu et al., 2020), to our knowledge FcRn, in any form, has not been localized or measured via an immunofluorescence technique to determine physical localization of the receptor within the intestinal tissue. This technique was utilized to identify only the small intestinal epithelial form of FcRn.

5.5.2 IgG Abundance

IgG intensity per cell in the intestinal villi, specifically in the distal jejunum, was 41%, 42%, and 15% greater than the duodenum, proximal jejunum, and ileum, respectively (duodenum, 4.98 ± 0.58; proximal jejunum, 4.86 ± 0.52; distal jejunum, 8.37 ± 1.71; ileum, 7.15 ± 0.67 A.U., $P = 0.03$, Figure 5.1 and Figure 5.3). There was no effect of treatment on IgG intensity per cell in the intestinal villi ($P = 0.60$), indicating that IgG abundance in the intestinal epithelium did not change over the first 24 hrs of life. However, IgG abundance measured as

intensity per cell in the intestinal villi of the distal jejunum at 1NC was 41%, 42%, and 15% higher compared to the duodenum, proximal jejunum, and ileum, respectively (duodenum, 4.98 ± 0.58; proximal jejunum, 4.86 ± 0.52; distal jejunum, 8.37 ± 1.71; ileum, 7.15 ± 0.67 A.U./cell, $P = 0.03$, Figure 5.1).

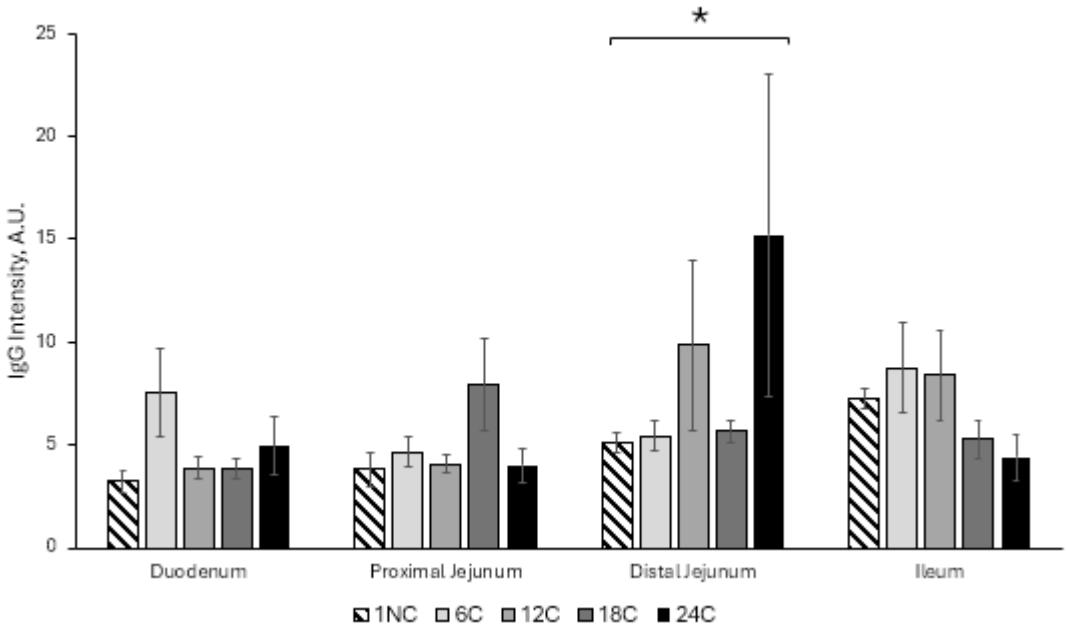


Figure 5.1 IgG intensity per cell in the intestinal villi, in non-fed neonatal calves at 1 hr of life and in calves treated with colostrum and euthanized at 6, 12, 18, or 24 hrs of life. Data is presented as mean ± SEM, n = 6/treatment. IgG intensity is presented as arbitrary units (A.U.) to identify relative changes in IgG abundance. IgG intensity was greatest in the distal jejunum ($P = 0.03$). IgG intensity did not differ by treatment ($P = 0.60$), indicating that the tissue content of IgG did not change over 24 hrs of life.

5.5.3 FcRn Abundance

Non fed calves at 1 hr post partum (1NC) had higher FcRn intensity per cell within intestinal villi by 34%, 37%, 70%, and 49% compared to 6C, 12C, 18C, and 24C calves, respectively (1NC, 128 ± 18.5; 6C, 84.4 ± 14.6; 12C, 80.6 ± 10.9; 18C, 37.8 ± 9.02; 24C, 64.9 ± 20.6 A.U., $P < 0.01$, Figure 5.2 and Figure 5.3). These results indicated that FcRn was of greatest abundance in the small intestinal epithelium at 1 hr post-partum and then declined over time, despite colostrum-IgG feeding and over time post-partum. The 18C calves ($P < 0.01$) and 24C calves ($P = 0.02$) had lower FcRn intensity per cell when compared to 1NC calves, suggesting that FcRn abundance decreases after 12 hrs of life. There were no differences in FcRn intensity per cell within intestinal villi between the intestinal tissue segments (duodenum, proximal jejunum, distal jejunum, ileum; $P = 0.92$, Figure 5.2), even though, as discussed in chapters 3 and 4, there were differences in IgG permeability between intestinal tissue segments. These results may indicate that IgG permeability is not necessarily linked directly to FcRn intestinal segment abundance, an indicator of high affinity receptor-selective transport, in the neonatal calf within the first 24 hrs of life. In addition, FcRn abundance decreasing over time is in opposition to the data shown in Ch. 3 where there was no difference between transport of IgG across the tissue between 1 hr and 24 hrs of life.

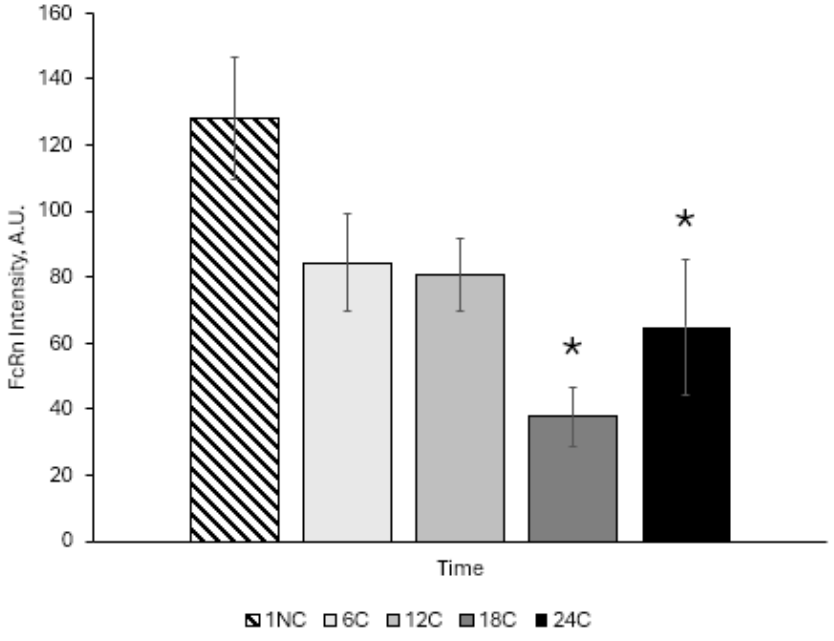


Figure 5.2 FcRn intensity per cell in the intestinal villi, in non-fed neonatal calves at 1 hr of life and in calves treated with colostrum and euthanized at 6, 12, 18, or 24 hrs of life. Data is presented as mean ± SEM, n = 6/treatment. FcRn intensity is presented as arbitrary units (A.U.) to identify relative changes in FcRn abundance. FcRn intensity per cell in the intestinal villi was lower in older calves (18C and 24C, $P < 0.01$) and there was no change in FcRn intensity per cell in the intestinal villi by intestinal tissue segment (duodenum, proximal jejunum, distal jejunum, or ileum; $P = 0.92$). Asterisk indicates significant difference from other treatment groups.

