Nutrient Impacts on Rumen Growth and Development

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Abstract

Our collective knowledge of calf nutrition has evolved over the past 100+ years, but there are still areas of improvement that merit further scientific inquiry. The work described herein explored different aspects of calf nutrition with a central focus on rumen growth and development. The first study performed used 8 Holstein bull calves to determine if calf starters differing in starch and neutral detergent fiber (**NDF**) content would affect calf growth, intake, rumen metabolites, blood metabolites, and gross rumen measurements when fed along with milk replacer (**MR**). The experiment used completely pelleted calf starters consisting of ground and pelleted barley, wheat, and corn grains. Besides the high-starch starter resulting in lower rumen pH, the hypothesis that completely pelleted calf starter diets differing in NDF and starch level would alter intake, growth, rumen metabolism, and rumen measurements was not supported. However, calves fed the high-NDF starter were $5.71 less expensive per calf to raise. Findings suggest a form of feed effect in today's calf starter diets that might be of physiological and economic importance. The second study tested custom-built rumen infusion, sampling, and evacuation devices. The main objectives were to build and confirm the successful use of the devices in one Holstein bull calf at 62 days of age, which determined a liquid passage rate out of the rumen at 40.2% of ruminal fluid/h. The third and final study examined the effects of form of diet (MR only, n = 5; MR and starter, n = 6) on rumen growth and development. More specifically, isocaloric and isonitrogenous diets were fed to neonatal and ruminally cannulated Holstein calves for 6 week. The hypothesis of MR and starter calves having altered gross rumen measurements, epithelial stem and progenitor cell number, and epithelial proliferation status was supported, but hypothesized changes in volatile fatty acid (**VFA**) transporter abundance and VFA absorption rate were not supported. These results indicate that form of diet, even one that

promotes rumen growth, does not equate to enhanced ability to absorb VFA, but there is an effect on rumen stem and progenitor cells as well as epithelial proliferation.

General Audience Abstract

Understanding the fundamental aspects of calf nutrition is of vital importance for enhancing efficiency of calf growth. Calves are the future of any dairy herd and to perform their best, calves should be managed and fed in ways that ensure efficient growth. Research in these related areas stem back to the 1890s. Questions examined in this body of work mainly focused on dietary impacts on rumen growth and development in young calves. The first trial investigated effects of nutrient composition of pelleted calf starter diets on growth. It was discovered that form of diet, rather than composition, may be more important for causing changes in rumen growth and development. The second experiment resulted in development of a rumen sampling and infusion device to refine calf growth research. The third research trial tested the importance of calf starter on rumen growth and absorptive function. Results indicate that presumptive stem and progenitor cells within the rumen epidermis are identifiable by their ability to retain labeled DNA long-term and change proliferative status in response to diet. Absorptive function was not influenced by calf diet. Starter was important for rumen growth but not its function. Overall this work contributes to the enhancement of growth efficiency in dairy calves.

Dedication

Dedicated to my parents, my friends, my mentors, and my dogs, who have gotten me this far in life and continue to push me further

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Table of Contents

List of Tables

List of Figures

Chapter 1 : Introduction

Understanding the fundamental aspects of calf nutrition is of vital importance for enhancing efficiency of calf growth. This growth is relevant at the whole animal level, but also at the organ and tissue level where there is much development occurring as the calf ages. The growth of the neonatal calf is dependent on receiving adequate nutrition to meet maintenance and growth requirements (Drackley, 2008). Most calves meet these nutrient requirements early in life through drinking a liquid diet that begins digestion in the abomasum (Drackley, 2008). This diet will shift to a solid feed as the calf gets older and will highlight the importance of the rumen to start digestion. Thus, the growth and development of the rumen tissue is of the utmost importance to support the progression of the metabolism of the calf from that of a non-ruminant to that of a ruminant.

The rumen epithelium is highly valuable for the growing calf, mainly because of its role in volatile fatty acid (**VFA**) absorption. Absorption of VFA in the rumen and subsequent metabolism either in the rumen epithelium (mainly butyrate), or by hepatic and peripheral tissues (propionate and acetate) grants the rumen a main role in supporting calf growth due to the use of these substrates for meeting energy requirements (Siciliano-Jones and Murphy, 1989; Bergman, 1990). What remains to be discovered in the area of rumen development is how the rumen epithelial tissue proliferates and adapts to absorb VFA, primarily acetate, propionate, and butyrate.

The changes in rumen structure and function that occur as the calf consumes increasing amounts of starter are poorly understood. There is a copious amount of evidence supporting the effect of diet, and the resultant VFA, on rumen growth and development (Flatt et al., 1958; Sander et al., 1959; Tamate et al., 1962; Sutton et al., 1963b; Stobo et al., 1966), but there is

limited data on how these structural differences affect function. Sutton et al. (1963a,b) explored the ability of the calf rumen to adapt to dietary changes and the subsequent absorption of VFA, but other than these results there is a lack of supporting research in this area.

Another area of interest regarding rumen growth and development that is poorly understood is the mechanism behind proliferation of the functional epithelial tissue. Evidence supporting the role of diet, and the resultant VFA, in stimulating rumen epithelial cell proliferation is known (Sakata and Tamate, 1978; 1979; Goodlad, 1981; Sakata and Yajima, 1984; Shen et al., 2004). What is left to comprehend is how nutrition is affecting proliferation and ultimately supporting homeostasis of the rumen epithelium. One possible explanation is that resident rumen stem and progenitor cells contribute to rumen structure and function through their ability to proliferate in response to extrinsic factors, such as ruminal VFA concentration. Previous work from our laboratory has initiated the conversation on putative stem and progenitor cells in the rumen (Yohe et al., 2016), but other reports in this area of research are lacking.

The aim of this work was to examine the possible connection between potential rumen epithelial stem and progenitor cells and VFA concentration through a coordinated study of rumen structure and function. The overall hypothesis is that potential rumen epithelial stem and progenitor cell abundance, rumen pH, total rumen VFA content, and proportions of individual VFA are coordinated in calves and are affected by diet. The long-term goal of this research is to develop nutritional management strategies that will improve feed efficiency of calves. When all experiments are complete, we will have the first known characterization of potential rumen stem and progenitor cells coupled with complementary VFA data. These findings will enhance our understanding of dietary effects on rumen epithelial growth and development and the ability of

the rumen epithelium to respond to VFA. In the future we will be able to use our results to design mechanistic studies regarding regulatory roles of VFA on rumen stem and progenitor cells.

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Chapter 2 : Literature Review

Calf nutrition overview

Dairy calves, like all animals, must ingest and metabolize food in order to meet nutrient requirements for maintenance and growth. The diet of the typical dairy calf changes drastically from birth to 10 weeks of age, a period of time that encompasses weaning. Briefly, dairy calves are born as functional non-ruminants and become true ruminants in roughly 10 weeks. Diet and digestive physiology both factor into this change.

Pre-weaned dairy calf nutrition and digestive physiology

In preweaned dairy calves, dietary nutrients needed for maintenance and growth are primarily supplied by a liquid diet of either milk or milk replacer (**MR**) (Drackley, 2008). Maternal milk provides a form of nutrients that almost perfectly match the neonatal calf's digestive abilities (i.e. nonruminant metabolism mainly utilizing the abomasum and intestines while bypassing the rumen) and nutrient requirements. As examples, intestinal lactase, lipase, and peptidase activities are high in dairy calves at birth (Davis and Drackley, 1998). These enzymes break down milk lactose, fat, and protein with near 100% efficiency without involvement of the rumen and endproducts are readily absorbed into the calf's bloodstream for use in meeting maintenance and growth needs (Davis and Drackley, 1998). Warner and Flatt (1965) supported the importance of the abomasum compared to the rumen in early life by showing the weights of the two organs at birth (49% and 38%, respectively). In contrast to milk components, the efficiency of digesting cellulose and complex carbohydrates, typical in ruminant diets, is near zero in dairy calves at birth. This ability is linked to rumen development that

involves many factors including dry feed intake, rumen microbial establishment, and the metabolism of fermentation endproducts that occur over time as explained in detail in later sections.

Calf nutrition and rumen development

Increased solid feed intake, commonly in the form of starter, is needed for the rumen to develop. In weaned animals, rumen metabolism directly supports 63 to 80% of an animal's maintenance and growth energy requirements (Siciliano-Jones and Murphy, 1989; Bergman, 1990). Rumen development is also important considering the diminishing importance of the abomasum as the animal grows, which is showcased by the abomasal weight percentage of the total forestomach at birth compared to weaning (~8 weeks): 49% vs. 15%, respectively, and the simultaneous increase of the rumen weight percentage of the total forestomach at birth compared to weaning (~8 weeks): 38% vs. 67%, respectively (Warner and Flatt, 1965). How best to develop the rumen has been an ongoing discussion for over a century with research from as early as 1897 showcasing the importance of fiber for developing the rumen compared to animals fed only milk (Davenport, 1897).

Impacts of inert materials on rumen development

Flatt et al. (1958), Sander et al. (1959b), and Harrison et al. (1960) demonstrated the ability of inert material to influence overall growth of the rumen, but this growth was focused on the muscular development of the organ and not on the epithelium that is important for nutrient absorption and metabolism. The mechanism of how inert material stimulates muscular development of the rumen comes from the bulky material activating stretch receptors in the rumen wall (Harding and Leek, 1972). This activation of the smooth muscle for contraction leads to development of the muscle and overall growth of the rumen.

Impacts of forages on rumen development

Early rumen physiologists presumed that forage was a key component needed for rumen development (Davenport, 1897). The forages used were often alfalfa hay or timothy hay and are generally described as being "coarsely ground" (Conrad et al., 1950; Conrad and Hibbs, 1953; Hibbs et al., 1953; Harding and Leek, 1972). However, after an experiment where diets with either predominantly hay or predominantly grain were fed to dairy calves, Warner et al. (1956) noted: "The influence of grain on fore-stomach development is somewhat surprising since it has been commonly believed that hay is required for the development of the normal rumen tissue". Further, when examined more closely, the effect of high-forage diets on rumen mass was found to be similar to the overall effect of inert materials in the rumen – increased muscle without an impact on papillae growth (Harrison et al., 1960). These observations in the 1950s and 1960s directed rumen development research in a different direction for the next few decades – one that focused on concentrates in the ration.

Impacts of concentrates on rumen development

In the mid 1950s scientists began more intense research on the impact of concentrate-based rations on rumen development. The concentrates used were often ground shelled corn, ground oats, wheat bran, soybean oil meal, or a combination of these (Conrad and Hibbs, 1953; Hibbs et al., 1954; Conrad and Hibbs, 1956). Other examples include pelleted diets often containing ground shelled corn, wheat bran, soybean oil meal, ground alfalfa hay, timothy hay, or a combination of these (Conrad and Hibbs, 1956; Hibbs and Conrad, 1958; Hibbs et al., 1961). Examination of the literature shows that by the mid 1960s, many scientists were feeding lower forage and higher pelleted/concentrate diets to calves (Addanki et al., 1966a,b). Further research in the area of calf nutrition connected rumen tissue/papillae growth (i.e. epithelial

growth) with higher concentrate/grain diets (Tamate et al., 1962; Sutton et al., 1963b; Stobo et al., 1966) compared to hay/forage that had a larger impact on muscular growth. More recently, Heinrichs (2005) demonstrated that papillae development in the rumen is much greater at 6 weeks in calves fed MR and grain compared to calves fed MR and dry hay or MR only.

Impacts of volatile fatty acids on rumen development

A major breakthrough in our understanding of rumen development came in the late 1950s after it was already established that inert materials alone do not support rumen papillae growth (Flatt et al. (1958), Sander et al. (1959b), and Harrison et al. (1960)). The breakthrough realization happened when Flatt et al. (1958), through the use of inert material and fatty acid solutions containing sodium salts of acetate, propionate, butyrate, and lactate, concluded that the act of fermentation and resultant fatty acids were of the utmost importance for rumen epithelial development. This was further demonstrated by Sander et al. (1959a), Tamate et al. (1962), Sutton et al. (1963b), and Stobo et al. (1966), who, in various ways, all showed that butyrate is the main VFA implicated in rumen epithelial development. The VFA butyrate, more so than acetate and propionate, is commonly an endproduct of concentrate fermentation (Bath and Rook, 1963; Sutton and Johnson, 1969). This finding supports the previous research where concentrates were directly fed. The association between concentrate feeds, butyrate, and rumen papillae growth is further supported by historic and contemporary experiments (Poe et al. (1971), Sakata and Tamate (1978), Shen et al. (2004), Gorka et al. (2009), and Malhi et al. (2013)).

Microbial fermentation, VFA, and rumen development

A complex population of microbes becomes established in the rumen within 2 days of birth (Anderson et al., 1987). After initial rumen colonization by aerobic organisms, anaerobic bacteria typical of adult ruminants become established. These bacteria perform typical fibrolytic,

amylolytic, cellulolytic, and proteolytic functions (Bryant et al., 1958; Anderson et al., 1987; Fonty et al., 1987; 1989). In addition to bacteria, other microbial populations, such as fungi, protozoa, and archaea can be found in the rumen of young ruminants as well. Fungi have been found in the rumen of lambs as early as the first week of life and primarily aid in fiber digestion (Fonty et al., 1987). Protozoa are introduced into the rumen of young ruminants via exposure to faunated ruminants (Veira, 1986). Methanogenic species of archaea have been found scavenging H_2 and CO_2 in the rumen of 2-d-old lambs (Fonty et al., 1987; Morvan et al., 1994). This indicates that introducing dry feed into the rumen at a young age has the ability to result in earlier microbial development and higher ruminal metabolic activity. This increased metabolic activity will result in increased VFA concentration in the rumen contents (Anderson et al., 1987) that will ultimately aid in developing the rumen tissue and allow the animal to function as a true ruminant (Sakata and Tamate, 1978; 1979; Sakata and Yajima, 1984).

The VFA profile produced from a given feed ingredient or from an overall diet is a function of microbial fermentation. Suarez et al. (2006a,b) fed pelleted concentrate diets differing in main carbohydrate source to veal calves and found a diet high in starch and low in neutral detergent fiber (**NDF**) (59.3% DM and 9.4% DM, respectively) resulted in increased ruminal butyrate concentration compared to a diet lower in starch and higher in NDF (10.8% DM and 49.3% DM, respectively). Interestingly, though, in the same study, they also included a diet high in pectin (starch = 2.24% DM and NDF = 34% DM) and a diet with all three carbohydrate sources combined (starch = 24.3% DM and NDF = 30.8% DM) and found that the pectin and combined diets resulted in the highest ruminal butyrate concentrations and had the highest numerical rumen epithelial:rumen muscle ratio indicating a more developed rumen epithelium (Suarez et al., 2006a,b). Additionally, Khan et al. (2008) fed isostarch, isonitrogenous, and

isocaloric pelleted calf diets that differed in main starch source (ground barley, ground corn, crimped oats, or ground wheat) and found that even with balanced diets, ruminal butyrate concentrations as well as rumen weight and rumen papillae measurements can differ. These data suggest the importance of other factors involved in the complicated relationship between dietary nutrients, VFA, and rumen growth and development.