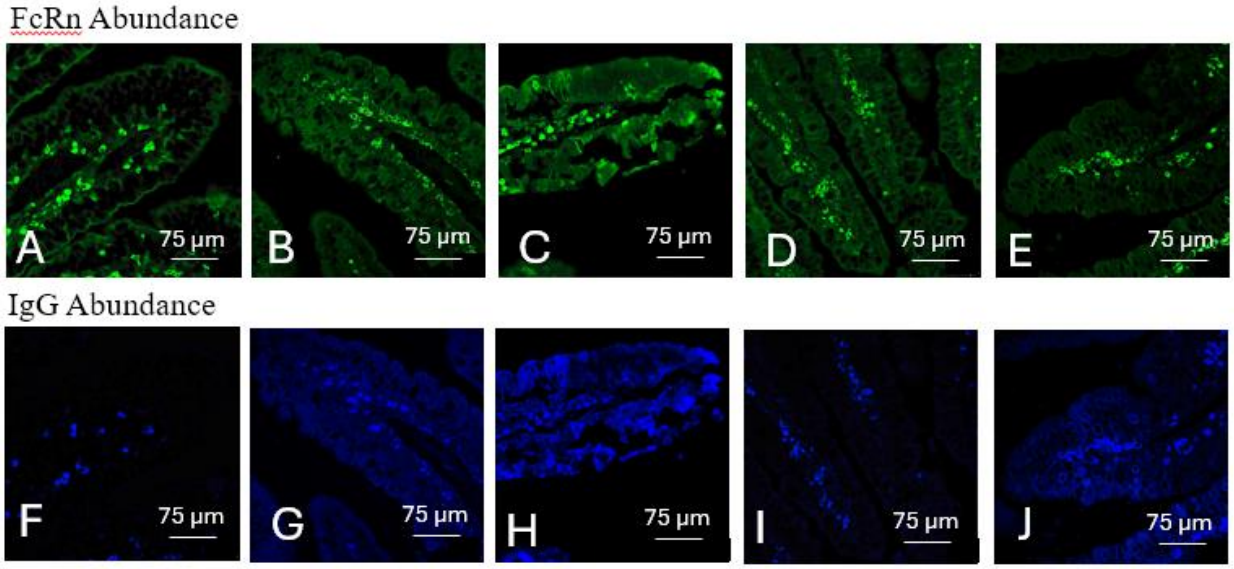


Figure 5.3 FcRn and IgG abundance in the distal jejunum villi, in non-fed neonatal calves at 1 hr of life and in calves treated with colostrum and euthanized at 6, 12, 18, or 24 hr24 hrs of life, n = 6/treatment. FcRn abundance in A) non-fed calves euthanized at 1 hr of life (1NC), B) fed calves euthanized at 6hrs of life (6C), C) fed calves euthanized at 12hrs of life (12C), D) fed calves euthanized at 18hrs of life (18C), and E) fed calves euthanized at 24 hr24 hrs of life (24C). IgG abundance in F) non-fed calves euthanized at 1 hr of life (1NC), G) fed calves euthanized at 6hrs of life (6C), H) fed calves euthanized at 12hrs of life (12C), I) fed calves euthanized at 18hrs of life (18C), and J) fed calves euthanized at 24 hr24 hrs of life (24C).

The imaging data is supported by the IgG protein concentration measured in tissues by ELISA (measurement of protein extracted from whole intestinal tissue), which shows IgG is in each intestinal segment (Figure 5.4). However, IgG concentration was higher in the proximal and

distal jejunum compared to the duodenum and ileum (Figure 5.4). IgG concentration overall was 99%, 14%, 69%, and 76% higher in 6C calves compared to 1NC, 12C, 18C, and 24C calves, (1NC, 0.84 ± 0.49; 6C, 92.6 ± 39.5; 12C, 80.4 ± 21.0; 18C, 29.2 ± 9.02; 24C, 22.4 ± 10.8 mg IgG / g tissue, $P < 0.01$, Figure 5.4), indicating that IgG abundance in the tissue increased with colostrum -IgG feeding and then declined over time, indicating clearance of IgG from the villus with time potentially indicating transport into villus and mesenteric lymphatics and the circulation. ELISAs have higher specificity given measuring protein but lower sensitivity than immunofluorescence techniques, and our data shows IgG located in abundance in all intestinal tissue segments for both techniques but higher for the immunofluorescence method. With the ELISA we could be missing some of the IgG signal, but with immunofluorescence we could be labeling something in addition to bovine IgG despite positive and negative controls (Menezes et al., 2020). Also, the immunofluorescence technique used was a measurement of IgG abundance in the intestinal villi only; the tissue extraction method was a measure of IgG in the entire tissue segment, including villi, lamina propria, musculature, and lymphatic and blood vessels.

IgG in the tissue as measured by ELISA was 99%, 55%, and 85% greater in the proximal jejunum than in the duodenum, distal jejunum, and ileum, respectively (duodenum, 5.51 ± 0.85; proximal jejunum 111 ± 33.2; distal jejunum, 50.8 ± 15.3; ileum, 16.6 ± 4.31 mg IgG/g tissue, $P < 0.01$, Figure 5.4), whereas the greatest amount of IgG as measured by immunofluorescence was found in the distal jejunum and ileum (Figure 5.1).

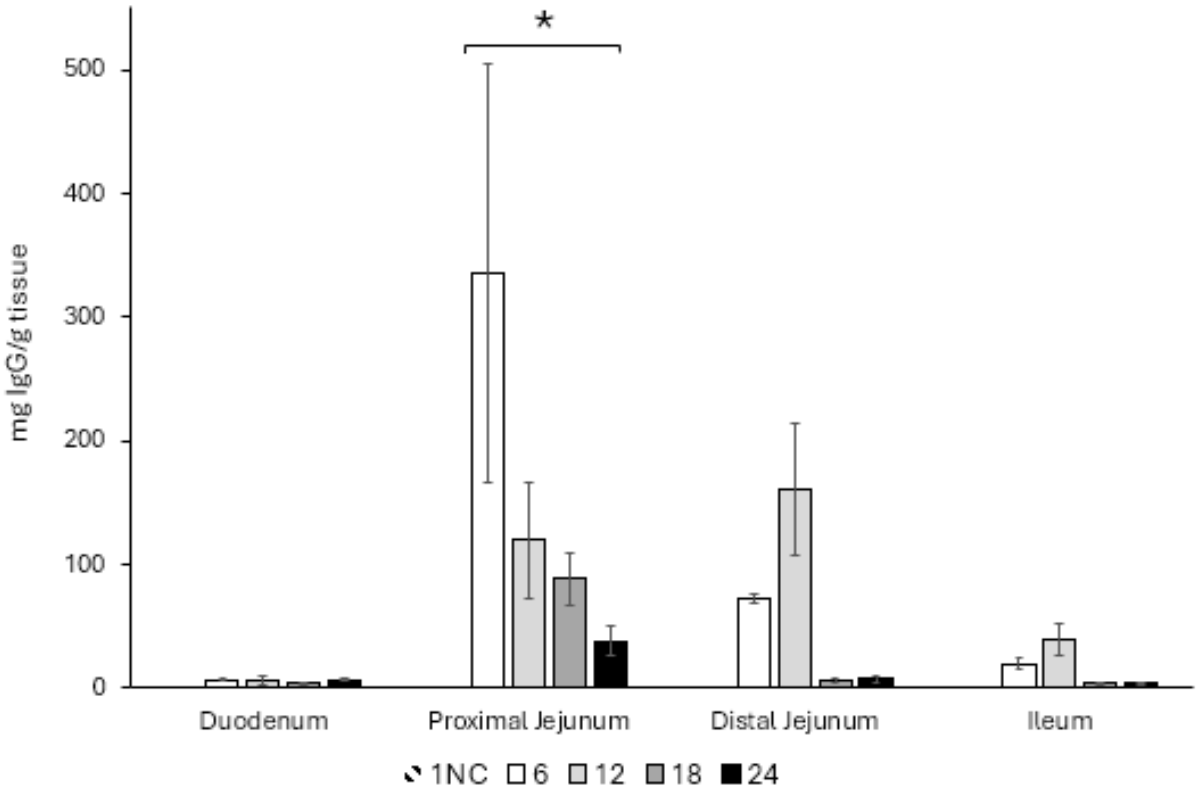


Figure 5.4 Tissue IgG concentrations from protein extracted from whole intestinal tissue samples and run on an ELISA for IgG, in non-fed neonatal calves at 1 hr of life and in calves treated with colostrum and euthanized at 6, 12, 18, or 24 hrs of life. Data is presented as mean ± SEM, n = 6/treatment. IgG concentrations were greater in 6C calves as compared to other treatment groups ($P < 0.01$) and were greatest in the proximal jejunum when compared to other intestinal tissue segments.

5.5.4 Colocalization of IgG and FcRn

IgG was strongly and positively colocalized with FcRn ($r = 0.84$, Figure 5.5 and Figure 5.6) and colocalization of IgG to FcRn did not differ by colostrum or time post-partum (1NC, 0.828 ± 0.01; 6C, 0.847 ± 0.01; 12C, 8.66 ± 0.01; 18C, 0.844 ± 0.01; 24C, 0.831 ± 0.018, $P = 0.13$, Figure 5.5 and Figure 5.6), indicating that colostrum feeding and age did not affect colocalization of IgG and FcRn.