Rumen structure, function, and cellular mechanisms that affect growth and development

The complex ruminant forestomach is what separates ruminants from other domestic mammals that only have a simple glandular stomach for an initial site of nutrient digestion. The ruminant forestomach is made up of four compartments: rumen, reticulum, and omasum (the nonglandular portions of the stomach), and abomasum (the glandular portion) (Nickel et al., 1979). Considering the rumen is the main site of nutrient digestion and absorption in the adult ruminant, it is important to consider aspects of its general structure, function, and growth and development to further enhance our understanding of dairy calf nutrition.

Rumen structure

The general structure of the rumen organ is shown in **Figure 2.1** and consists of two outer muscle layers (tunica muscularis) that stimulate contraction of the rumen to mix and move contents into the reticulum for eventual eructation and chewing of cud. Moving further away from the surface there is the submucosal region harboring connective tissue and blood vessels that allows movement of nutrients, oxygen, CO_2, etc. The innermost, luminal, region of the rumen is comprised of the epithelium that line the papillae structures and increase surface area to aid in the main function of the rumen, which is absorption of VFA.

The absorptive surface of rumen papillae, the rumen epidermis, is described as keratinized stratified squamous epithelia that contains four cell layers, or strata, that is shown in

Figure 2.2. From the basement membrane side to the luminal side, they are stratum basale, stratum spinosum, stratum granulosum, and stratum corneum, respectively (Henriksson and Habel, 1961; Steven and Marshall, 1970; Graham and Simmons, 2005). Cells within the stratum basale are attached to the basement membrane. This strata may contain stem and progenitor cells (Yohe et al., 2016) that, upon division, either remain stem cells or differentiate into progenitor cells that will further differentiate and migrate through the epidermal cell layers (Steven and Marshall, 1970; Goodlad, 1981; Graham and Simmons, 2005).

Potential stem cells aside, the keratinized stratified squamous structure of the rumen epidermis is strikingly similar to that of skin (Neogrady et al., 1994; Costa et al., 2008a,b). Corroborating the similar structures between rumen and skin, the process of epithelial cells (specifically keratinocytes) migrating up through the strata is documented in human and mouse epidermal cells (Brody, 1959; Schon and Rheinwald, 1996; Tron et al., 1998) and is known as kertatinizaton. The process of keratinization consists of epithelial cells migrating from the stratum basale layer to the stratum corneum layer where at each stage they incorporate an increasing amount of keratin proteins while simultaneously losing cellular components until they die and become keratinized squames that will act as an abrasion-resistant protective layer and microbial barrier (Brody, 1959; Lavker and Matoltsy, 1970).

Interestingly, while previous thought as to the embryonic origin of the rumen pointed to another stratified epithelium, the esophagus, it has been shown that all chambers of the ruminant compound stomach arise from a spindle-shaped gastric enlargement that is unrelated to the esophagus (Warner, 1958) whereas cells of the abomasum become simple columnar epithelia (Warner, 1958).

The other major structural components of the rumen are tight-junctions and gap junctions. These aid in forming the physical barrier of the rumen and the functional syncytium needed to foster cell-to-cell communication, respectively. Tight junctions are mostly located between the outermost layers of rumen epithelial cells (i.e. stratum granulosum and stratum corneum) with the main protein, claudin-1, declining in density toward the stratum basale (Graham and Simmons, 2005). Unlike tight junctions, which serve a barrier function, gap junctions exist between cells for communication. Intercellular passage of small ions such as K^+ and Na^+ and cell-to-cell biochemical coupling occurs through members of the connexin gene family that aid in the sharing of small molecules (metabolites, sugars, lactate, butyrate, etc.) (Graham and Simmons, 2005). The location of the most prevalent gap junction protein, connexin 43, is localized to the stratum granulosum, stratum spinosum, and stratum basale in adult ruminants (Graham and Simmons, 2005). Both claudin-1 and connexin 43 have been shown to be present in the calf rumen epithelium (4 and 8 weeks old) via real-time PCR (Yohe, 2014), but the location of the proteins has not been confirmed.

Rumen function

A functional rumen must absorb fermentation end products, namely VFA, and perform ketogenesis (mainly utilizing butyrate as substrate). Ruminal ketogenesis will be discussed briefly, but because it is not the focus of this dissertation, a comprehensive review is not provided. A major component of this dissertation, rumen VFA absorption, will be discussed in detail below.

Rumen ketogenesis

Rumen catabolism of VFA centers on the production of ketone bodies. This process of ketogenesis in the rumen epithelium is limited in the young ruminant, but increases with age

(Baldwin and Jesse, 1992; Lane et al., 2000; 2002) and concentrate feeding (Penner et al., 2011; Connor et al., 2013). Lane et al. (2000; 2002) linked the increase in ketogenic ability (i.e. beta-hydroxybutyrate production) with the increase in mRNA expression of the gene for the rate-limiting enzyme HMG-CoA synthase, noting an ontogenic effect without dietary implications. Interestingly, Penner et al. (2011) and Connor et al. (2013) discussed results regarding dietary influence (mainly higher concentrate/grain feeding) having an influence on increased expression of rate-limiting ketogenic genes, but demonstration of activity via beta-hydroxybutyrate production was lacking. Nonetheless, during the preweaning stage of the calf's life, it appears that ketogenesis is not substantial; as such, rumen ketogenesis will not be further discussed in this dissertation.

Rumen VFA absorption

When we consider enhancing the ability of the rumen to absorb VFA, it is logical to think that increasing surface area via overall organ growth and papillae size/number should be a main goal. Shen et al. (2004) and Anderson et al. (1982) both demonstrated that feeding young ruminants a high-concentrate diet resulted in increased papillary size and density. Similarly, Zitnan et al. (1998) fed Holstein male calves a concentrate diet consisting of barley and soybean meal and observed an increase in the absorptive surface area of the papillae (papillae surface area/number of papillae in a specific area; mm^2/cm^2) compared to calves fed alfalfa only. As discussed in earlier sections, when calves are fed forages or other bulky feeds (such as alfalfa hay), papillae development is not stimulated but muscularization of the rumen is increased (Heinrichs, 2005) and thus it is thought, but not known, that the ability to absorb VFA is decreased.

Existing data suggest that VFA entry into ruminal epithelial cells can occur via facilitated transport and passive diffusion (Muller et al., 2000; Connor et al., 2010; Aschenbach et al., 2011) where facilitated transport requires membrane transport of the dissociated VFA and passive diffusion is relevant for the undissociated VFA. A diagram depicting the rumen epithelial layers and placement of protein transporters is shown in **Figure 2.2**.

In dairy cattle, commonly studied VFA transporters include members of the solute carrier (**SLC**) family. These include down-regulated-in-adenoma (**DRA**; *SLC26A3*), putative anion transporter 1 (**PAT1**; *SLC26A6*), and monocarboxylate transporters 1, 2, and 4 (**MCT1**, *SLC16A1*; **MCT2**, *SLC16A7*; **MCT4**, SLC16A3) (Connor et al., 2010; Laarman et al., 2012; Naeem et al., 2012; Schlau et al., 2012; Steele et al., 2012; 2013). DRA and PAT1 are both believed to help maintain pH in the rumen via transporting bicarbonate into the rumen (Connor et al., 2010). The MCT1 protein was localized to the basal membrane of ruminal epithelial cells in the stratum basale of calves (Kirat et al., 2005). The MCT1 protein is suspected to be involved with VFA, lactate, and ketone body exit from ruminal epithelial cells to portal circulation (Graham et al., 2007). Laarman et al. (2012) found greater *MCT1* mRNA abundance in calves fed MR, hay, and calf starter, compared to those fed only MR and hay. MCT2 is also localized to the basal membrane of ruminal epithelial cells; however, in addition to being localized to the stratum basale, MCT2 is also diffusely located in cells comprising the stratum granulosum and stratum spinosum (Connor et al., 2010). The relative abundance of *MCT2* compared to *MCT1* is much less (Connor et al., 2010), but both seem to have a similar function. Naeem et al. (2012) observed a diet by time interaction for *MCT2*; in general mRNA abundance for this transporter was more highly expressed in 5-week-old (compared to 10-week-old) calves fed a diet with more concentrate than the comparison group. Kirat et al. (2007) located the MCT4 protein in the

corneum and granulosum layers of the rumen epithelium, which Connor et al. (2010) suggested an apical cell membrane location that fits the narrative for transporting VFA from the rumen lumen by MCT4 and then subsequent transport to lower epithelial cell layers via MCT1 on the basal membrane of cells in the stratum basale layer.

Passive diffusion of VFA is enhanced at a lower rumen pH (Dijkstra et al., 1993; Aschenbach and Gabel, 2000; Penner et al., 2011). Many factors affect rumen pH: feed ingredients, feed composition, particle size of feed, VFA concentration, lactate concentration, salivary buffer present, and bicarbonate and hydrogen ions secreted from the rumen epithelium to name a few (Nagaraja and Titgemeyer, 2007). Once undissociated forms of VFA enter ruminal epithelial cells, intracellular dissociation can occur which can increase intracellular H^+ concentration. This, coupled with the loss of intracellular HCO_3^- in exchange for VFA can further lower intracellular pH (Kramer et al., 1996), which can lead to damage of the rumen epithelial cells (Huhn et al., 2003). To regulate intracellular pH, ruminal epithelial cells have Na^+/H^+ exchangers (**NHE**). Graham and Simmons (2005) identified 3 ruminal isoforms of NHE; these are known as NHE1, NHE2, and NHE3. These NHE secrete H^+ [obtained after dissociation of VFA] from ruminal epithelial cells in exchange for Na^+. In goats, Yang et al. (2012) showed greater mRNA abundance of *NHE1* and *NHE3* in animals fed highly fermentable, as opposed to lowly fermentable diets. The NHE1 protein is mainly at the apical end of cells in the stratum granulosum (Graham et al., 2007). The authors suggested that this exchange protein may contribute to maintenance of the rumen permeability barrier and may also reduce the pH of the extracellular space surrounding cells of the stratum granulosum, which would promote protonation of VFA and uptake by diffusion (Graham et al., 2007). The NHE2 protein is located intracellularly in the cytosol of cells in the stratum granulosum, stratum spinosum, and stratum

basale (Graham et al., 2007). Graham et al. (2007) suggested that NHE2 functions to excrete H^+ into the extracellular space surrounding cells, rather than into the lumen of the rumen. This too would aid in diffusion of VFA down a concentration gradient. The NHE3 protein is localized to the apical ends of ruminal epithelial cells (Connor et al., 2010). Laarman et al. (2012) noted that mRNA abundance of *NHE3* was lower in calves fed MR and hay compared to those fed MR, hay, and starter. Schlau et al. (2012) observed more *NHE3* mRNA abundance in rumens of steers classified as "acidosis resistant" as opposed to "acidosis susceptible". Overall expression and localization patterns of ruminal VFA transporters (DRA, PAT1, MCT1, MCT2, MCT4) and transporters that aid in VFA uptake (NHE1, NHE2, and NHE3) are not well characterized in young calves, and little is known about the impact of age in addition to diet on their abundance. This is a serious gap in our understanding because it appears that they play important roles in VFA uptake and intracellular pH regulation.

VFA absorption in mature ruminants

There has been considerable work looking into VFA absorption in the mature rumen. Phillipson and McAnally (1942) demonstrated in the ewe that administering different carbohydrate sources (e.g. glucose, lactate, and sodium acetate) directly into the fistulated rumen caused disappearance of each carbohydrate within the rumen as well as very little to no appearance of the carbohydrates reaching the abomasum over time. These results led the researchers to conclude that there must be absorption of carbohydrates occurring in the rumen (Phillipson and McAnally, 1942). The work of Phillipson and McAnally was continued and further supported by Barcroft et al. (1944) who utilized an anaesthetized ewe and took blood samples from veins draining specific compartments of the alimentary tract. This work showed that the vein draining the rumen contained more VFA than that of the cecum, reticulum, and

omasum, whereas the abomasum and small intestine contained little to no VFA, leading the researchers to conclude that the rumen is the main site for fermentation of carbohydrate and subsequent VFA absorption in the ruminant. Danielli et al. (1945), Gray (1948), Parthasarathy and Phillipson (1953), and Annison et al. (1957) all further demonstrated the ability of the ewe rumen to absorb VFA either through measuring a decrease in concentration of VFA in the rumen, appearance of VFA in the portal blood, or a combination of both results. In the time since these early studies were conducted, much research interest has focused on optimization of diets for maximal VFA production and absorption.

VFA production is largely a function of diet and the complement of rumen microbes present whereas rumen VFA absorption depends more on body tissues. Several factors are known to influence VFA absorption in the rumen. Danielli et al. (1945) utilized a washed rumen technique in ewes and found that pH alters the mechanism of absorption. It was found that a lower rumen pH (~5.8) caused an increase in absorption of the intact VFA alone (i.e. passive diffusion) compared to a higher rumen pH (~7.5) where absorption of the VFA anion accompanied by a hydrogen ion (i.e. facilitated transport) was more prevalent; this has since been confirmed by many researchers (Dijkstra et al., 1993; Aschenbach and Gabel, 2000; Penner et al., 2011).

In addition to pH, rate of blood flow affects rumen VFA absorption (Storm et al., 2011; 2012), which has been shown to be influenced by butyrate (Dobson et al., 1971), rumen vein blood flow rate (Remond et al., 1993; Storm et al., 2011), dietary crude protein intake (Storm et al., 2011), and rumen epithelial metabolism of VFA (Storm et al., 2012). Bidirectional flux of VFA is another factor in rumen VFA absorption that is regulated by epithelial blood flow. Bidirectional flux of VFA (i.e. VFA transfer from the rumen lumen to the epithelium to the

blood as well as VFA transfer from blood to the epithelium to the rumen lumen) has been demonstrated by Kristensen et al. (1996) and Storm et al. (2012).

VFA absorption in preweaned calves

Much less is known about the ability of the calf rumen to absorb VFA compared to the mature cow/ruminant. The absorption of VFA by the ruminant animal to meet energy needs is not the primary source for obtaining energy until after the calf has been weaned from a liquid diet. That being said, the ability of the rumen to absorb VFA is present during the preweaning phase of the calf's life (Khouri, 1969), thus warranting further investigation. Data regarding VFA absorption in dairy calves is sparse. Conrad et al. (1956) showed a decrease in acetic acid, propionic acid, and butyric acid concentrations between the rumen/reticulum and abomasum of a 5-week-old calf consuming 0.45 kg of a pelleted diet/d (acetic acid: 26.1 vs. 0.31 meq/L, propionic acid: 14.3 vs. 0.07 meq/L, and butyric acid: 4.0 vs. 0.04 meq/L). Khouri (1969) showed that the calf's rumen has the ability to absorb VFA within the first week of life and this is due to the presence of acetate in peripheral blood. Khouri's work corroborated that of Sutton et al. (1961) who utilized the washed rumen technique in calves and showed a decreased ability to absorb VFA in the rumen of calves fed only a milk diet (week 1: 20.5 meq % acid/h, week 4: 26 meq % acid/h, and week 8: 21.5 meq % acid/h) compared to that of a milk, alfalfa pellet, and grain diet (week 1: 29.5 meq % acid/h, week 4: 162.5 meq % acid/h, and week 8: 178.5 meq % acid/h). Calves that continued to consume only milk maintained the same rate of absorption at 35 weeks of age whereas if the calf switched to a diet of milk, alfalfa pellets, and grain, the rate of absorption increased to 248 meq % acid/h by 35 week. Other research by the same group demonstrated calves fed only milk had a decreased maximum ability to absorb acetate from week 1 to 34 of age (stayed constant around 30 mg acetate/100 mL solution/h) compared to calves fed

increasing amounts of milk, grain, and hay from week 1 (about 30 mg acetate/100 mL solution/h) to 34 (about 210 mg acetate/100 mL solution/h) (Sutton et al., 1963a). Sutton et al. (1963a) also showed a decreased rumen pH increased VFA absorption in calves just as it does in adult ruminants. This illustrates that calves are born with the ability to absorb VFA in their rumen (albeit a limited ability), but any factors that influence VFA absorption in calves have yet to be delineated. This represents a gap in knowledge that this dissertation work aims to address.

Further, Storm and Kristensen (2010) demonstrated that lactating dairy cows can absorb around 100 mol of VFA per day (50 mol acetate if assuming a three-pool ratio of: 50% acetate, 35% propionate, and 15% butyrate). A similar absorption rate is not well documented in calves. One of the only traces of information on this exists in the literature of Sutton et al. (1963a). The researchers added a buffer containing 127 m*M* VFA (specific VFA concentrations were not specified, but assuming a three-pool ratio of: 50% acetate, 35% propionate, and 15% butyrate - that yields a buffer containing: 63.5 m*M* acetate, 44.5 m*M* propionate, and 19.0 m*M* butyrate) to the empty rumens of calves fed different diets. Calves fed only milk absorbed up to 0.18 mol of acetate per day at 8 weeks of age whereas calves fed milk, grain, and hay absorbed 1.63 mol of acetate per day at 8 weeks of age (Sutton et al. (1963a). Questions arise from inspecting the work by Sutton et al. (1963a), such as: was the concentration of the buffer used in the first experiment the same used by Sutton et al. (1962)? This is not clear. Why did calves fed milk, grain, and hay receive rumen inoculations of adult rumen contents? Also, what were the absorption rates for the other two VFA (propionate and butyrate)? This work was thorough and delicately executed, but considering the advancement of time and lack of corroborating data, it is worth revisiting the topic of dietary effects on rumen VFA absorption in preweaned calves.

Cellular mechanisms that affect rumen growth and development

There have been many different hypotheses as to how the rumen epithelium is influenced at the cellular level to undergo proliferation and differentiation. Our initial research supports existence of rumen epidermal stem and progenitor cells (Yohe et al., 2016) but, in general, this is an understudied area of research.

Stem and progenitor cells

Stem and progenitor cells have been mislabeled and misunderstood since they were discovered. Stem cells are defined as either totipotent (capable of producing cell types from any of the three embryonic germ layers: ectoderm, mesoderm, and endoderm) or pluripotent (capable of producing more than one cell type, but not an entire organism) (Potten, 2006). Embryonic stem cells are totipotent and they are not the focus of this dissertation. Pluripotent cells capable of populating the rumen epidermis are the main interest. These cells are further classified as adult stem cells which are known to help replenish cell populations within tissues (Potten, 2006). Progenitor cells are broadly defined as committed cells deriving from a stem cell (Potten, 2006).

Rumen epidermal stem and progenitor cells likely serve the same general purposes of corresponding cells in similar organs/tissues. Because of the lack of data on rumen epidermal stem and progenitor cells and because of suspected similarities with corresponding cells in other epithelial tissues (e.g., skin epidermis, intestinal epithelia, and mammary epithelia), findings related to these other organs are presented next. Where possible, bovine references are highlighted.

Skin epidermal stem and progenitor cells

Given its similar tissue architecture and function as a protective barrier, further examination of skin epidermis literature represents a potential starting point for the study of

rumen epidermal stem and progenitor cells. Both organs having a keratinized, stratified squamous epithelial structure (Neogrady et al., 1994; Costa et al., 2008a,b). Much research has been put into characterizing and understanding the function of epidermal stem and progenitor cells, including the processes of aging, wound healing, and in skin disease (Bell and Van Zant, 2004; Giangreco et al., 2010; Arwert et al., 2012; Plikus et al., 2012; Li et al., 2017).

So far, epidermal stem cells are known to reside in the hair follicle bulge and replenish the functional compartments of the epidermis (Miller et al., 1993; Yang et al., 1993; Lavker et al., 2003). From this hair follicle bulge, it is believed that stem cells create progenitor cells that differentiate into follicular cells, sebaceous glands, and the epidermis (Taylor et al., 2000; Oshima et al., 2001; Morris et al., 2004).

Zouboulis et al. (2008), Ambler and Maatta (2009), and Eckhart et al. (2013) identified the following epidermal stem and progenitor markers: beta 1-integrin (**ITGB1**), tumor protein p63 (**TP63**), keratin-14 (**KRT14**), and Notch-1 (**NOTCH1**). Integrins are a class of proteins that are important for cell adhesion (Jones and Watt, 1993; Janes and Watt, 2006), which demonstrates the importance of ITGB1 as a stem cell marker due to its role in adhering the stem cell located in the basale epidermal layer to the basement membrane (Jones and Watt, 1993; Janes and Watt, 2006; Shen et al., 2017). Tumor protein p63 is of interest due to previous findings of its importance for maintaining proliferative potential of epidermal stem cells through controlling genes relevant for stem cell function (Yang et al., 2006; Senoo et al., 2007; Suzuki et al., 2015). Keratin-14 is a part of the keratin family of proteins that comprise the intermediate filaments that make up the cytoskeletal system of cells (Moll et al., 2008). There are a multitude of keratins that are expressed throughout many epithelial cells in the body with KRT14 known to be an important type I filament that combines with the type II filament keratin-5 to form a

heteromeric filament (Moll et al., 2008; Wei et al., 2016). This heteromeric complex has been shown to bind to desmosomes and hemidesmosomes to support cell-to-cell adhesion and extracellular matrix adhesion and have been found to be strongly expressed in the undifferentiated basale cell layer of the epidermis (Fuchs and Green, 1980), which has been shown to include the stem cell population as well (Zhang et al., 2016). NOTCH1 is a cell signaling protein found in many processes, but relevant to stem cell biology it is known to be implicated in regulating stem cell self-renewal and differentiation mainly via altering cell-to-cell adhesion (Watt et al., 2008; Zhang et al., 2013). It is important to note that the epidermis is not the rumen, which means that although comparisons between the two similarly structured tissues is an acceptable basis for initial questions, their functions are still different, which will ultimately influence the proteins expressed and thus any potential stem cell markers.

Intestinal stem and progenitor cells

Looking to the intestinal epithelium for clues about rumen stem and progenitor cell markers makes sense for two reasons. First, the shared location within the gastrointestinal tract may portend shared lineage markers. Second, both the intestinal epithelia and rumen epithelia perform absorptive functions; this might also forecast shared lineage markers. Unlike the rumen, the intestine is not a stratified squamous epithelium, but rather is a simple columnar epithelium comprised of villi and crypts (Potten, 2006).

There has been a considerable amount of research looking into intestinal stem and progenitor cells, which are known to reside in the crypts of the small intestine and migrate upward to become an enterocyte (nutrient absorption), goblet cell (mucin secretion), enteroendocrine cell (hormone secretion) or move to the base of the crypt and become a paneth cell (mucosal immunity) (Potten, 2006). Leucine-rich repeat-containing G protein-coupled

receptor 5 (**LGR5**) and musashi-1 (**MSI1**) have emerged as potential intestinal cell markers (Barker, 2014; Clevers et al., 2014; Tan and Barker, 2014). LGR5 has been shown to be important for acting as a coreceptor in the Wnt signaling pathway (Tan and Barker, 2015), which is known to be an important pathway regulating intestinal epithelial homeostasis (Potten, 2006). MSI1 is an RNA-binding protein shown to be important for asymmetric division in *Drosophilia* in precursor cells for organs (Kaneko et al., 2000) and it has been found in undifferentiated cells located in the intervillus region (non-crypt area between villi) supporting the notion that it labels an undifferentiated stem cell population (Kayahara et al., 2003; Nishimura et al., 2003; Potten et al., 2003). Other potential markers include those involved in the Wnt signaling pathway, Notch signaling pathway, and the Hedgehog signaling pathway to name a few (Potten, 2006). The intestine may prove a useful reference to start searching for markers and potential mechanisms, but any comparison should be noted with caution considering the exposure to different environments for each tissue and varied patterns of cell renewal within each tissue.

Mammary epithelial stem and progenitor cells

Another tissue that has been the subject of much stem and progenitor cell research is the mammary gland. Mammary epithelial ducts have a stratified cuboidal epithelium. In relevance to the rumen epithelium, the main connection resides in the previous research conducted in stem cell biology wherein cattle themselves were used in research (Capuco and Ellis, 2005; Capuco, 2007; Capuco et al., 2009; Daniels et al., 2009) as opposed to epidermal and intestinal work that has mainly used rodents or *in-vitro* models.

Bovine mammary epithelial stem and progenitor cells have been identified with an in vivo DNA labeling technique (Capuco, 2007; Capuco et al., 2009; Daniels et al., 2009) that may prove useful for the study of rumen stem and progenitor cells. *In-vivo* DNA label-retaining

studies have utilized 5-bromo-2`-deoxyuridine **(BrdU**) as well as ^{3}H-thymidine (Gunduz, 1985; Langer et al., 1985) incorporation into replicating DNA strands during the S-phase of the cell cycle (Potten et al., 2002; Capuco et al., 2009; Daniels et al., 2009). Why label-retaining cells are promising stem cell candidates has to do with the "immortal DNA strand hypothesis" that essentially states that during asymmetric cell division adult stem cells will retain the oldest DNA strand, which allows for evasion of any misrepair during DNA replication by DNA polymerases (Cairns, 1975; Potten et al., 1978; Janion, 2001; Friedberg, 2003). This retention of DNA without alteration will supposedly ensure constant renewal of cells that retain the functionality of the tissue of interest.

Rumen epithelial stem and progenitor cells

The only previous work looking into potential rumen epithelial stem cells was performed by this laboratory (Yohe et al., 2016) where real-time PCR was utilized to assess the abundance of suggested stem cell markers from the epidermis (*ITGB1*, *TP63*, *KRT14*, and *NOTCH1*) and intestine (*LGR5* and *MSI1*) in the rumen of Holstein heifer calves fed differing preweaning diets (Geiger et al., 2016a,b). The diets differed in amount of energy and protein intake via liquid diet that resulted in an increase in expression of *ITGB1* gene in the rumen epithelium of calves fed a higher plane of nutrition (Geiger et al., 2016a,b). It is unclear how the diet would have had an effect on *ITGB1* expression, but considering *ITGB1* is important for cellular adhesion it can be speculated that an increase in expression may mean an increased number of stem cells adhering to the basement membrane. It should be noted that the expression of these genes was from the entire rumen epithelium and not just a stem and progenitor cell population so any inference cannot be directed solely to the stem and progenitor cells. That being said, this is just initial speculation attempting to make sense of some preliminary data.

Evidence of the rumen epithelium responding to external dietary stimuli was demonstrated by Goodlad (1981) who showed increased turnover rates of rumen epithelial cells in sheep fed a diet transitioning from forage to concentrate (4.3 ± 2.8 days turnover time) compared to forage-based (16.5 ± 0.7 d) and concentrate-based (10.9 ± 2.0 d) diets. These results are focusing on the proliferation of the rumen epithelium and not a specific stem/progenitor cell population, but considering the proliferating cells are derived from the stem and progenitor cells these data are relevant. Whether or not a specific stem cell is present in the rumen epithelium is to be determined, but there must be a population of cells that is supporting the renewal of cells in the rumen epithelium; the question is where is this population and how can it be characterized?

Knowledge gaps addressed in research for this dissertation

There is much less knowledge regarding ruminant metabolism in the calf compared to the adult ruminant. This makes sense considering the nutritional needs of the preweaned calf, but enhancing our understanding of nutritional concepts surrounding the calf will be beneficial to better understand how to effectively feed and manage these young ruminants.

The work relevant to starch and NDF digestion as well as the detailed technical work will help add to our collective knowledge on how to feed calves as well as manage them in a research setting.

A main concept that will be explored is the connection between preweaned calf nutrition and rumen growth and development with an emphasis on the ability of diet to affect the ability of the rumen tissue to absorb VFA. Within this objective there will be some novel insight into localization of VFA transporters that has previously not been demonstrated in calves.

The last main focus of this research is to further our understanding of potential stem and progenitor cells in the rumen epithelium. There is very limited research on this topic, which

makes initial findings difficult to interpret, but any findings here will hopefully be useful for future research in this area. It should be noted, though, characterizing and identifying a true stem cell may be impossible due to the nature of altering the environment during testing and observation, but the most accurate and useful identifier of a stem cell is its ability to regenerate stem cells and non-stem cells of a tissue for as long as the organism is alive

Noting the previous work cited throughout this review there are still gaps of knowledge that can be filled. Throughout the remaining chapters of this dissertation an explanation to some of the questions will be attempted.