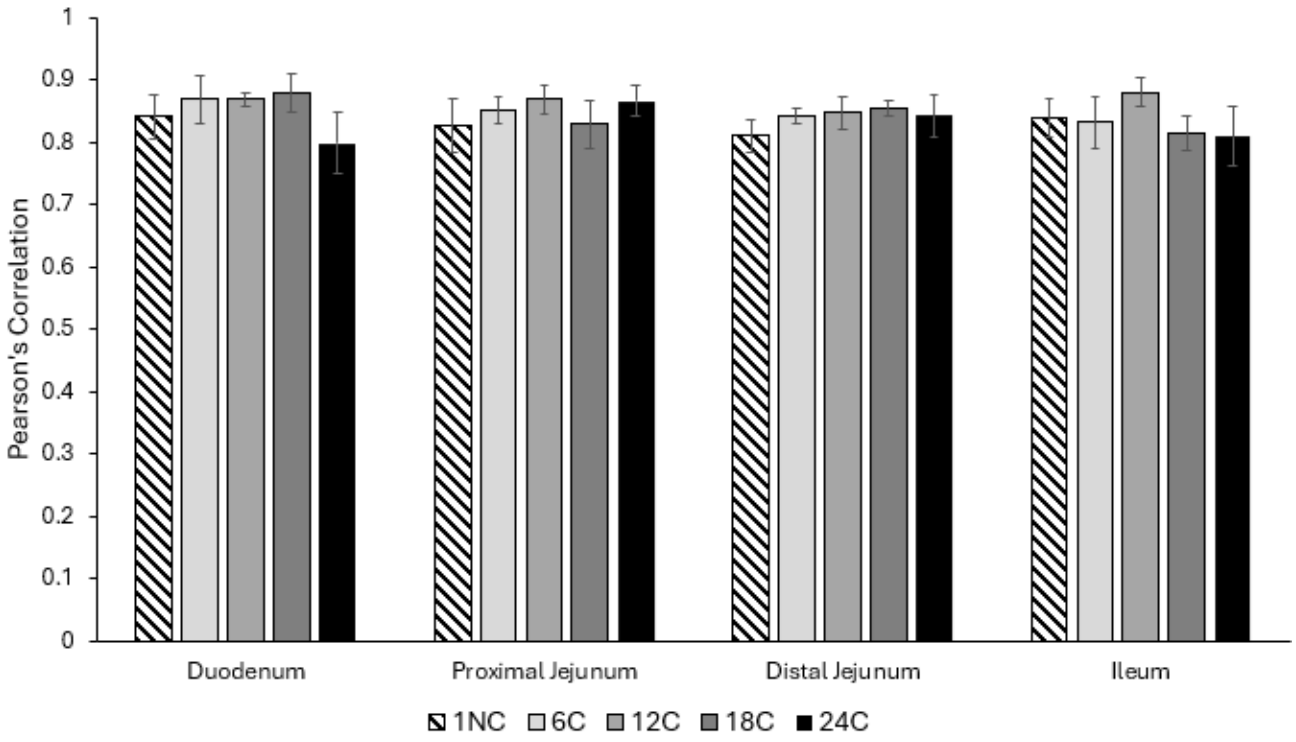


Figure 5.5 FcRn to IgG colocalization coefficient, in non-fed neonatal calves at 1 hr of life and in calves treated with colostrum and euthanized at 6, 12, 18, or 24 hrs of life. Data is presented as mean ± SEM, n = 6/treatment. IgG was highly positively colocalized to FcRn (r = 0.84 average across all treatments) and did not change with treatment ($P = 0.13$) or with intestinal tissue segment ($P = 0.72$).

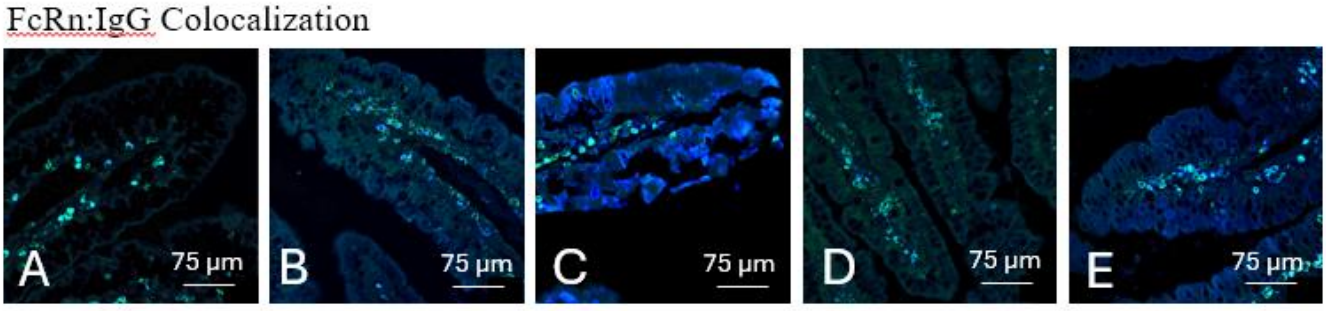


Figure 5.6 FcRn to IgG Colocalization, in non-fed neonatal calves at 1 hr of life and in calves treated with colostrum and euthanized at 6, 12, 18, or 24 hr24 hrs of life, n = 6/treatment. Blue = IgG, Green = FcRn. A) non-fed calves euthanized at 1 hr of life (1NC), B) fed calves euthanized

at 6hrs of life (6C), C) fed calves euthanized at 12hrs of life (12C), D) fed calves euthanized at 18hrs of life (18C), and E) fed calves euthanized at 24 hrs of life (24C). IgG was highly positively colocalized to FcRn (r = 0.84 average across all treatments) and did not change with treatment (treatment (P = 0.13) or with intestinal tissue segment (P = 0.72).

5.5.5 Localization of FcRn within the Intestinal Villi

To examine if FcRn abundance differed from the tip of the villi to the base of the villi, FcRn intensity data were analyzed by top, middle, and bottom 1/3 of each villi. This technique allowed us to determine the localization of FcRn within the villi and if that location changed over time or by intestinal tissue segment. FcRn localization within villi did not differ among top, middle, or bottom third of villi (top of villi, duodenum, 0.07 ± 0.01; proximal jejunum, 0.06 ± 0.01; distal jejunum, 0.05 ± 0.01; ileum, 0.06 ± 0.01 A.U. | middle of villi, duodenum, 0.07 ± 0.01; proximal jejunum, 0.06 ± 0.01; distal jejunum, 0.056 ± 0.01; ileum, 0.07 ± 0.01 A.U.| bottom of villi, duodenum, 0.06 ± 0.01; proximal jejunum, 0.059 ± 0.01; distal jejunum, 0.06 ± 0.01; ileum, 0.07 ± 0.01 A.U., P = 0.95, Figure 5.7 and Figure 5.8) indicating that FcRn abundance did not change anteriorly or posteriorly within the villi with age over the first 24 hrs of life. There was also no change observed between the intestinal tissue segments (P = 0.52).

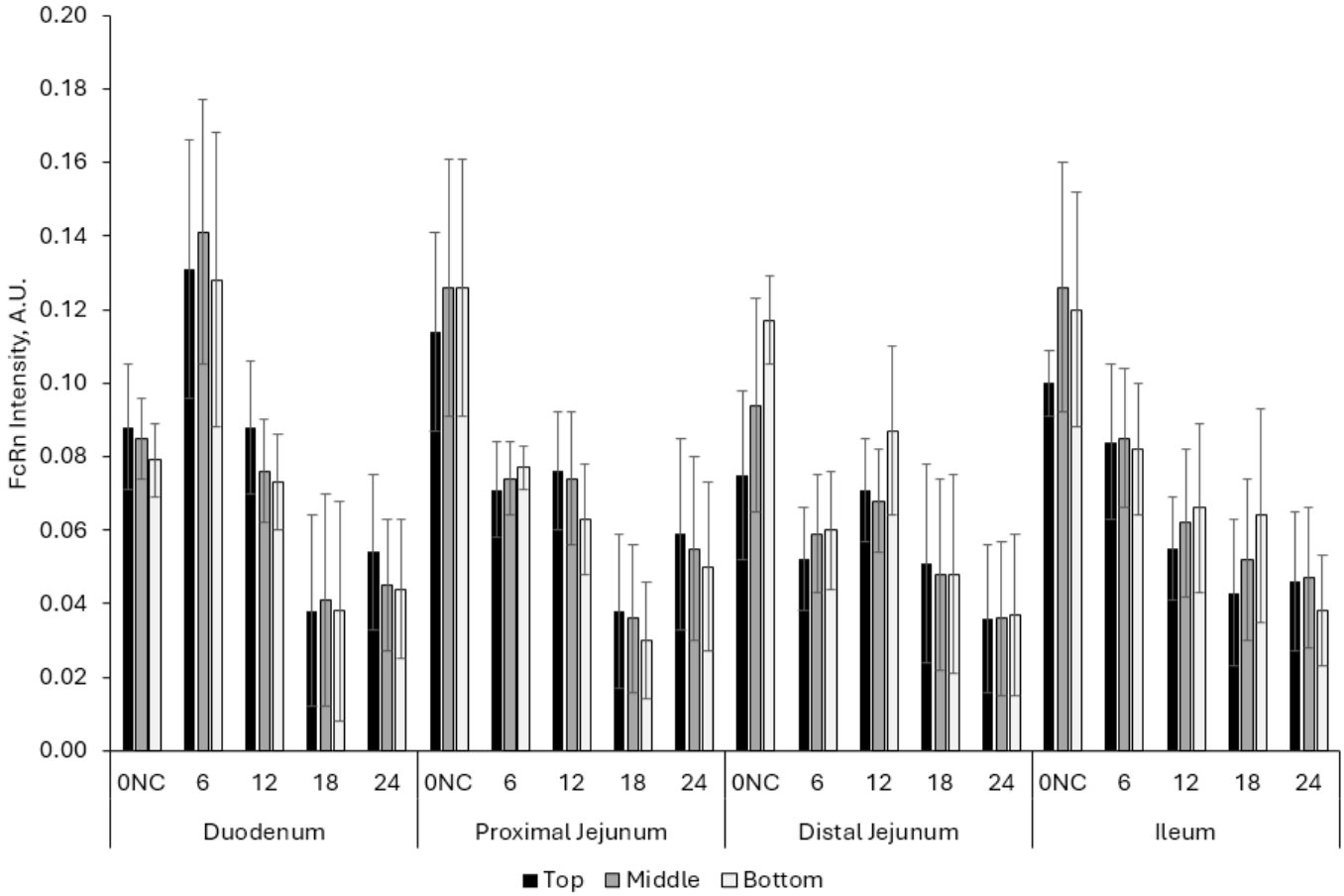


Figure 5.7 FcRn intensity by location within each villi, in non-fed neonatal calves at 1 hr of life and in calves treated with colostrum and euthanized at 6, 12, 18, or 24 hrs of life. Data is presented as mean ± SEM, n = 6/treatment. FcRn intensity did not differ by location within the villi ($P = 0.95$) nor with intestinal tissue segment ($P = 0.52$) and thus we can conclude that FcRn abundance did not change with location within the villi throughout the first 24 hrs of life in the neonatal calf.

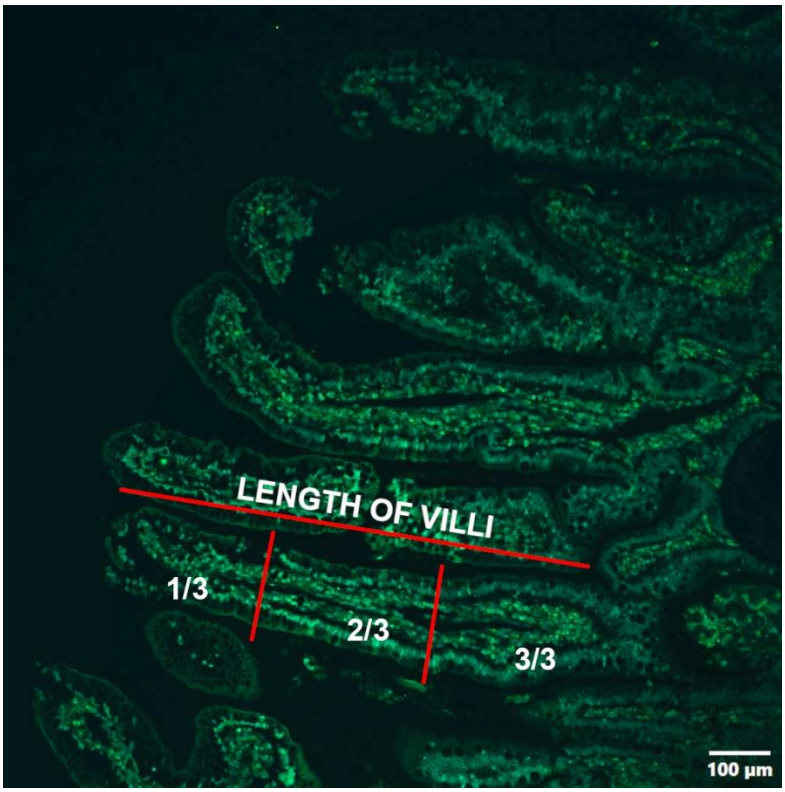


Figure 5.8 Example of FcRn localization analysis in distal jejunum villi in non-fed neonatal calves at 1 hr of life and in calves treated with colostrum and euthanized at 6, 12, 18, or 24 hrs of life. Each villi was split into even thirds and the FcRn intensity was measured in each third of the villi. In each image, 2 to 3 villi were measured and 3 images were taken per tissue type per calf.

5.5.6 Lymphatic and Blood Vessel Marker Colocalization with FcRn

In order to investigate the potential for uptake of IgG into the blood capillaries or lymphatic venules in the villus, the colocalization FcRn with blood vessel marker (VEGFR2) and lymphatic vessel maker (PROX1) was determined. Positive colocalization was determined by correlation, ranging between 0 and 1, indicates the degree of the fluorescent signal for FcRn and the additional marker (either PROX1 or VEGFR2) are co-located in the villus. Specifically, it indicates that pixels that are positively fluorescing for the FcRn marker are also fluorescing for the co-stained marker. Negative correlation, ranging between -1 and 0, indicates that pixels that are positively fluorescing for the FcRn marker are not also fluorescing for the co-stained marker. Positive colocalization in this technique suggests that FcRn signal is coming from the same physical location as the co-stained marker but does not determine if the two markers are

interacting or bound together in any way. The closer a colocalization correlation is to 0, the smaller the association between the two markers.

FcRn was positively co-localized to PROX1 (average Pearson's correlation of 0.58) and colocalization of PROX1 to FcRn did not differ by colostrum feeding or overtime in each group (1NC, 0.56 ± 0.04; 6C, 0.52 ± 0.03; 12C, 0.64 ± 0.03; 18C, 0.60 ± 0.04; 24C, 0.59 ± 0.03, P = 0.22, Figure 5.10 and Figure 5.11), showing that FcRn is located in lymphatic venules in the villi. There was no difference in co-localization in intestinal tissue segments (P = 0.73), showing that FcRn and PROX1 are consistently co-localized throughout the intestine.

FcRn was also positively colocalized to blood vessel marker VEGFR2, though the colocalization correlation was weaker by approximately 10 to 50% compared to PROX1 (r = 0.39). Co-localization of VEGFR2 to FcRn did not differ by colostrum feeding or overtime (1NC, 0.41 ± 0.02; 6C, 0.39 ± 0.03; 12C, 0.39 ± 0.02; 18C, 0.33 ± 0.03; 24C, 0.42 ± 0.02, P = 0.22, Figure 5.10 and Figure 5.11). There was also no difference in intestinal tissue segment on VEGFR2 colocalization with FcRn (P = 0.31). The colocalization of PROX1 to FcRn was significantly greater than that of VEGFR2 to FcRn (P < 0.01, Figure 5.10 and Figure 5.11), indicating that FcRn is located more abundantly in lymphatic vessels compared to blood vessels.

There are limitations to colocalization analysis. These data should be interpreted with caution, however, as a colocalization value close to 1 or to 0 suggests the presence or absence of a relationship between the two markers, but data in the middle of 1 to 0 could indicate a null result as much as a positive result (Dunn et al., 2011). For example, VEGFR2 and FcRn had positive colocalization but it is unclear if this directly indicates a physical relationship within the epithelial tissue. FcRn was also positively identified in the mesenteric lymph nodes via immunofluorescence staining (Figure 5.9), though it was not quantified in the lymph nodes, further suggesting that IgG is transported via the lymphatic vessels in neonatal calves.