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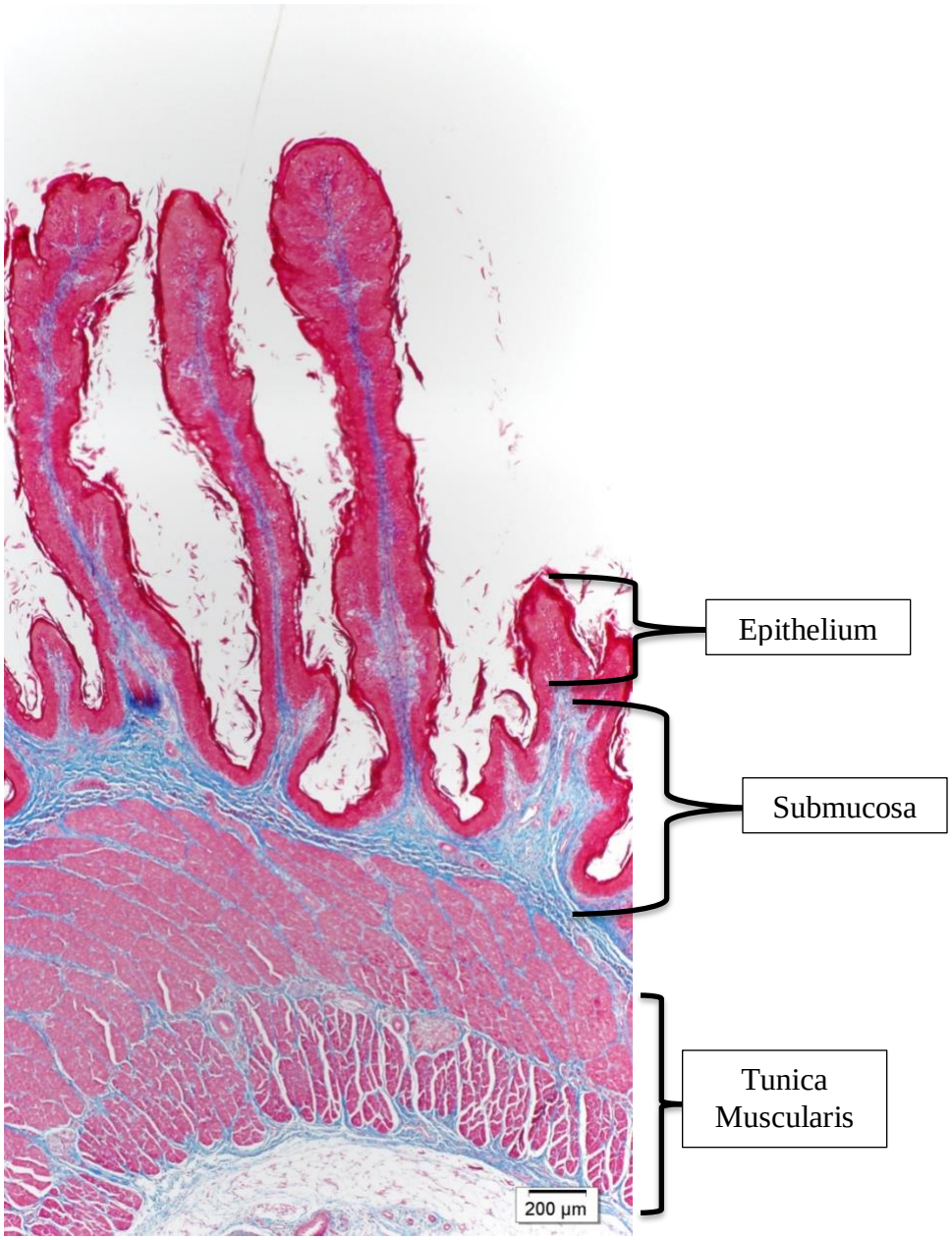

Figure 2.1. Rumen tissue stained with Masson's Trichrome stain. The colors stained in the following manner: dark purple = nucleus, red/pink = cytoplasm, and blue = connective tissue.

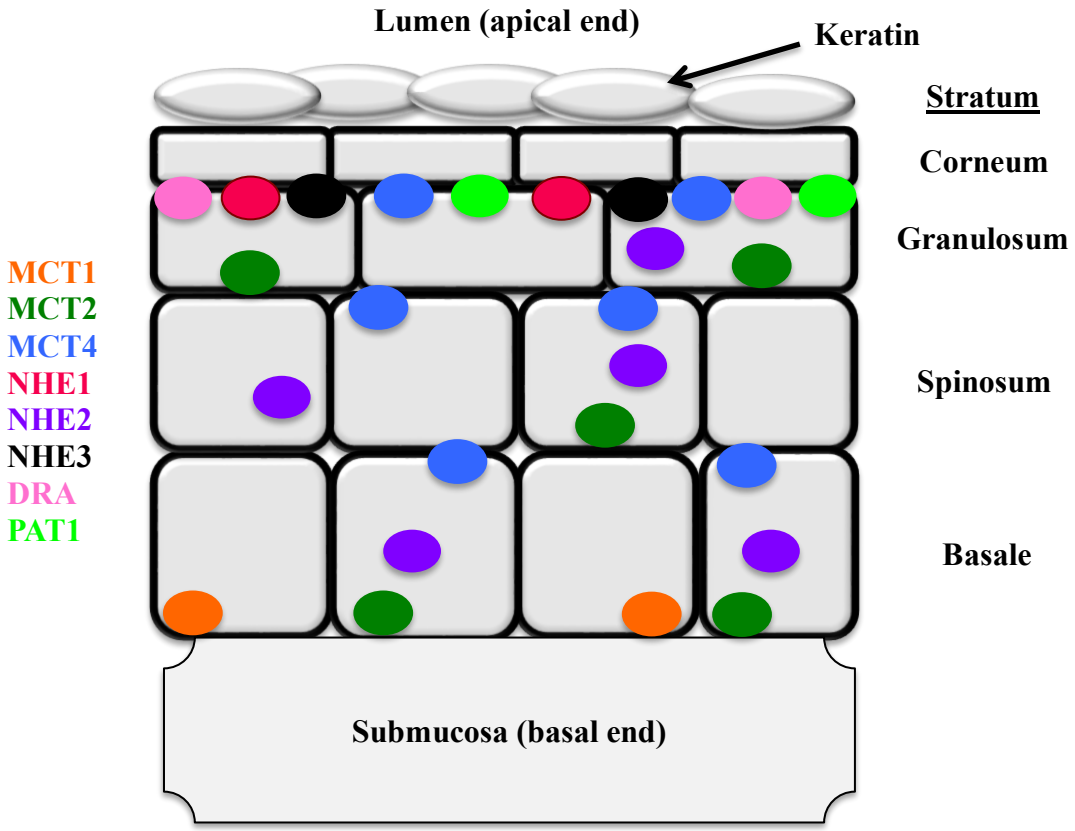

Figure 2.2. Rumen epithelial cell layers depicting location of protein transporters. MCT = monocarboxylate transporter, NHE = sodium-hydrogen exchanger, DRA = down-regulated-in-adenoma, and PAT = putative anion transporter.

Chapter 3 : Effects of completely pelleted calf starter diets differing in starch and NDF content

Abstract

Nutrient composition of ingested feed partly determines the profile of volatile fatty acids (**VFA**), produced in the rumen via microbial fermentation. High starch feeds typically yield a high molar proportion of the VFA butyrate, whereas high fiber feeds typically yield high molar proportions of acetate and propionate. Ingestion of high starch diets and subsequent butyrate production and metabolism are sometimes, but not always, associated with increased rumen development in dairy calves. Objectives were to determine if pelleted calf starter diets differing in levels of starch and neutral detergent fiber (**NDF**) would result in altered feed intake, growth, rumen metabolism, and rumen morphometric analyses. The hypothesis was that feeding young dairy calves completely pelleted, high-starch diets would increase: DMI, growth, ADG, rumen metabolism, blood BHB concentration, and rumen morphometric analyses. Eight neonatal Holstein bull calves were blocked into two dietary treatments based on body weight (40.3 ± 1.0 kg) and serum total protein (5.80 ± 0.21 g/dL). Treatments were diets containing high-starch, low-NDF diet (**HS**, n = 4) or high-NDF, low-starch diet (**HN**, n = 4). Both starters were completely pelleted. All calves were fed the same non-medicated milk replacer containing 22.7% CP, 21.9% fat (DM basis). Milk replacer was reconstituted to 13% solids and fed by bottle until calves were bucket trained. Pelleted starter and water were available *ad libitum* to all calves from day 0. Feed and water intake were recorded daily. Body measurements, rumen fluid, and blood samples were obtained weekly and calves were euthanized at 7 weeks of age to collect gross rumen measurements. The only treatment differences noted were that HS had lower overall rumen pH compared to HN calves (HS = 5.37 ± 0.08, HN = 5.81 ± 0.07) as well as some

treatment × week interaction effects on rumen lactate concentrations. Besides HS resulting in lower rumen pH, the hypothesis that completely pelleted calf starter diets differing in NDF and starch level would alter feed intake, growth, rumen metabolism, and rumen measurements was not supported. However, calves fed the high-NDF starter were $5.71 less expensive per calf to raise. These findings suggest a form of feed effect in today's calf starter diets that may be of physiological and economic importance.

Key words: Starch, NDF, Rumen development, VFA, Calf nutrition

Short Communication

As dairy calves mature they increase dry feed consumption, which leads to an increase in available substrate in the rumen for microbial populations to ferment into VFA (acetate, propionate, butyrate) (Bergman, 1990). High-starch diets are known to yield more butyrate than high-NDF diets; butyrate (and to a lesser extent propionate) is implicated in stimulating rumen epithelial growth (Sander et al., 1959; Sakata and Tamate, 1978; Shen et al., 2004; Gorka et al., 2009; Malhi et al., 2013). These are two reasons that high-starch diets are recommended for young dairy calves and high-NDF diets are not. Despite these feeding guidelines, contemporary data that show enhanced rumen growth effects when high amounts of starch (as opposed to high-NDF), are fed to calves are lacking. Therefore, our objectives were to determine if completely pelleted calf starter diets differing in levels of starch and NDF would result in altered feed intake, growth, rumen metabolism, and rumen morphometric analyses. We hypothesized that feeding young dairy calves completely pelleted, high-starch diets would increase: dry matter intake (**DMI**), growth, average daily gain (**ADG**), rumen metabolism, blood β-hydroxybutyrate (**BHB**) concentration, and rumen morphometric analyses.

This experiment was approved by the Virginia Tech (**VT**) Animal Care and Use Committee (protocol #15-165) and conducted from September 2015 to January 2016. Eight VT-born Holstein bull calves were used. Calves were enrolled as they were born and were individually housed and fed in outdoor hutches on gravel with sawdust bedding. Hutch placement prevented calf-to-calf contact. Within 8 hours of birth each calf received 4 L of colostrum over 2 feedings; body weight, hip height, and withers heights also were recorded. On day 1, calves were balanced by BW (40.3 ± 1.0 kg) and serum total protein (5.80 ± 0.21 g/dL) into 2 treatment groups: high-starch, low-NDF (**HS**, n = 4), and high-NDF, low-starch (**HN**, n =

4). All calves were fed the same non-medicated milk replacer (**MR**; Amplifier Max; Land O'Lakes Animal Milk Products Co., Shoreview, MN) containing 22.7% CP, 21.9% fat (DM basis). Protein sources in the MR were all milk-based; the fat source was primarily edible lard. At each feeding, 283 g of MR (as-fed) were reconstituted to 13% solids and fed by bottle until calves were bucket trained. Calves began a 5-day weaning process when 0.91 kg of starter was consumed for 3 consecutive days or at day 42, whichever came first. Calves were fed MR at morning (0700 h) but not evening (1900 h) feedings during the weaning process. Pelleted starter (Southern States Cooperative; Richmond, VA) and water were available *ad libitum* to all calves on day 0. Calves on HS received a starter that was 25.13% starch, 26.27% NDF (DM basis; **Table 3.1**). Calves on HN received a starter that was 40.53% NDF, 8.30% starch (DM basis; **Table 3.1**). Both starters contained: ground soybean hulls, high-protein soybean meal, cottonseed hulls, wheat middlings, molasses, limestone, and a vitamin and mineral premix (**Table 3.1**). The HS diet included ground barley and ground wheat whereas the HN diet excluded these ingredients, but included ground corn (**Table 3.1**). Weekly MR and starter samples were taken, combined, mixed, and sent to Cumberland Valley Analytical Services (Hagerstown, MD) for analysis. Resultant feed analyses are presented in **Table 3.1**. Also, *in situ* disappearance of DM, CP, NDF, and starch were determined for the HS and HN pellets. Approximately 10 g of pellets were placed in 10 × 20 cm polyester bags (Ankom Technology, Macedon, NY) with a pore size of 50 ± 15 μm and suspended in a large nylon mesh bag that was placed in the rumen of a steer (Cyriac et al., 2008). Residual feed in nylon bags were obtained at 0, 8, and 24 h, finely ground and sent to Cumberland Valley Analytical Services (Hagerstown, MD) for analysis.

Calf was the experimental unit. Body growth and rumen metabolites were measured

weekly; MR, starter, and water intake were measured daily. Fecal and respiratory scores were assessed twice daily (Yohe et al., 2015) and did not differ by treatment (data not shown).

Rumen fluid samples (20 to 50 mL) were obtained weekly until 7 weeks of age resulting in 7 rumen samples per calf. Samples were collected via oroesophageal tube at 0900 hours using an attached 60 mL syringe for aspiration as performed by Geiger et al. (2014); pH measures were taken at sampling after any excess saliva was removed. Approximately 15-20 mL of rumen fluid were strained through four layers of cheesecloth and stored at -20°C in glass screwtop tubes for VFA and lactate analyses. Analysis of VFA was done by gas chromatography (**GC**) using an adapted method from Kristensen (2000). *D*- and *L*- lactate analysis was by enzymatic determination (Boehringer Mannheim D/L-Lactic acid Test-Combination; R-Biopharm AG, Darmstadt, Germany) on triplicate samples. Jugular blood (5 mL) was collected into evacuated sodium heparin tubes from each calf weekly, two hours after the morning feeding (0900 h), to determine BHB concentrations. For this, plasma was harvested, stored at -20°C, and analyzed in triplicate for BHB via enzymatic colorimetric assay (β-Hydroxybutyrate Colorimetric Assay Kit; Cayman Chemical, Ann Arbor, MI).

At 7 weeks of age calves were euthanized and full stomachs (reticulorumen, omasum, abomasum) were removed. Briefly, the ligated full stomach was weighed, individual portions evacuated (reticulorumen, omasum, and abomasum), and weighed again. Reticulorumens underwent morphometric analyses according to our previously described methods (Yohe et al., 2015). In brief, rumen samples were stored in saline overnight, dried the following day and then weighed. Ten representative papillae were plucked from the cranial ventral biopsy and measured for 2-dimensional surface area.

Growth, intake, health, blood BHB, and rumen data were analyzed using the MIXED

procedure of SAS 9.4 (SAS Institute, Cary, NC). Growth, intake, health, blood BHB, ruminal VFA, pH, and lactate models included the fixed effects of treatment, week, and their interaction. Rumen morphometric measurement models included the fixed effect of treatment. Calf nested within treatment was a random effect in all models. All analyses included the best-fit covariance structure; denominator degrees of freedom were not specified. Best-fit covariance structures were used for each variable: body weight, total VFA, acetate, propionate, and BHB measurements used heterogeneous compound symmetry, ADG, total DMI, starter DMI, water intake, butyrate, *D*-Lactate, *L*-Lactate, and rumen pH measurements used heterogeneous autoregressive, withers height and MR DMI measurements used autoregressive, and hip height measurements used toeplitz. Significance was declared when $P \leq 0.05$. Orthogonal polynomial contrasts were performed for variables involving a significant time effect. Least squares means ± standard errors of the mean are reported.