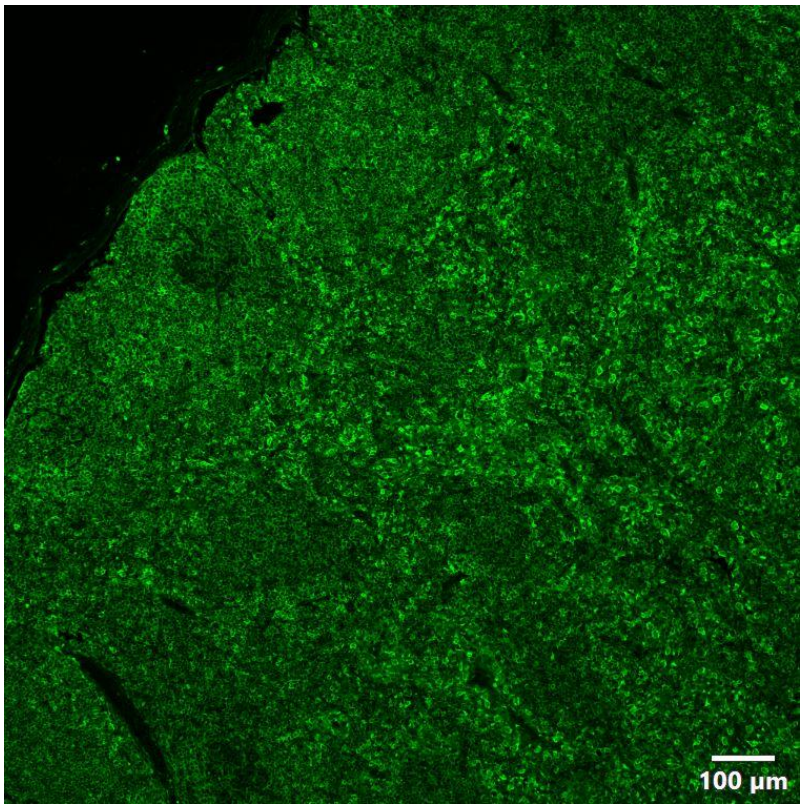


Figure 5.9 FcRn signal in the mesenteric lymph node at 1 hr of life in non-fed neonatal calves. The bright spots indicate FcRn within the mesenteric lymph node at 1 hr post-partum, suggesting that FcRn is involved in lymphatic IgG transport from birth.

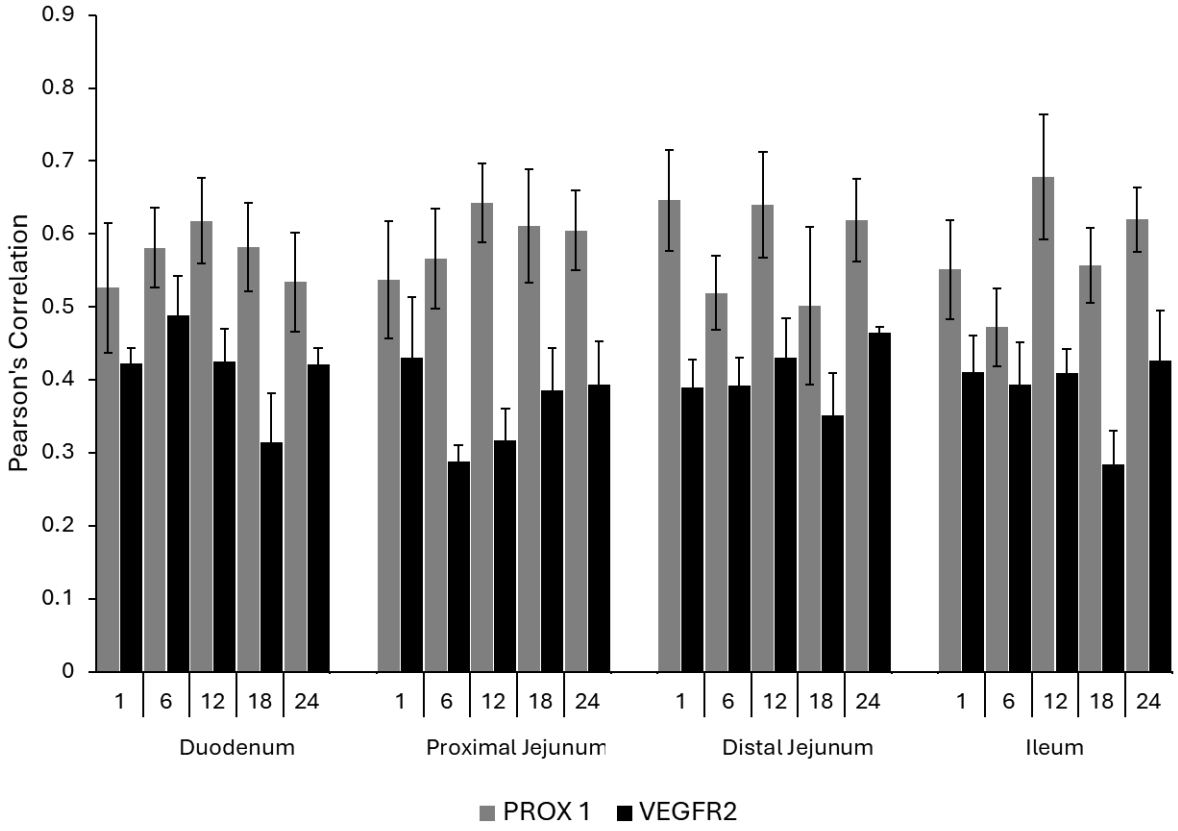


Figure 5.10 FcRn colocalization to PROX1 and VEGFR2 in non-fed neonatal calves at 1 hr of life and in calves treated with colostrum and euthanized at 6, 12, 18, or 24 hrs of life. Data is presented as mean ± SEM, n = 6/treatment. FcRn was more strongly colocalized to PROX1 (average Pearson's correlation, r = 0.58) than VEGFR2 (average Pearson's correlation, r = 0.39; $P < 0.01$), suggesting that FcRn is located more abundantly in lymphatic vessels compared to blood vessels in the small intestinal villi.

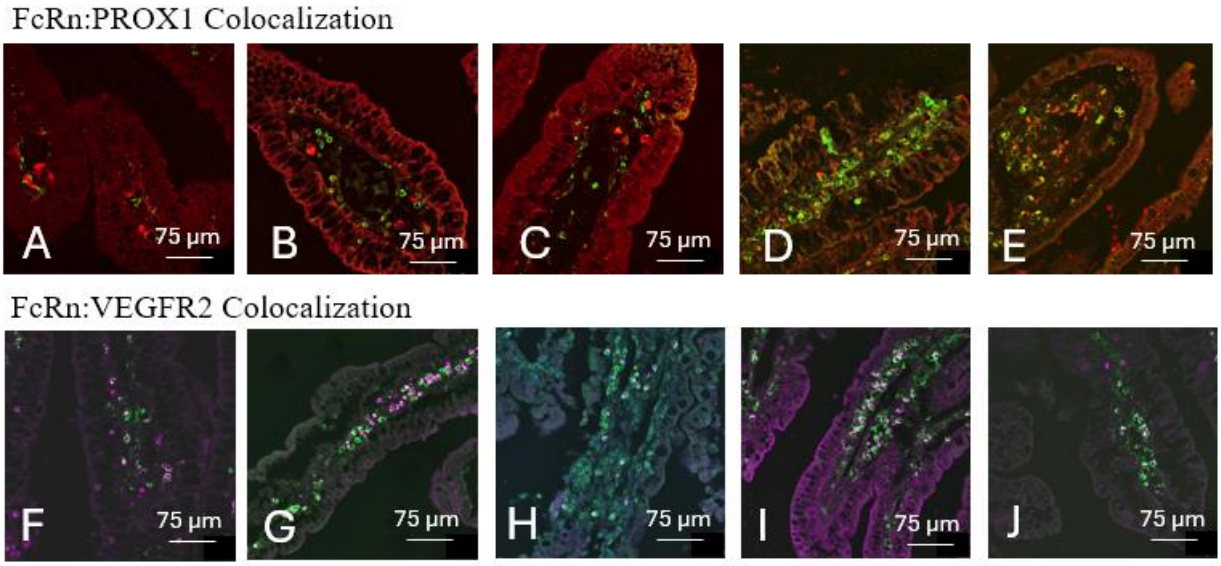


Figure 5.11 FcRn colocalization to PROX1 and to VEGFR2 in non-fed neonatal calves at 1 hr of life and in calves treated with colostrum and euthanized at 6, 12, 18, or 24 hr24 hrs of life, n = 6/treatment. FcRn to PROX1 (FcRn = Green, PROX1 = Red) colocalization is depicted in the distal jejunum villi of A) non-fed calves euthanized at 1 hr of life (1NC), B) fed calves euthanized at 6 hrs of life (6C), C) fed calves euthanized at 12 hrs of life (12C), D) fed calves euthanized at 18 hrs of life (18C), and E) fed calves euthanized at 24 hr24 hrs of life (24C). FcRn to VEGFR2 colocalization (FcRn = Green, VEGFR2 = Purple) is depicted in the distal jejunum villi of F) non-fed calves euthanized at 1 hr of life (1NC), G) fed calves euthanized at 6 hrs of life (6C), H) fed calves euthanized at 12 hrs of life (12C), I) fed calves euthanized at 18 hrs of life (18C), and J) fed calves euthanized at 24 hr24 hrs of life (24C).