For the majority of variables measured, the hypothesis that completely pelleted calf starter diets differing in NDF and starch level would alter growth, intake, rumen metabolism, and rumen morphometric analyses was not supported. **Table 3.2** shows there were no differences for treatment × week or the main effect of treatment for: body weight ($P = 0.577$, $P = 0.585$), withers height ($P = 0.751$, $P = 0.127$), hip height ($P = 0.243$, $P = 0.888$), or ADG ($P = 0.728$, $P = 0.941$). These same variables all differed for the main effect of week, as expected for growing calves (**Table 3.2;** $P < 0.0001$ for each). As designed, milk replacer DMI did not depend on any model effects (**Table 3.2**); calves typically drank all MR that was offered. In this experiment, calves had *ad libitum* access to one of two completely pelleted calf starters. Characteristics of each starter are shown in **Table 3.1**. There was no treatment × week interaction effect on starter DMI (**Table 3.2**; $P = 0.700$) but starter DMI intake increased as calves aged (**Table 3.2**; $P < 0.0001$),

which is expected. Starter DMI was not impacted by treatment (**Table 3.2**; $P < 0.985$). Calf growth measures were biologically normal for calves on each treatment given diet ingredients (**Table 3.1**) and DMI (**Table 3.2**).

Calves on treatment HS had lower overall rumen pH (5.37 ± 0.08) than calves on treatment HN 5.81 ± 0.07 (**Table 3.2**; $P = 0.006$). This finding supports previous research showing that high-starch diets contribute to lower rumen pH (Steele et al., 2012; Danscher et al., 2015). Despite the noted effect on rumen pH, diets that differed in starch and NDF content resulted in similar molar proportions of rumen acetate, propionate, and butyrate 2 hours after feeding in all 7 week of this experiment (**Table 3.2**). This was unexpected, but given that empty reticulorumen weights were also not different by treatment when measured in all calves at 7 week of age (HS = 1.11 ± 0.13 kg; HN = 1.14 ± 0.13 kg; $P = 0.861$), this makes sense. Because rumen VFA concentrations did not differ by treatment but a pH effect was detected (**Table 3.2**), we suggest that lower rumen pH in HS may have stemmed from underlying *D*-lactate and *L*-lactate concentration effects (**Table 3.2**). Our reasoning for suggesting this possibility is increased lactate concentrations in the rumen are known to have a strong negative effect on rumen pH (Gentile et al., 2004), mainly due to the pKa of both lactate isoforms in comparison to pKa of individual VFA. Further, it is evident that the assay kits we used for detecting *D*-lactate and *L*-lactate concentrations generated data that were more variable than our VFA measurements obtained by GC (**Table 3.2**). This likely obscured real *D*-lactate and *L*-lactate effects on rumen pH.

This experiment used completely pelleted calf starter diets that differed in NDF and starch content. We designated our two calf starters either “HS” to reflect its higher starch content or “HN”, to reflect that diet’s proportionally higher NDF. When our diet compositions are

compared to the wider body of literature on starch and NDF content of pre-ruminant and ruminant diets, two points of clarification are warranted. First, for mature dairy cattle, the prevailing recommendation is for dietary starch to range between 25 and 30% of DM. Research data indicate that young dairy calves can tolerate more dietary starch (Suarez et al., 2006a,b; Kosiorowska et al., 2011; Vestergaard et al., 2013) than lactating cows, although an upper inclusion limit of starch in the calf diet is not known. It should be noted that the amount of starch in HS (25.13% of diet DM as starch) was triple that of HN (8.30% of diet DM as starch), but HS did not contain an excessive amount of starch. Huntington (1997) suggested that, while there is no apparent limit to the capacity of rumen microbes to digest starch, it is generally accepted that too much starch can negatively affect rumen health because rumen bloat, rumen acidosis, and liver abscesses occur most often in animals fed high-starch diets. In our study, no cases of ruminal bloat, clinical ruminal acidosis, or liver abscesses at slaughter were observed (data not shown). Further, we note that compared with recently published nutrient analyses of calf starters averaging around 35% of diet DM as starch (Suarez et al., 2006a,b; Khan et al., 2007; 2008; Kosiorowska et al., 2011; Vestergaard et al., 2013; Hill et al., 2016), our HS diet was below this level. It should also be noted that, for mature dairy cattle, the recommended dietary NDF range is 25 to 40% of diet DM as NDF depending on stage of lactation (NRC, 2001). Calf research studies that used "high-NDF diets" fed calf starter that was around 25% NDF on DM basis (Porter et al., 2007; Terre et al., 2013). Our HN diet, with 40.53% of diet DM as NDF was on the high end of recommended NDF content for mature cows and the NDF content of HS (26.27% of diet DM) was comparable to recorded "high-NDF" calf diets (Porter et al., 2007; Terre et al., 2013), but still 1.5 times lower than HN.

Ruminal fermentation of starch is a function of rate of fermentation and retention time of feed in the rumen. The barley, wheat, and corn grains used in this experiment were all ground before further processing into pellets (**Table 3.1**). This amount of grain processing most likely made all starch sources more available to rumen microbes, likely resulting in similar rate and extent of starch digestion, as our data seem to suggest (**Figure 3.1**). This was an unintended but informative finding. Had a different form of grain processing been chosen, such as cracking instead of grinding, or had the starter been a texturized feed instead of a completely pelleted feed, there may have been more treatment effects linked to rate and extent of rumen starch digestion, such as a papillae growth response.

By first grinding grain ingredients and then pelleting both of the calf starters in this experiment, we appear to have negated rumen starch effects attributed to individual diet ingredients (e.g., wheat, barley, corn), resulting in similar total VFA production (**Table 3.2**), absorption (inferred), and rumen growth (**Table 3.3**) across the two diets. Put another way, the calf starters, while varied in ingredient composition, appear to have been fermented at the same rate, as evidenced by the lack of significance between the treatment least squares means for starter DMI intake (**Table 3.2**), acetate, propionate, and butyrate measured 2 hours after morning feeding (**Table 3.2**), and the lack of difference between *in situ* disappearance of CP, starch, and NDF (**Figure 3.1**). It should be noted that *in situ* digestion was performed in an adult ruminant. If the digestion would have occurred in a young calf we may have observed different results based on microbiota and substrate present. Our data suggest that the same amount of starch was ruminally fermented in HS and HN calves (based on rate of starch digestion for both diets). Because the total starch was higher in HS calves, more starch presumably flowed to the small intestine of HS calves as well. In our experiment, neither total tract digestibility measures nor

intestinal enzyme activities were measured. Because calves grew the same, HS may have had higher fecal starch content, lower intestinal enzyme activity, or both, compared to HN calves. If true, that would represent a digestive inefficiency and wasted nutrients in HS calves. This may be worthy of further investigation.

Time after feeding is another important consideration in this experiment because large daily drops in rumen pH are known to occur within 3 hours after meal ingestion, coincident with VFA production from mainly starch degradation (Laarman et al., 2012; Kim et al., 2016). The calf starters also appear to have been retained the same length of time in the rumen. Indirect evidence for similar rumen retention times of the two diets comes from our observation that starter DMI within 24 hours of slaughter (data not shown) and rumen content weighs at slaughter (difference between full and empty reticulorumen weights; **Table 3.3**) were similar.

Concerns when feeding high-NDF diets are decreased DM digestibility (Beckman and Weiss, 2005) and increased gut fill (Dado and Allen, 1995), which were not reflected in our DM digestibility results (**Figure 3.1**). We detected no treatment differences in starter DMI (**Table 3.2**), calf BW (reflective of gut fill; **Table 3.2**), or full reticulorumen weights at 7 weeks of age (**Table 3.3**). Also, the high amount of NDF in HN compared to HS diets did not negatively impact rumen development in this experiment (**Table 3.3**).

Suarez et al. (2006a,b) also fed pelleted concentrate diets differing in main carbohydrate source to veal calves. They found that a diet high in starch (59.3% diet DM) and low in NDF (9.4% diet DM) resulted in increased ruminal butyrate concentration compared to a diet lower in starch (10.8% diet DM) and higher in NDF (49.3% diet DM), which is in contrast to our butyrate findings ($P = 0.851$; **Table 3.2**). Rumen observations by Suarez et al. (2006b) noted lighter empty rumens at 8 weeks in calves fed a higher starch compared to a higher NDF diet (0.89 ±

0.08 kg and 1.23 ± 0.08 kg respectively; $P < 0.05$). Looking closer at the tissue layers, they observed thicker total epithelial and muscle layer in the ventral rumen of higher starch compared to higher NDF calves (1.32 ± 0.09 mm and 0.97 ± 0.09 mm, respectively; $P < 0.05$), but a thinner muscle layer in higher starch compared to higher NDF calves (1.51 ± 0.07 mm and 1.29 ± 0.07 mm, respectively; $P < 0.05$). Results of the current study do not agree with the results of Suarez et al. (2006b) in respect to biopsy weights given there were no differences between treatment for total biopsy, epithelial, and muscle weight ($P = 0.210$, $P = 0.180$, and $P = 0.158$, respectively). Taken together, these diets differing in NDF and starch concentrations both seemingly benefit rumen tissue growth with NDF supporting structural growth of the tissue and starch supporting growth of the functional epithelium.

Considering the overall lack of differences observed between the two diets in our experiment, the cost of each starter diet might be of practical calf management interest. In 2015, the HN diet cost $1.38/kg of DM whereas the HS diet cost $1.62/kg of DM (**Table 3.1**). Starter DMI was not different between treatments (**Table 3.2**) and resulted in a total (7 week) starter cost of $32.83/ HN-calf and $38.54/HS-calf. Our HN calves were $5.71 less expensive per calf to raise and feeding HN resulted in similar DMI, ADG and rumen parameters as the more expensive HS.

In conclusion, results support the idea that not all changes in calf starter nutrient composition (i.e. starch and NDF) result in effects on intake, growth, and rumen measurements. The lower rumen pH in HS compared to HN was of no apparent functional significance in this 7 week experiment. These findings are valuable because they suggest a form of feed effect in today's calf starter diets that might be just as important as, or even supersede, diet ingredient effects on rumen papillae growth. Furthermore, our findings may have additional implications on

starch digestion in the small intestine as well as potential economic factors in creating pelleted feeds differing in starch and NDF contents. Form of diet (i.e. pelleted vs. textured starter), which will impact digestibility of the diet, seems to be an area of opportunity when trying to alter rumen metabolism and subsequent rumen development compared to just altering diet nutrient composition.

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Table 3.1. Ingredient and chemical composition of pelleted calf starter diets

Composition	High-starch Low-NDF (HS)	High-NDF Low-starch (HN)
Ingredient, % of DM		
Barley, ground	23.00	--
Wheat, ground	23.00	--
Corn, ground	--	6.83
Soybean hulls, ground	15.16	31.22
High protein soybean meal	25.70	27.28
Cottonseed hulls	4.01	10.00
Wheat middlings	4.01	19.70
Molasses	1.05	1.44
Limestone, ground	1.88	1.79
Vitamins and minerals	2.19	1.74
Dry matter, % of as-fed	88.07	87.47
Chemical composition, % of DM		
CP	22.90	23.10
NDF	26.27	40.53
ADF	16.10	26.73
Starch	25.13	8.30
Ether extract	1.55	2.14
Ash	8.48	8.54
Cost, $/kg of DM (2015 prices)	1.62	1.38

Table 3.2. Growth, intake, rumen metabolite, and blood metabolite data of calves fed diets differing in starch and NDF composition

	Week									Test of fixed effects, *P*-value[3,4]		
Item[1]	0	1	2	3	4	5	6	7	SEM[2]	Trt	Wk	Trt*Wk
Body weight (kg)	40.36	42.53	43.00	44.51	48.64	53.43	58.59	63.54	2.06	0.585	<0.0001†‡	0.577
Withers height (cm)	75.56	76.50	77.63	78.00	78.94	80.63	82.06	83.25	0.47	0.127	<0.0001†	0.751
Hip height (cm)	79.50	81.19	81.63	82.38	83.69	85.19	86.69	87.56	0.56	0.888	<0.0001†‡	0.243
Total DMI (kg/wk)	--	3.72	4.05	4.85	6.06	7.66	9.51	8.97	1.15	0.974	<0.0001†	0.800
MR DMI (kg/wk)	--	3.43	3.61	3.65	3.65	3.58	2.64	0.49	0.13	0.824	<0.0001†‡	0.824
Starter DMI (kg/wk)	--	0.30	0.45	1.20	2.41	4.08	6.87	8.48	1.18	0.985	<0.0001†‡	0.700
Water Intake (kg/wk)	--	3.48	6.51	6.35	7.14	10.61	20.45	27.25	4.46	0.995	<0.0001†‡	0.176
ADG (kg/d)	--	0.31	0.07	0.22	0.58	0.69	0.74	0.71	0.23	0.941	<0.0001†	0.728
Rumen Metabolites												
Total VFA (m*M*)	--	33.60	57.55	135.09	118.79	155.59	138.76	136.66	39.58	0.921	<0.0001†‡	0.811
Acetate (m*M*)	--	20.91	34.53	75.48	62.88	84.88	69.58	68.97	19.14	0.277	<0.0001†‡	0.876
Propionate (m*M*)	--	9.48	17.82	46.31	40.88	45.46	49.40	50.71	12.61	0.293	<0.0001†‡	0.433
Butyrate (m*M*)	--	3.21	5.65	15.47	15.03	25.25	19.77	16.98	12.37	0.851	0.0004†	0.696
D-Lactate (m*M*)												
HS	--	4.58^{ab}	1.97^{ab}	13.18^{a}	1.66^{b}	9.15^{ab}	3.79^{ab}	2.82^{b}	4.19	0.949	0.004	0.0002
HN	--	3.86^{ab}	9.47^{ab}	2.79^{b}	5.02^{b}	5.82^{ab}	6.33^{ab}	3.19^{b}				
L-Lactate (m*M*)												
HS	--	5.98^{ab}	3.00^{ab}	15.28^{a}	1.19^{b}	12.93^{ab}	3.95^{ab}	5.52^{ab}	5.07	0.400	0.041	0.016
HN	--	4.63^{ab}	8.70^{ab}	4.41^{ab}	4.49^{b}	3.44^{ab}	6.31^{ab}	4.32^{b}				
Rumen pH												
HS	--	5.36^{abc}	5.59^{abc}	5.66^{abc}	5.26^{bc}	5.03^{c}	5.32^{bc}	5.39^{abc}	0.50	0.006	<0.0001†	0.521
HN	--	6.35^{abc}	6.22^{a}	5.69^{ab}	5.67^{ab}	5.38^{bc}	5.49^{abc}	5.89^{ab}				
Blood BHB (m*M*)	--	0.05	0.05	0.05	0.06	0.06	0.06	0.08	0.008	0.158	0.0003†‡	0.221

[a-d]LSmeans compared between treatments for *D*-Lactate, *L*-Lactate, and Rumen pH with different superscripts differ ($P \leq 0.05$)

[1]Treatments: HS = high-starch, low-NDF (25.13% starch, 26.27% NDF; DM basis), HN = high-NDF, low-starch (40.53% NDF, 8.30% starch; DM basis).