5.5.7 Digesta pH

Digesta pH was not different among treatments for duodenum ($P = 0.34$), proximal jejunum ($P = 0.24$), distal jejunum ($P = 0.14$), ileum ($P = 0.15$), and cecum ($P = 0.33$), but there was a difference between treatments in the abomasum ($P = 0.01$, Figure 5.12). For the abomasum, 6C calves had the highest pH, and pH declined with increasing age over the first 24 hrs of life (1NC, 4.19 ± 0.47; 6C, 5.09 ± 0.43; 12C, 3.36 ± 0.43; 18C, 3.11 ± 0.53; 24C, 2.7 ± 0.61 pH, Fig. 5.12). On average, the proximal intestine digesta pH was lower than the distal intestine digesta pH by about 1 pH unit (duodenum, 6.08; proximal jejunum, 6.17; distal jejunum, 7.08; ileum, 7.35; cecum, 6.91). There was likely no effect of digesta pH on transport as these were not significantly different across tissue segments.

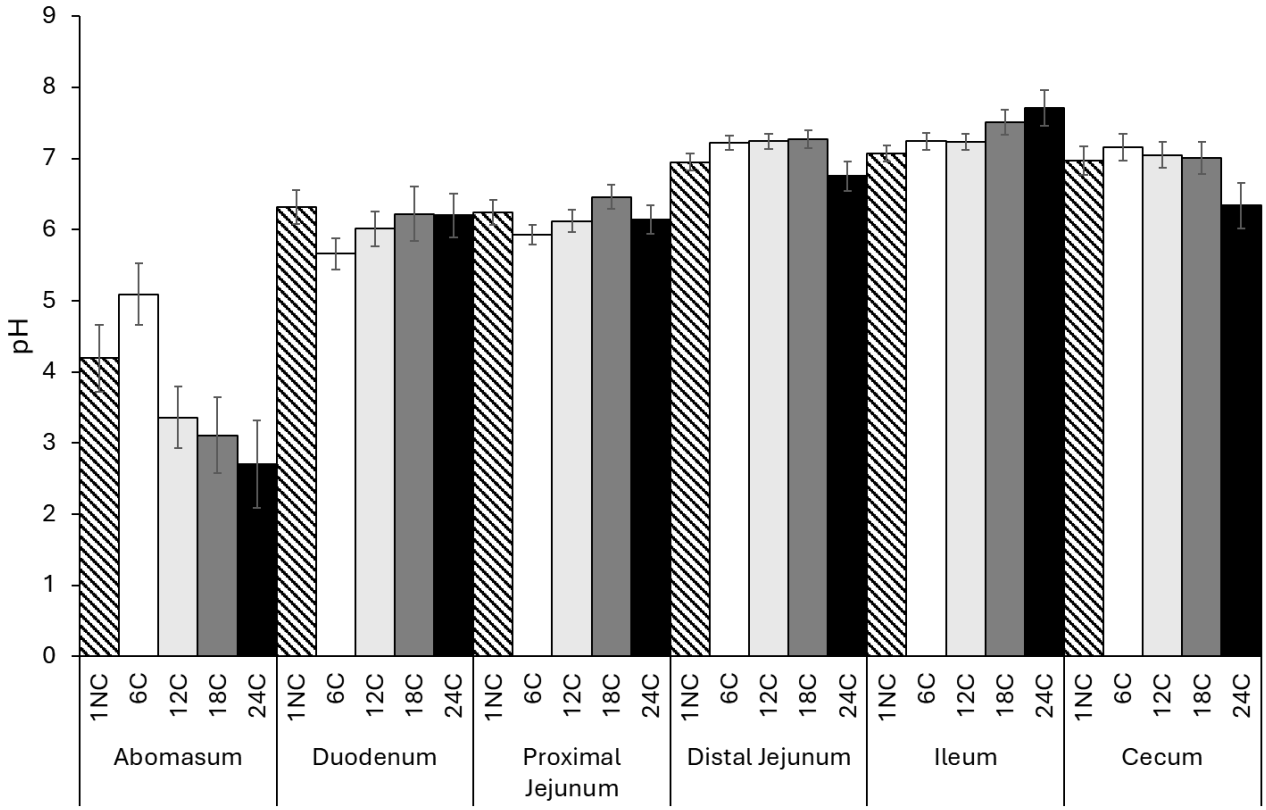


Figure 5.12 Intestinal luminal content (digesta) pH in non-fed neonatal calves at 1 hr of life and colostrum treated calves at 6, 12, 18, and 24 hrs of life. Data is presented as mean ± SEM, n = 6/treatment. There were no differences between treatment groups for duodenum, proximal jejunum, distal jejunum, ileum, or cecum. In the abomasum, pH increased with feeding at 6hrs of life and then declined with age thereafter ($P < 0.01$)

5.6 Discussion

5.6.1 FcRn and IgG Abundance in the Small Intestine of Neonatal Calves

Older calves (18C and 24C) had reduced FcRn abundance in the intestinal villi as compared to younger calves (1NC), suggesting that FcRn abundance in the small intestinal epithelium decreases with age in the first 24 hrs of life. In contrast, IgG abundance, as measured by immunofluorescence, did not change with age during the first 24 hrs of life. FcRn abundance is not affected by IgG abundance (Latvala et al., 2017), likely because FcRn also functions as a salvage and transport protein for albumin and therefore is not IgG dependent (Pyzik et al., 2019).

IgG is observed in the serum of some calves at birth - one calf in the current study had detectable circulating IgG at birth - but thus far IgG has not been measured in the intestinal tissue at birth. Calves have the ability to produce IgG at birth, though in relatively small amounts (0.85 g produced per day as measured in circulation, Devery et al., 1979) as compared to the abundance of IgG in colostrum (74.4 g / L average, Urie et al., 2018). The IgG concentration in colostrum is greater than the measured amount of IgG in the intestinal tissue at birth (0 mg IgG / g tissue in the current study as measured via ELISA). IgG measured via immunofluorescence showed IgG within the tissue at birth in all sections of the small intestine suggesting that while IgG is not present in the circulation of all calves at birth, it may be present in the intestinal epithelium at birth. It is unknown if the IgG in the tissue at birth is endogenous or maternally derived, and whether the IgG is present in other tissues as well. Immunofluorescence is known to be a more sensitive but less selective technique than ELISA (Tayde et al., 2020), so it is possible that the immunofluorescence was able to pick up more IgG signal than the ELISA. It is also important to note that the immunofluorescence technique examined IgG abundance in the villi only, whereas the ELISA technique used the entire intestinal tissue, including villi, musculature, and vasculature. Due to the reduced sensitivity of the ELISA technique and possible dilution of IgG due to use of the entire tissue segment, it is not surprising that we see conflicting results between the two techniques.

A comparison of the immunofluorescence and ELISA IgG results is interesting as it suggests that 1) IgG is present in the intestinal tissue at birth (immunofluorescence) and 2) when fed, IgG has differential retention in the distal jejunum and ileum as compared to the duodenum and proximal jejunum during the first 24 hrs of life. Taken together, these data suggest that some IgG from colostrum remains in the tissue instead of all of it being transported into circulation, and suggests that calves are born with intestinal tissue-bound IgG but not necessarily circulating IgG. The presence of IgG within neonatal tissues in calves not fed colostrum is a novel finding, and the presence of IgG in the neonate at or before birth requires further investigation.

5.6.2 FcRn Association with Blood Capillaries and Lymph Venules

In this study we sought to better understand the relationship between FcRn, IgG, and the intestinal epithelium in the neonatal calf. FcRn-positive cells were more closely colocalized to PROX1, a marker of lymphatic vessels, than they were to VEGFR2, a marker of blood vessels. In the small intestine, blood vessels interact closely with lymph vessels in the villi. It has been

assumed that IgG is transported directly into the bloodstream in calves (Rath et al., 2013). Data from the current study suggest that IgG undergoes exocytosis by FcRn into the lymph vessels instead, as it is in other mammals, or into both the lymph and the blood simultaneously. If the IgG undergoes exocytosis into both blood and lymph, it appears to be preferentially transported via the lymph as FcRn was more closely colocalized to PROX1 instead of VEGFR2. In addition to FcRn colocalization to lymph vessels, the current study found that FcRn is present in the mesenteric lymph nodes, demonstrating that IgG migrates there too. Transport of maternal IgG to the lymph nodes induces "immunological imprinting effects" that are traceable into adulthood, where maternal antibodies influence the specific B cell repertoire of the neonate (Lemke et al., 2009). These imprinting effects transfer immunological memory from mother to offspring, and are therefore an epigenetic inheritance for the calf (Lemke et al., 2009). If maternal antibodies are specific and can help develop the immune memory of the calf via specific B cell production, it may be that shunting of maternal IgG through the lymphatic system allows for initial development of the calf's immune memory.

5.6.3 Intestinal Luminal pH and FcRn to IgG Binding

The pH of the intestinal digesta in the calf differs across the sections of the small intestine and hindgut in the first 24 hrs of life, ranging from 6.0 to 7.3 in the current study. FcRn binding to IgG is highly dependent on pH (Jensen et al., 2017) and on IgG subtype, where IgG_1 binds more strongly than IgG_2 (Stapleton et al., 2019). FcRn binds to both subtypes of IgG at pH 6 to 6.5 only; IgG does not bind to FcRn at pH 7 or more (He et al., 2008). In the distal jejunum, ileum, and cecum, IgG binding to FcRn should not be favored based on the pH of the digesta (He et al., 2008), and IgG could even be exocytosed into the lumen from inside the epithelium due to the pH gradient (Lencer and Blumberg, 2005). The presence of an acidic pH microclimate at the cell surface may explain this phenomenon (Shiau et al., 1985; Said et al., 1987), though this has not been directly identified in the bovine intestine. The intestinal surface acid microclimate is present in suckling, weanling, and adult rats - it is possible that the lower pH of the microclimate (5.8 to 6.7 in the small intestine, Said et al., 1987) enhances uptake of colostrum by providing the optimal binding pH for FcRn and IgG. Further investigation into the mechanism of endocytosis for IgG in relation to FcRn is needed, with special consideration for the pH fluctuations at the site of binding.

5.7 Conclusion

FcRn is colocalized to the lymph vessel marker PROX1 more strongly than it is colocalized to blood vessel marker VEGFR2, suggesting an alternative pathway for IgG transport in the neonate than what has been previously assumed and reported in research on dairy calves. FcRn abundance decreased with age but IgG abundance in the tissue did not, indicating that IgG exists in the tissue regardless of colostrum feeding. The pH of the digesta does not appear to affect FcRn to IgG binding, despite literature stating that they do not interact above a pH of 7, providing evidence for a pH microclimate at the surface of the epithelium. There is still much to unravel about the pathway of IgG absorption in bovine neonates and the role of the FcRn receptor in colostral IgG absorption.

6.0 General Discussion

6.1 Study Summary

This research investigated the kinetics and histological features of the neonatal bovine intestine during the critical absorption period of the first day of life. Previously, many studies in the field of dairy calf nutrition have examined the effects of modulating feeding status on serum IgG concentrations. Feeding guidelines for calves have been identified and provide colostrum quality, administration method, and administration timing recommendations to producers. Yet, despite following best practices, some calves still have FTPI as defined by serum IgG concentration and we do not know why. The study described in this thesis sought to dive deeper into the subject of IgG absorption and gut closure mechanisms in the first day of life in the neonatal calf by investigating IgG at the tissue level and comparing the findings with that of serum IgG. Strengths of this research include examination of IgG absorption in the calf using a variety of sampling techniques to understand the time-course and localization of IgG transport in the intestine, combining kinetic measurements with histological analysis, and a focus on basic physiology to confront long-standing assumptions in the field of neonatal calf nutrition. Novel findings include intestinal epithelial vesicle counts, acidification of the abomasum throughout the first 24 hrs of life, the kinetics of transport of IgG through the first day of life, and absorption potential in the calf were reported.

One study was carried out and the resulting data were examined to meet four different objectives. The first objective was to develop a novel ELISA system for quantifying biotinylated IgG within complex matrices. This novel ELISA system would allow for differentiation between colostrum-fed IgG and experimentally dosed IgG in an ex-vivo setting, enhancing the nuance with which we can examine IgG uptake in the neonatal intestine. The second objective was to examine the effect of feeding status on kinetic and histological parameters within the bovine small intestine during the first day of life. Two groups of calves were euthanized at 24 hrs of life, one having been fed colostrum and one not fed colostrum. These two groups allowed us to examine the effect of colostrum feeding and thus nutritional stimulation on kinetics and histological features – namely, PAS-positive vesicles – within the first 24 hrs of life in the neonatal calf. The third objective was to investigate kinetic and histological features in the small intestine in a sequential time-course throughout the first day of life, to examine critical points in

the maximal absorption to gut closure window. This time-course, in conjunction with different sections of the small intestine, provided insight into the absorptive process during the first day of life in the calf. The fourth objective was to examine the relationship between FcRn and IgG, and their relationship to lymphatic and blood vessel localization, within the context of the first day of life. This objective began to examine a receptor-based transport mechanism for IgG during acquisition of passive transfer. These data provide information on the absorptive state of the calf within the critical first day of life during the period of colostrum absorption.

Objective 1 provided the technical knowledge to quantify non-radioactive, labeled IgG in Ussing chamber samples containing neonatal bovine tissues. We hypothesized that a "shortened" version of a typical sandwich ELISA would allow for detection of a biotinylated IgG within a mixed sample with unmodified IgG. When samples and standards have the same ratio of modified to unmodified IgG, competition within the assay is predictable and biotinylated IgG can be quantified – this is, however, a limitation of the method. Without a set ratio of biotinylated to unmodified IgG, competition for binding sites prevents accurate quantification. However, it is possible that this ELISA technique could be applied to many other biotinylated proteins to measure the tissue export kinetics of other components of bovine colostrum. In the current study, the shortened ELISA system would allow us to measure the difference between IgG that is absorbed into the tissue after colostrum feeding, and IgG that is added to the Ussing chamber during the experiment. Therefore, the kinetics of IgG efflux from colostrum and experimentally supplemented IgG transcytosis could be examined separately.

Objective 2 examined the effects of feeding status on IgG uptake, IgG tissue release, and histological features in the small intestine. One limitation of this analysis is that, in the Ussing chamber, whole neonatal intestine tissue was mounted instead of just the epithelium. In neonates, the intestine is too fragile to remove the muscularis layer without damaging the epithelium. Therefore, all kinetics data is trans-tissue instead of trans-epithelial flux. Mucosal IgG influx did not differ between treatments or intestinal tissue segments, indicating that neither feeding status not age had an effect on IgG uptake in neonatal intestinal tissues up to 24 hrs old. Serosal IgG efflux was highest in 24C calves for the first hour of the experiment, likely due to IgG present in the epithelium from colostrum feeding, however, 1NC calves had the same efflux profile during the second hour of the experiment. Age and feeding status did not affect trans-epithelial flux in neonatal calves. FITC-dextran, used to measure intestinal permeability, exhibited opposite efflux

location within the neonatal intestine as compared to IgG, suggesting that different sections of the small intestine are preferential locations for paracellular vs. transepithelial flux in neonates. A limitation of using FITC-dextran as a quality control marker was that it only informed about increased passage of small molecules and was unable to indicate tissue reuptake pathways. Large, PAS-positive vesicles, indicating presence of glycoproteins, were observed in fed calves but not in unfed calves. The small intestine of the calf has the ability to absorb IgG (macromolecules, transcytosis) and FITC-dextran (micromolecules, paracellular) regardless of feeding status within the first 24 hrs of life.

Objective 3 investigated the effect of a sequential time-course throughout the first 24 hrs of life on the histological features and IgG transport kinetics in the neonatal intestine. Again, mucosal IgG influx was not affected by age of the calf, but serosal IgG efflux was highest in the 18C and 24C calves. Abomasal pH increased with feeding but then steadily dropped until 24 hrs of life. Digesta IgG also peaked after feeding but passed quickly out of the small intestine or was absorbed, and thus was only found in the cecum at 18 and 24 hrs of life – a limitation to this study is that we only fed one meal of colostrum, where generally two meals would be fed in a production setting. A second meal, however, would have been confounding for many of the results in this study. Serum IgG reached minimum recommended concentrations by 12 hrs of life. PAS-positive vesicle count followed a similar time-course to serum IgG absorption, peaking at 12 hrs of life. A limitation of the PAS-positive vesicle counting is that we did not directly determine that the glycoprotein indicated by the stain is IgG. There was more FITC-dextran efflux in younger calves than in older calves, and more efflux in the proximal intestine than in the distal intestine. The analysis of sequential timepoints within the first day of life gives a snapshot of IgG uptake from a kinetic and intestinal level and brings into question whether the gut actually “closes” at 24 hrs of life.

Objective 4 examined the relationship between FcRn and IgG within the context of the neonatal small intestine. Older calves had decreased FcRn abundance as compared to younger calves, but FcRn abundance did not appear to be related to IgG abundance. FcRn was highly colocalized with IgG, suggesting an interaction of the two proteins. A limitation of this analysis is that, while highly colocalized via immunofluorescence, a direct interaction of FcRn and IgG could not be determined. In addition, FcRn was more closely colocalized to lymph venules than to blood capillaries, suggesting that the uptake of IgG may be preferentially shunted to the

lymphatic system over the bloodstream; again, this could not be directly determined. Though uptake of IgG has been termed “non-selective”, evidence from the current study suggests that FcRn is closely linked to IgG during the first day of life.