[2]Largest standard error of the mean for all weeks is reported.

[3]Significance declared when $P \leq 0.05$. Linear effect denoted by † and quadratic effect denoted by ‡ when $P \leq 0.05$.

[4]Trt = treatment (either HS or HN); Wk = week.

Table 3.3. Effect of calf diets differing in starch and NDF composition on gross rumen measurements

Item	Treatment[1] HS (n = 4)	HN (n = 4)	SEM[2]	Test of fixed effects, *P*-value[3] Trt[4]
Full Stomach (kg)	7.01	8.97	0.94	0.188
Full Reticulorumen (kg)	5.44	6.96	0.93	0.291
Empty Reticulorumen (kg)	1.11	1.14	0.13	0.861
Rumen Papillae Area (mm^2)	4.37	4.75	0.70	0.719
Rumen Biopsy				
Whole Biopsy (g)	2.26	1.20	0.13	0.210
Epithelium (g)	1.65	1.33	0.15	0.180
Muscle (g)	0.61	0.66	0.02	0.158
Epithelium/Muscle ratio	3.10	2.26	0.39	0.172

[1]Treatments: HS = high-starch, low-NDF (25.13% starch, 26.27% NDF; DM basis), HN = high-NDF, low-starch (40.53% NDF, 8.30% starch; DM basis).
[2]Largest SEM reported.
[3]Significance declared when $P \leq 0.05$.
[4]Trt = Treatment (either HS or HN).

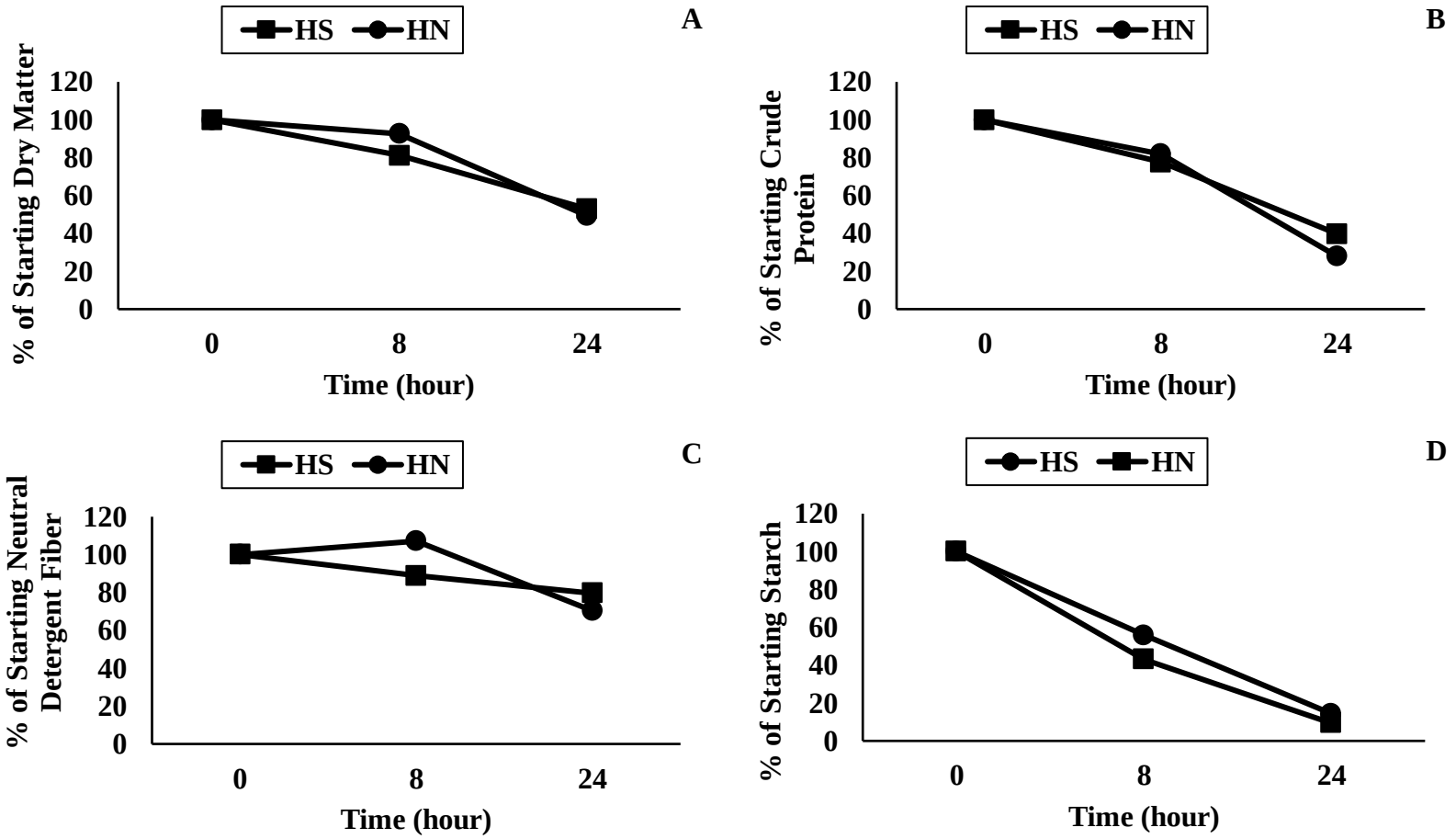

Figure 3.1. In situ disappearance of A) DM, B) CP, C) NDF, and D) starch of pelleted calf starter diets that either contained high-starch, low-NDF (HS) or high-NDF, low-starch (HN).

Chapter 4 : Technical Note: Infusion, sampling and vacuum-assisted collection devices for use in ruminally cannulated calves

Abstract

Calves can be ruminally cannulated at young ages, but equipment size limitations preclude use of an infusion and sampling apparatus in these small animals. Likewise, a means to easily evacuate rumen contents in young calves is lacking. Overcoming technical complications related to assessment of ruminal passage kinetics, nutrient digestion and volatile fatty acid (**VFA**) absorption would advance knowledge of dairy calf nutrition. The first objective was to design and fabricate two devices (one apparatus for infusion and sampling, and another for vacuum-assisted collection) suitable for use in young ruminally-cannulated dairy calves. The second objective was to test the utility of these tools when performing tasks commonly used in ruminant nutrition research. A single 62-d-old ruminally cannulated calf was used to evaluate the ability to infuse a solution of LiCoEDTA and sample rumen contents through the cannula cap over a period of 2 hours to assess the rumen liquid passage rate (task 1). The device was capable of infusing the LiCoEDTA and sampling the rumen fluid, as evidenced by the presence of elevated Co concentrations in the sampled rumen fluid. Using the fluid samples obtained, liquid passage rate within the calf was estimated to be 40.2% of ruminal fluid/h. The second task tested the vacuum-assisted collection device and consisted of vacuum-assisted removal of rumen contents, which is considered a key preparatory step in washed reticulorumen technique experiments that aim to measure nutrient absorption. The vacuum-assisted collection device was used to evacuate the rumen and collected content weight was recorded. In agreement with existing literature, evacuated rumen contents represented approximately 4% of the calf's body weight. In conclusion, custom-built devices for infusion, sampling and vacuum-assisted

collection were efficacious when tested in a 62-d-old ruminally cannulated calf fed a diet of 100% texturized starter (18% CP, as-fed). Fellow scientists may employ and further modify these techniques to suit their needs when assessing passage kinetics, nutrient digestion and VFA absorption in calves.

Key words: calf, rumen, washed rumen technique

Technical Note

Ruminal passage kinetics (Azevedo et al., 2016), nutrient digestion and VFA absorption (Conrad et al., 1956; Sutton et al., 1963; Khouri, 1969) are difficult to assess in young dairy calves. These measurement challenges directly limit full comprehension of dairy calf nutrition and nutrient utilization. Overcoming technical complications related to assessment of ruminal passage kinetics, nutrient digestion and VFA absorption are therefore essential steps toward improving our understanding of dairy calf nutrition.

Experiments that measure ruminal passage kinetics, nutrient digestion and VFA absorption in mature ruminants are often facilitated by use of ruminally cannulated animals fitted with a specialized indwelling infusion and sampling apparatus (Annison et al., 1974; Sutton et al., 2003), or employ the washed reticulorumen technique. The latter requires an evacuated rumen (Gaebel et al., 1987; Kristensen and Harmon, 2004; Storm et al., 2011). Calves can be ruminally cannulated at young ages (Lesmeister and Heinrichs, 2004; Kristensen et al., 2010; Suarez-Mena et al., 2015; 2016), but equipment size limitations preclude use of an infusion and sampling apparatus in young calves. Likewise, an efficient means to easily evacuate rumen contents in young calves is lacking.

Our first objective was to design and fabricate an infusion and sampling apparatus and a separate vacuum-assisted rumen contents collection device suitable for use in young ruminally cannulated dairy calves. The second objective was to test the effectiveness of these devices in a ruminally cannulated calf. Two tasks common in ruminant nutrition research were selected to evaluate the performance of our newly created devices. The first task was to infuse a solution of LiCoEDTA into the rumen and sample rumen contents through the cannula cap over time to assess rumen liquid passage rate (Udén et al., 1980; Dijkstra et al., 1993; Krizsan et al., 2010).

The second task was to remove and collect the content of the rumen, which is considered a key preparatory step in washed reticulorumen technique experiments that aim to measure nutrient absorption (Gaebel et al., 1987; Kristensen and Harmon, 2004; Storm et al., 2011).

Our device design approach for the infusion and sampling apparatus was to identify important functional properties of systems used in mature animals (such as prevention of backflow of large particles into sampling and infusion lines) and scale down components using commercially available materials. The design of the vacuum-assisted rumen collection system is essentially a scaled-up version of the common laboratory vacuum trap, using durable commercially available components. **Figures 4.1** to **4.4**.

The design, manufacture and evaluation of our two devices were conducted from September 2016 to November 2016. One Virginia Tech (**VT**) born Holstein bull calf was utilized in the evaluation of the devices. The following procedures were approved by the VT Animal Care and Use Committee (protocol #15-181). The bull calf was healthy (serum total protein 60 mg/mL after colostrum-feeding) and underwent rumen cannulation surgery on day 5 of age. The rumen cannulation surgery was performed similarly to that described by Kristensen et al. (2010). The surgically placed rumen cannula (2.75 cm i.d.) was the same design used in Lesmeister and Heinrichs (2004), Suarez-Mena et al. (2015), and Suarez-Mena et al. (2016). In between uses, the cannula opening was plugged with a #6 rubber laboratory stopper.

The calf was fed twice daily at 0700 hours and 1900 h. Prior to weaning, the calf was fed a diet of milk replacer (Southern States Cooperative; Richmond, VA) containing 22% CP and 20% fat (as-fed basis) and texturized calf starter (Southern States Cooperative; Richmond, VA) containing 18% CP (as-fed). Water was available at all times. The calf was housed in an individual calf hutch bedded with sawdust. Hay was not offered. The calf's birth weight was 43

kg and on day 62 of life, which coincided with the procedures described next, the calf weighed 80 kg. The calculated ADG (0.597 kg/d) agrees with NRC (2001) data for calves consuming milk replacer and starter with similar DMI (intake data not shown) i.e. 0.6 kg/d.

On day 62 of life, immediately after the evening feeding, the calf was haltered and walked approximately 90 m to a 2.13 m long × 1.32 m wide pen in a nearby barn. The halter was tied so the calf could lie down but not turn around. Drinking water and starter were available to the calf unless noted otherwise.

To verify that our custom-made rumen infusion and sampling device (**Figures 4.1 and 4.2)** worked as desired, our rumen infusion and sampling apparatus was adapted from similar devices used in mature dairy cows (Annison et al., 1974; Sutton et al., 2003). The infusion and sampling apparatus was manually guided into the rumen through the open rumen cannula. As shown in **Figure 4.1**, we used 0.64 cm o.d. × 0.48 cm i.d. plastic tubing for both the infusion line and the sampling line. Both lines were passed through a #6 rubber laboratory stopper; this stopper acted as a cannula plug during the infusion and sampling period. The rumen content sampling line terminated with a paint sprayer filter (Graco, Minneapolis, MN) shielded by a fenestrated 15-mL plastic conical tube (**Figure 4.1**). This design feature filtered out large feed particles and prevented sampling lines from clogging. The infusion line also terminated in a paint sprayer filter (**Figure 4.1**). Filters were secured to tubing by passing a piece of flexible wire (similar to a cotter pin) through holes drilled through the overlapping sections of the tubing and filter collar. To lower the risk of accidental animal puncture, orthodontist's wax was applied to any exposed wire during use. An IV set (Primary PlumSet; Hospira, Lake Forest, IL) fed into the top of the electronic clinical infusion pump (Abbott Lifecare 5000; AIV, Harmans, MD) with the infusate (described later) contained within a 1 L Kangaroo gravity feeding bag (Covidien,

Dublin, Ireland) (**Figure 4.2**). The free end of the infusion line (~1.45 m in length) was joined to the down-stream line of the IV set. The free end of the sampling line was clamped between sample collections (**Figure 4.1**). When a rumen fluid sample was taken, a 60 mL syringe was placed at the end of the sampling line, the clamp was released, and the syringe was used to manually aspirate rumen fluid. The first 5 to 10 mL (sample line dead-volume) were discarded at each sampling.

In many rumen metabolism experiments, continuous infusion of a CoEDTA compound has been used to assess rumen liquid passage rate (Udén et al., 1980; Dijkstra et al., 1993; Krizsan et al., 2010). Thus, we rationalized that LiCoEDTA would be a suitable infusate for us to test with our rumen infusion and sampling apparatus. The apparatus would be considered a success if the infusate was recovered in all rumen fluid samples and the calculated rate of liquid passage considered plausible when compared with existing data for calves fed a similar diet. To assess rumen liquid passage rate, a continuous infusion of a LiCoEDTA solution (0.45 mg LiCoEDTA/mL) was administered at a rate of 20 mL/h starting approximately 8 hours before sampling to bring the concentration of LiCoEDTA to a plateau (Krizsan et al., 2010). In our case, the LiCoEDTA infusion began at 0045 hours and the first rumen fluid sample was collected at 0900 h. The infusion pump was calibrated in our laboratory prior to use. Beginning at 0900 hours on the day of the experiment, rumen fluid samples (~5 to 10 mL) were obtained through the sampling line every 15 min for 2 h. Rumen pH of each sample was measured immediately (EcoSense pH100A Meter; YSI Inc., Yellow Springs, OH) and the remaining samples were transferred to 15-mL conical tubes and frozen at -20°C until analysis. For analysis of LiCoEDTA these 8 samples underwent nitric acid digestion using method 3030H via EPA (1998). Cobalt

determination was by inductively coupled plasma atomic emission spectroscopy completed at the VT Soil Testing Lab.