6.2 Impact of Results

6.2.1 IgG Kinetics Indicate IgG Uptake Does Not Decrease with Age

Historically, the capacity of the neonatal intestine to uptake IgG is assumed to decrease with age, declining rapidly in the first 6 to 12 hrs of life (Stott et al., 1979a). These assumptions are based on studies that analyze serum IgG levels, not intestine tissue level kinetics. At 24 hrs of life, even unfed calves had the same capacity to uptake IgG as 1-hour-old calves, within the setting of the Ussing chamber. It has been noted that when neonatal epithelial cells mature, they lose the capacity to uptake IgG and other large macromolecules (Jochims et al., 1994a), but this process is said to take about 3 days in the mature intestine (Barker, 2014). From our data the neonatal intestine retains the ability to uptake IgG until 24 hrs of life. Instead, the abomasal pH may be the “closure” or cessation point of IgG uptake. The pH of the abomasum is higher in the calf at birth (4.2; Parrish and Fountaine, 1952) than in a mature animal (2.0; Winden et al., 2002) and from our data, decreases rapidly after the first colostrum meal. Most proteins denature between pH 2 and pH 5 (Whitten et al., 2001) and by 12 hrs of life in this study, the abomasal pH was already 3.3. A pH of 3.3 suggests that IgG may not be able to pass intact into the small intestine after the first colostrum feeding. Despite this, others have seen positive results with extended colostrum feeding (Pyo et al., 2020). Further research should focus on the ability of IgG to survive the low pH of the abomasum after the first 12 hrs of life in the calf.

6.2.2 The Relationship Between FcRn and IgG in Neonates is Unclear

FcRn has been identified in the intestine and in the mammary gland of the cow (Zhu et al., 2020). The role of FcRn in IgG uptake in calves is unclear, as uptake has long been called “nonselective”, but the relationship between FcRn and IgG is apparent in this study. In addition, the presence of PAS-positive vesicles indicates the presence of IgG within the epithelial tissue. When IgG was directly measured in tissue, the greatest amount of IgG was found at 12 hrs of life which coincides with the largest number of PAS-positive vesicles in the tissue. FcRn and IgG are highly colocalized within the tissue, suggesting that FcRn is involved of transport of IgG into

these vesicles. Further investigation into FcRn and IgG binding in the lumen of the gut, as well as the transport mechanism into the vesicles is needed to determine if FcRn is responsible for transporting IgG into the cell.

6.3 Future Research

The research presented in this thesis begins to elucidate the relationship between IgG uptake, tissue maturation, FcRn presence, and colostrum absorption to the bloodstream and lymphatics of the neonatal bovine intestine. Most importantly, IgG transport through the tissue was found to be active at 24 hrs of life in colostrum-fed calves; this is opposite of our current understanding of gut closure in neonatal calves. Future research should focus on timing of colostrum feeding or multiple colostrum feedings and their effect on IgG transport kinetics in neonatal calves. It would also be pertinent to record the gestation age of each calf (number of days in utero, based on day of breeding the dam), as this may account for some of the variation seen between calves in terms of IgG absorption capacity and kinetic transport.

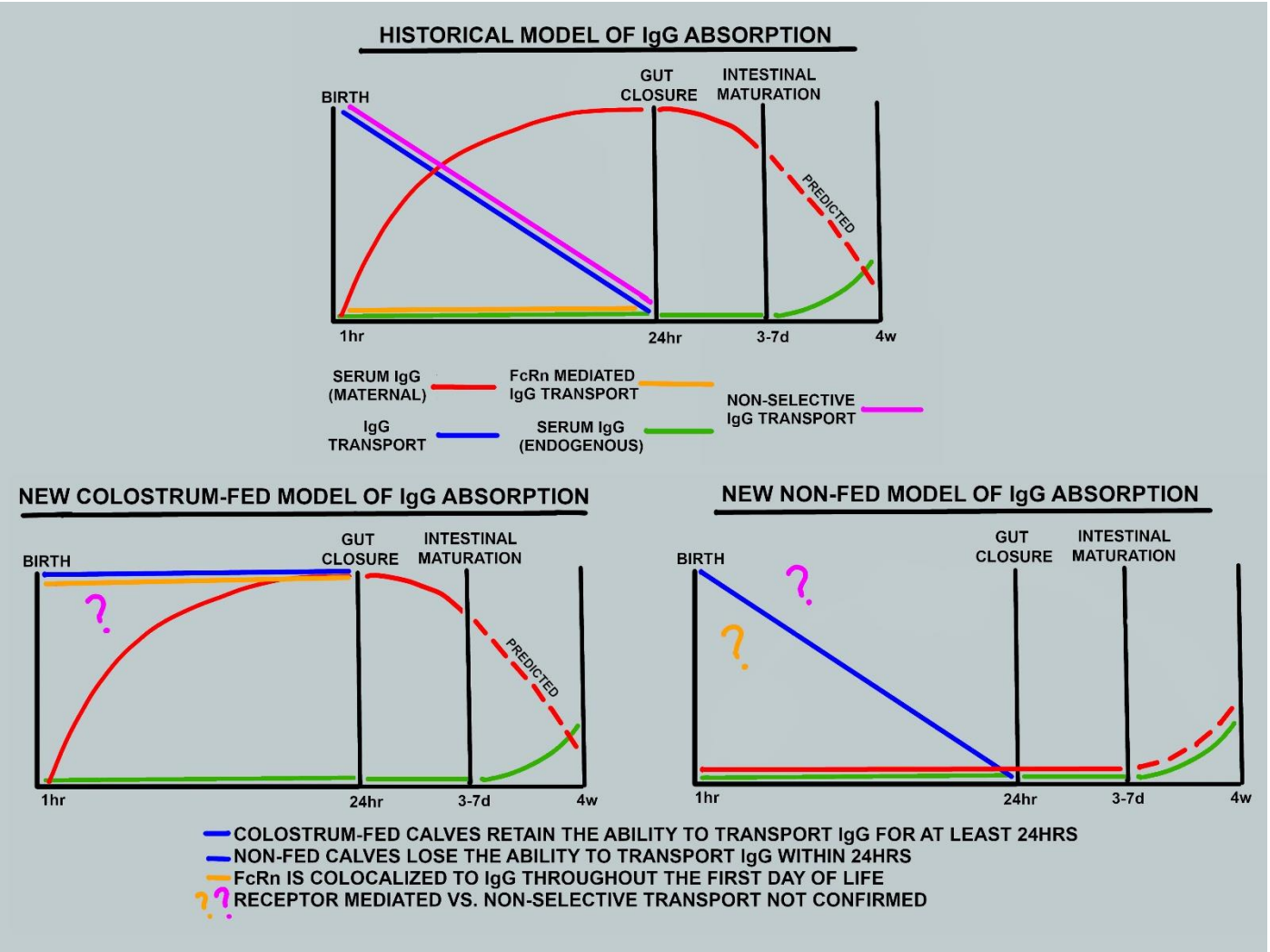
FcRn was found in the lymph node and was more closely colocalized with the lymphatic marker PROX1. In addition, IgG was found in the small intestinal tissue at birth, and an IgG reservoir was noted in the tissue up to 24 hrs of life. These data suggest that IgG transport and accumulation may follow a different pattern than previously understood in the dairy calf. Future research should focus on lymphatic concentration of IgG, the tissue reservoir, and lymph node accumulation of FcRn and IgG. It is also possible that neonatal dairy calves have IgG transfer through the placenta, typically considered to not occur, but that the Igs passed through remain in the tissues and lymphatics rather than being transported into blood circulation.

Understanding interaction between FcRn and uptake of IgG into the epithelium may help explain the decreased apparent efficiency of colostrum absorption seen in dairy calves. Though the research presented in this thesis only looked at colocalization of FcRn, other methods could be used to examine if IgG and FcRn are interacting. A Western-blot based co-immunoprecipitation assay, or a pull-down assay for the FcRn-IgG complex, or even something like FRET would inform on the IgG-FcRn interactions.

In addition, continued kinetics research with varying colostrum feeding programs will help us understand quality colostrum feeding programs on an intestinal tissue level instead of

depending on blood-based assays alone. The abomasum provides an additional point of study, as the pH drops rapidly and may affect IgG reaching the small intestine after birth. These data only show a snapshot of the development and maturation of the neonatal gut at birth – a deeper dive into this subject should touch upon IgG transport mechanics, FcRn abundance disappearance, and the physical filling and emptying of epithelial vesicles. I urge future researchers to think past serum IgG when examining TPI in calves – serum, after all, is the end point for IgG, and there is so much happening before it gets to the serum – and to consider tissue and lymphatic markers to continue to advance this area of study.

6.4 Graphical Abstract of Findings



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