The Co results in **Table 4.1** show that both the infusion and sampling method were successful. Further, over a 2hour period, the rumen liquid passage rate equaled 40.2%/h. Because the continuous infusion did not achieve a steady-state within the sampling period, a non-steady state solution was required to estimate fluid passage rate. In this case, a two pool model with entry and exit fluxes was constructed. The first pool represented the rumen fluid volume (L) and the second pool represented the Co mass (mg) within the rumen fluid. Entry of fluid into the rumen fluid pool was assumed to be 0.020 L/h and entry of Co into the Co pool was set at 0.52 mg/h. Exit of Co was assumed to be equal to the current Co concentration (mg Co/L rumen volume) multiplied by the fluid exit rate (L/h). The fluid exit rate was equal to a fractional fluid passage rate (h^{-1}) multiplied by the current fluid volume (L). The fractional fluid passage rate was derived by matching the modeled Co concentration to the measured Co concentration using the FME package of R version 3.4.0 (Soetaert and Petzoldt, 2010).

Suarez et al. (2007) measured the fractional rumen liquid passage rate in 10-week-old calves fed diets containing milk replacer and differing amounts of concentrate and roughage and found a rate of liquid passage at 46%/h. The difference in these values may be explained by the nature of Co administration into the rumen where the current study infused Co directly into the rumen and Suarez et al. (2007) mixed CoEDTA into the milk replacer to assess ruminal drinking. Both of the liquid passage rates estimated in calves (40.2%/h in the present study and 46%/h in Suarez et al. (2007)) are much higher than those found in adult ruminants (13% ruminal fluid/h (Seo et al., 2007)). The reasoning for the increase in liquid passage rate is not clear, but may be

explained by insufficient rumen size to hold large volumes of liquid, thus allowing increased passage of liquid to the rest of the gastrointestinal tract.

After the infusion and sampling protocol was completed, calf starter was removed from the pen, but the calf had continuous access to water. The goal of this task was to verify efficacy of our custom-built vacuum-assisted collection device (**Figures 4.3 and 4.4**) to evacuate typical rumen contents of a young dairy calf for ruminant nutrition experiments. To evacuate the rumen, the cannula plug was removed and the vacuum hose from a custom-built vacuum device (**Figure 4.3**) was manually guided into the rumen through the cannula and used to remove rumen contents. For the vacuum device, we connected an 18.93 L bucket to a vacuum pump (Model DOA-P704-AA; Gast Manufacturing, Benton Harbor, MI). The rumen was evacuated by suction through a tube slightly smaller in outer diameter than the cannula opening with the contents being collected in the bucket. The vacuum trap was created by installing tubing ports in the lid of the bucket. The vacuum OUT port was built using a brass 0.95 cm i.d. male hose barb × 0.64 cm male iron pipe, a 0.50 cm male iron pipe × 0.25 cm female iron pipe brass pipe bushing, and 1 to 2 custom washers cut from rubber packing sheets (**Figure 4.4**). The sample IN port was built using a nylon 0.64 cm i.d. × 1.91 cm male iron pipe male hose barb, a nylon 1.91 cm female iron pipe threaded union fitting, one custom rubber washer, and a 15.24 cm section of 3.18 cm i.d. vinyl hose (**Figure 4.4**). The connection between the vacuum OUT port and the pump was 0.95 cm i.d. vinyl tubing, and the sample tubing was 1.91 cm i.d. vinyl (**Figure 4.3**). Vacuum-assisted removal of rumen contents took approximately 2 min and ceased when contents were no longer observed exiting through the vacuum tube. Rumen emptiness was confirmed visually by passing an inspection scope (model DE-1012; Volador Technology Co., Ltd, Shenzhen, China) through the rumen cannula and manually maneuvering it throughout the various sacs of the rumen. After

removal, rumen contents were weighed (3.15 kg), and then the rumen was washed using a warm (37 °C) wash buffer adapted from Storm et al. (2011). The buffer contained:106 m*M* NaCl, 24 m*M* $NaHCO_3$, 20 m*M* KOH, 2 m*M* K_2HPO_4, 1.5 m*M* $CaCl_2$, and 1.5 m*M* $MgCl_2$ (Storm et al. (2011). The buffer remained in the rumen for 5 min and was removed via our vacuum device. The original rumen contents were then replaced into the rumen. The entire evacuation, inspection, and washing procedure required 70 min to complete. Rumen content pH changed from 5.62, measured when rumen contents were removed, to 5.60 when rumen contents were replaced in the rumen. Evacuated rumen contents represented about 4% (3.15 kg / 80 kg) of the calf's BW, which is slightly lower than what has been reported previously (5 to 8% of BW) in calves between 6 and 8 week of age (Suarez et al., 2006; Kristensen et al., 2010; Yohe et al., 2015). This difference might be explained by the collection methods used. In the present study, despite efforts, complete evacuation of the rumen was likely not achieved. This can be accomplished in slaughter studies and likely accounts for the noted difference.

In conclusion, we demonstrated that our custom-built infusion and sampling apparatus and the custom-built vacuum apparatus were effective tools when tested in a 2-mo-old ruminally cannulated calf. Fellow scientists may employ and further modify our techniques to suit their needs when performing nutrition trials in calves.

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Table 4.1. Fractional rate of rumen liquid passage, rumen content weight, and rumen pH for an 8 week old Holstein calf weighing 80 kg at sampling, fed a diet of 18% CP calf starter and no hay

Item	Rate of liquid passage[1], %/h	Rumen content weight (kg)	Rumen pH pre-content removal	Rumen pH post-content removal
Calf	40.2	3.15	5.62	5.60

[1]Rate of liquid passage was determined by LiCoEDTA administration and subsequent Co determination by inductively coupled plasma atomic emission spectroscopy, using the formula: rate of liquid passage = fluid exit rate (L/h)/current fluid volume (L).

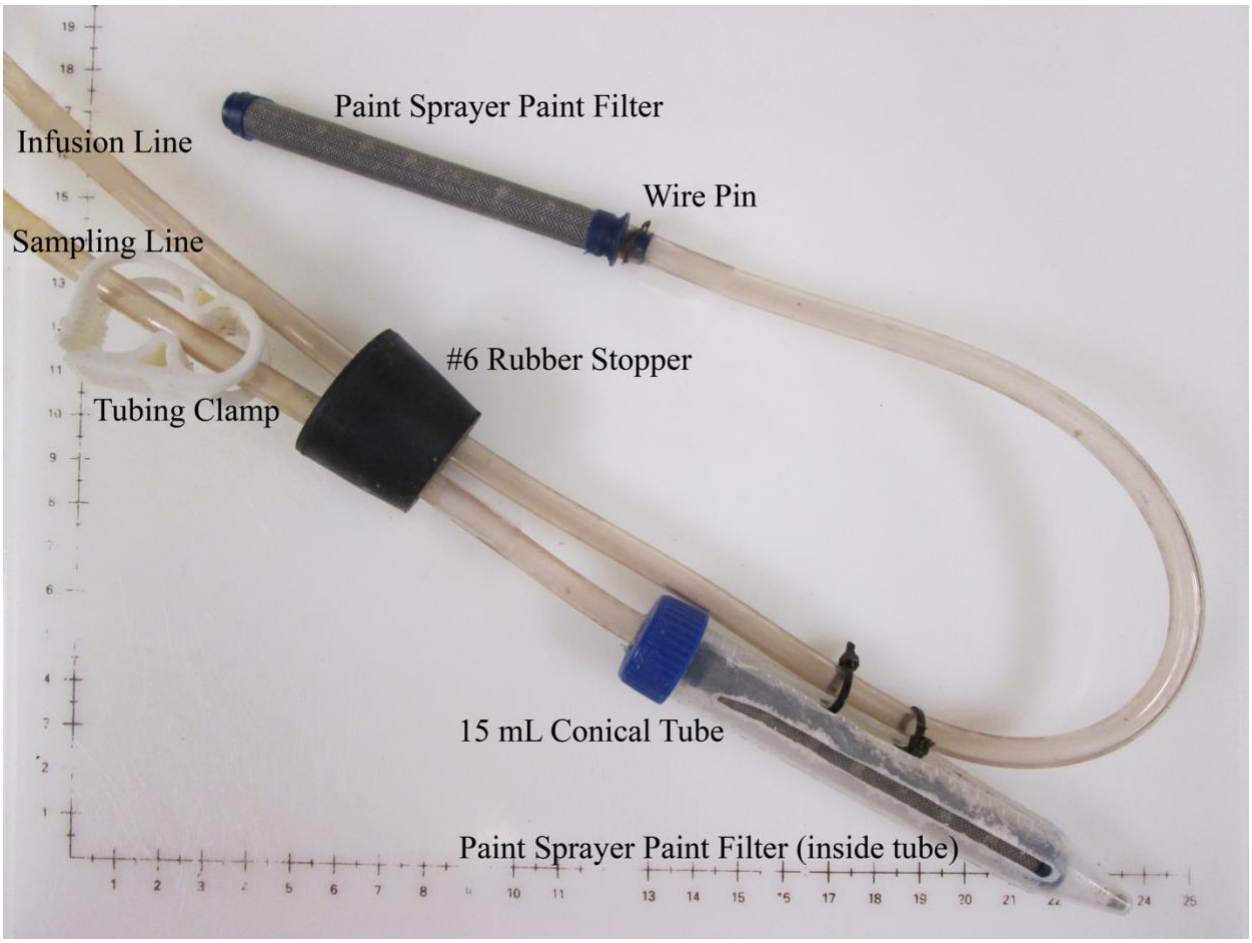

Figure 4.1. Custom-made rumen infusion (upper line) and sampling (lower line) device built and used to administer infusate and sample rumen fluid, respectively, in the ruminally cannulated calf. See text for specifications.

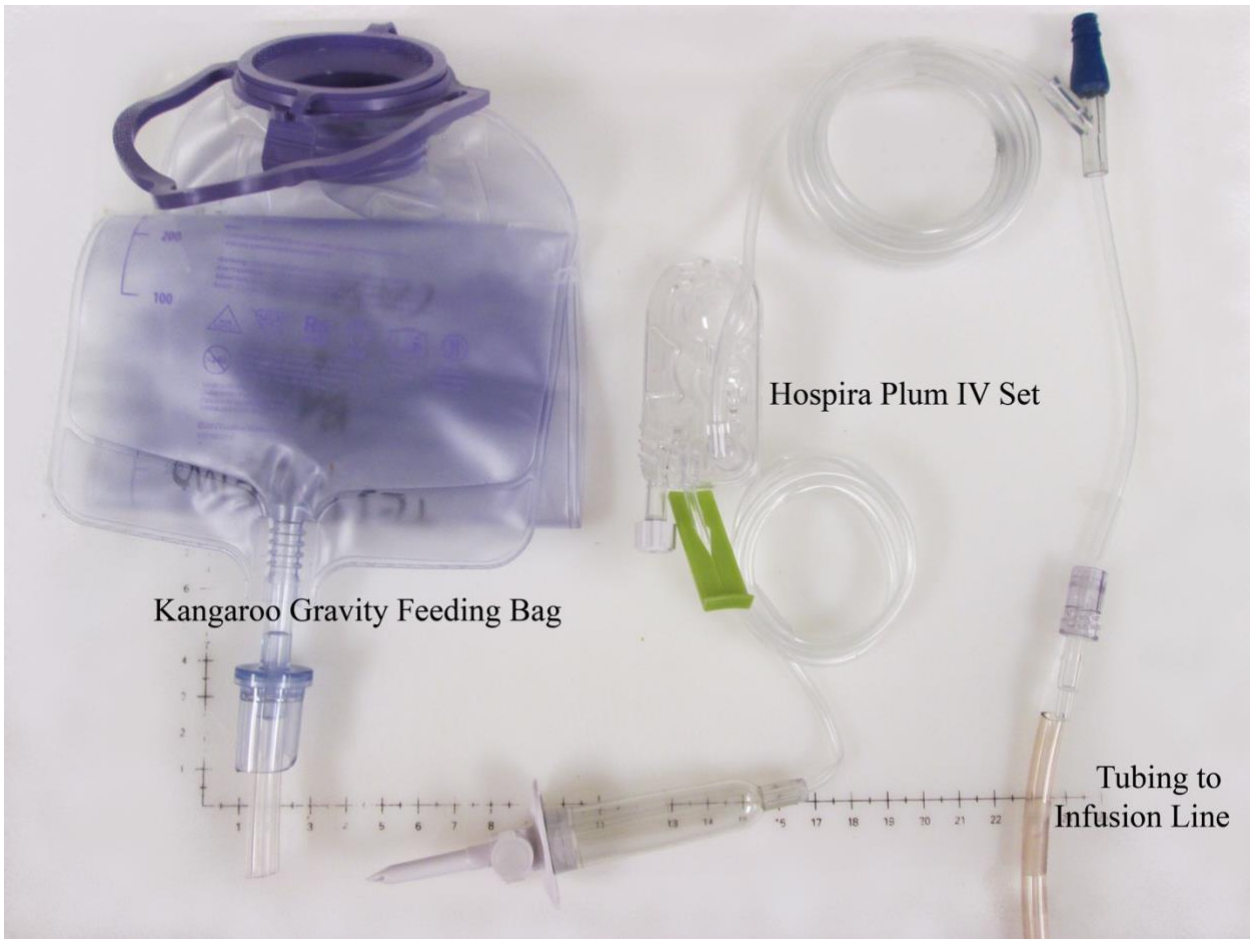

Figure 4.2. Modified rumen infusion bag and infusion line used to administer infusate in the ruminally cannulated calf. See text for specifications.

Figure 4.3. Fully assembled custom-made vacuum-assisted collection device used to suction rumen contents out of the rumen and temporarily store rumen contents. See text for specifications.

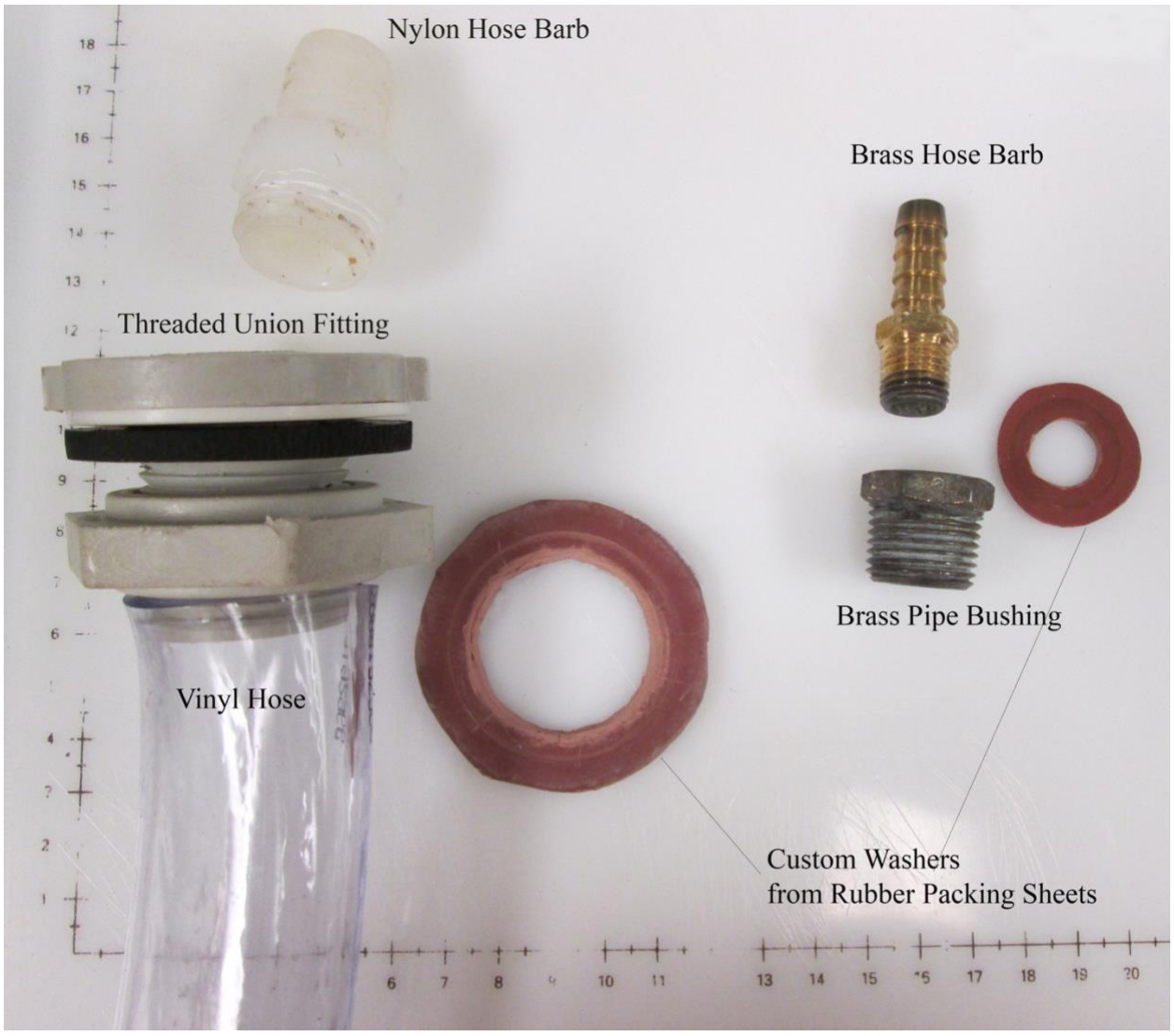

Figure 4.4. Assembly parts for the custom-made vacuum-assisted collection device used to suction rumen contents from the ruminally cannulated calf. See text for specifications.

Chapter 5 : Effects of form of diet on VFA absorption in the rumen of preweaned calves

Abstract

Diet is known to affect rumen growth and development. Calves fed an all liquid diet have smaller and less developed rumens and have previously been shown to have a decreased ability to absorb volatile fatty acids (**VFA**) compared to calves fed both liquid and dry feed. However, it is unknown how these two types of rumens respond when challenged with a defined concentration of VFA. The objective of this study was to assess the effects of two different feeding programs on VFA absorption in preweaned calves. Neonatal Holstein bull calves were individually housed and randomly assigned to one of two treatment diets. The treatment diets were milk replacer only (**MRO**; n = 5) or milk replacer with starter (**MRS**; n = 6); diets were isoenergetic (3.87 ± 0.06 MCal of ME/d) and isonitrogenous (0.17 ± 0.003 kg/d of apparent digestible protein). Milk replacer was 22% CP, 21.5% fat (DM basis) while the textured calf starter was 21.5% CP (DM basis). Feed and ad libitum water intakes were recorded daily. Calves were exposed to defined concentration of VFA buffer (acetate: 143 m*M*, propionate: 100 m*M*, butyrate: 40.5 m*M*) 6 hours prior to euthanasia on day 43 ± 1. Rumen fluid samples were obtained every 15 to 30 min for 6 hours to measure rate of VFA absorption (data forthcoming). Rumen tissues were obtained from the ventral sac region and processed for morphological and immunohistochemical analyses of the VFA transporters monocarboxylate transporter 1 (**MCT1)** and 4 (**MCT4**). Data were analyzed using SAS 9.4. Body growth did not differ between treatments, but empty reticulorumens were heavier in MRS than MRO calves

(0.67 kg vs. 0.39 kg ± 0.04 kg; $P = 0.001$) and MRS calves had larger papillae area (0.76 mm^2 vs. 15 mm^2 ± 0.08 mm^2; $P = 0.001$). No differences between treatments in protein abundance of MCT1 and MCT4 per unit area were observed. These results indicate that the extrapolated increase in total abundance of MCT1 or MCT4 in MRS calves is not due to increased transporter density per unit area. Modeling of VFA absorption data will help determine the proportion of VFA undergoing protein-mediated transport via MCT1 and MCT4 versus those that are passively diffused.

Key words: dairy, calf nutrition, ruminant physiology

Introduction

The dairy calf has an undeveloped rumen at birth (Tamate et al., 1962; Warner and Flatt, 1965) that must grow and develop to support ruminant metabolism. Both rumen growth (an increase in organ size) and rumen development (a change in function) are stimulated when the calf starts consuming dry feed (i.e. calf starter) (Warner et al., 1956; Flatt et al., 1958; Connor et al., 2013). Calves that remain on a liquid only, or primarily liquid diet, have underdeveloped rumens. Physical characteristics of underdeveloped rumens include small overall rumen size and low papillae dimensions and mass (Lengemann and Allen, 1959; Harrison et al., 1960; Tamate et al., 1962). In the face of this, contemporary research shows benefits of providing calves with a large amount of liquid feed in early life (Soberon et al., 2012; Castells et al., 2015; Kiezebrink et al., 2015). Studies by Soberon et al. (2012), Kiezebrink et al. (2015), and Castells et al. (2015) all linked early life ADG, achieved through feeding a primarily liquid feed diet, to increased milk production during the first lactation. Economics aside, this feeding strategy seems to oppose the goals of supporting rumen growth and development through promotion of early life dry feed intake (Warner et al., 1956; Flatt et al., 1958; Connor et al., 2013). Consequences of these two differing feeding strategies on the ability of the rumen to absorb VFA are unknown.

Most of the existing data on rumen VFA absorption in calves was generated in the 1960s. While informative, given the advances in calf nutrition and genetic progress made via selection for high-yielding dairy cows since the 1960s, this topic is worthy of a second look, as the efficiency surrounding VFA absorption, our understanding of physiology, or both may have changed.

Khouri (1969) demonstrated the ability of < 1-week-old calf rumen to absorb VFA when fed a milk diet. Similarly, Conrad et al. (1956) used a 5-week-old calf consuming a pelleted diet to show a difference in VFA concentrations between the rumen and abomasum, thereby demonstrating VFA absorption in the reticulorumen (and potentially the omasum). Sutton et al. (1963a) showed that calves fed only milk had a decreased maximal rate of rumen acetate absorption compared to calves fed milk, grain, and hay. Considering the limited capacity of the pre-weaned calf rumen (Tamate et al., 1962; Warner and Flatt, 1965) one might assume that the ability of the rumen to absorb VFA is decreased when compared to a developed calf rumen. The assumption may be incorrect and was the chief motivating factor for performing this research. What remains to be seen is whether an undeveloped rumen has the ability to absorb VFA at the same rate of a developed rumen when exposed to a high concentration of VFA. This study aimed to test that question, with the objective of assessing the "maximal" ability of the rumen tissue to absorb VFA when comparing undeveloped vs.. developed rumens of calves around 6 weeks of age. The hypothesis was that calves fed a diet of liquid and solid feed meant to stimulate rumen development would have an enhanced ability to absorb VFA compared to calves fed a liquid-only diet when exposed to defined concentrations of VFA. The findings have the potential to influence the way calves are fed if it is found that either of these feeding strategies results in more efficient VFA metabolism.

Materials and Methods

Animals, treatments, and experiment

This experiment was approved by the Virginia Tech (**VT**) Institutional Animal Care and Use Committee (protocol #16-165) and conducted from February 2017 to May 2017. A total of 12 healthy Holstein bull calves were purchased from a single farm and transported approximately 240 km to VT. Within 12 hours of birth at the source farm each calf was fed a total of two 470 g bags of colostrum replacer (≥ 200 g IgG; Bovine IgG Land O Lakes Colostrum Replacement; Land O Lakes Animal Milk Products Co., Shoreview, MN) mixed in 3.79 L of hot water and the navel of each calf was dipped in an iodine tincture. Where applicable, subsequent feedings were of milk replacer (**MR**; Ledger, 22% CP as-fed, 20% fat as-fed; Southern States Cooperative Inc., Richmond, VA). Calves arrived at VT in one of two arrival periods (n = 6/period); period 1 calves were on site from February 17, 2017 to April 2, 2017. Period 2 calves were on site from April 11, 2017 to May 25, 2017.

Upon arrival to VT, a single jugular blood sample was collected from each calf into 10 mL glass tubes (cat# 367921; BD Vacutainer; Franklin Lakes, NJ) and used for serum IgG determination (to assess passive transfer of immunity); calf body weight, hip height, and withers height were measured. After initial measurements were obtained, each calf was moved into an individual pen (122 cm × 183 cm) in a temperature controlled room (21°C to 22°C) in the basement of Litton-Reaves Hall at VT where they remained for the duration of the experiment. Each pen was placed on top of a solid rubber mat (122 cm × 183 cm) topped with a perforated rubber mat (91.44 cm × 152.4 cm) for traction and hygiene purposes. Additionally, cotton towels were used as bedding and

were laundered daily. Calves had visual and auditory contact with other calves but no physical contact.

Within 24 hours of arrival to VT, calves were balanced by age (2.33 ± 0.15 days vs. 2.66 ± 0.15 d, for treatments 1 and 2, respectively), body weight (43.17 ± 1.70 kg vs. 43.74 ± 1.70 kg), serum IgG (12.48 ± 0.94 mg/mL vs. 13.52 ± 0.94 mg/mL), and dam lactation number (2.83 ± 0.32 vs. 2.33 ± 0.32) and split into two dietary treatments.

The dietary treatments were: milk replacer only (**MRO**; n = 5) and milk replacer and calf starter (**MRS**; n = 6). The trial started with six calves allocated to the MRO treatment, but one calf died at 2 weeks of age from septicemia and was not replaced; the death was deemed unrelated to dietary treatment. Initially, a power analysis was performed using data for rate of absorption of acetate, propionate, and butyrate in the rumen of steers and rumen surface area measurements from 4-weeks-old preweaned calves either fed milk replacer and calf starter or milk replacer only. The data from these variables were used to run a power test that resulted in a power of 0.91 when n = 5 and a power of 0.96 when n = 6. The diets were formulated to be isocaloric and isonitrogenous. Both groups of calves were fed MR (Ledger, 22% CP as-fed, 20% fat as-fed; Southern States Cooperative Inc.) twice daily at 0700 hours and 1900 hours and water was available ad libitum. The calf starter offered to MRS calves was an 18% CP (as-fed) textured feed (Southern States Cooperative Inc.). Milk replacer only calves were offered 0.884 kg of powder mixed in 7.537 kg of water (10.5% solids) daily. In order to maintain isocaloric and isonitrogenous diets, MRS calves were offered a decreasing amount of MR throughout the trial to accommodate the planned increase in calf starter intake. The weekly feeding schedule for MRS calves for both MR (always fed at 14% solids) and calf

starter was (as-fed): week 1: 0.875 kg of MR powder mixed in 5.373 kg of water and 0.018 kg of starter per day; week 2: 0.867 kg of MR powder mixed in 5.328 kg of water and 0.031 kg of starter per day; week 3: 0.785 kg of MR powder mixed in 4.824 kg of water and 0.160 kg of starter per day; week 4: 0.689 kg of MR powder mixed in 4.236 kg of water and 0.311 kg of starter per day; week 5: 0.564 kg of MR powder mixed in 3.466 kg of water and 0.509 kg of starter per day; and week 6 and days 42, 43, and 44: 0.415 kg of MR powder mixed in 2.330 kg of water and 0.746 kg of starter per day. Any refusals were recorded daily. Based on equations from the Dairy NRC (2001), these diets fed according to the feeding schedule described, should have provided each calf with 4.0 Mcal of ME/d and 0.198 kg of apparent digestible protein (**ADP**)/day on average throughout the trial to fulfill the isocaloric and isonitrogenous conditions. Fresh MR powder and calf starter samples were taken at three time points equally spaced throughout each period, pooled, and sent to Cumberland Valley Analytical Services for nutrient analysis (**Table 5.1**).

At 10 days of age the calves underwent rumen cannulation surgery. The rumen cannulation surgery was performed similarly to that described by Kristensen et al. (2010). The surgically placed rumen cannula (2.75 i.d.) was the same design used in Lesmeister and Heinrichs (2004), Suarez-Mena et al. (2015), and Suarez-Mena et al. (2016). The cannula opening was plugged with a #6 rubber laboratory stopper.

The trial lasted 42 to 44 days depending on the calf. For each period, two calves were harvested each day (one per treatment) starting on day 42 and ending on day 44. On the final day of the trial (43 ± 1 days), each calf underwent a washed rumen procedure adapted from Storm et al. (2011). The procedure involved evacuating the rumen using a

vacuum-assisted collection device, washing the rumen with warm tap water, warm wash buffer (**Table 5.2**), and then filling the rumen with an experimental buffer (**Table 5.2**). Subsequently, a bolus dose of LiCoEDTA (15 mg/mL) was administered into the rumen. After an initial baseline sample, the rumen was sampled for the next 6 h, which then ended in harvesting the calf for final sample collection. After initial dosing of LiCoEDTA, a continuous infusion of LiCoEDTA was administered through the rumen cannula for the duration of sampling. In total, 19 samples of rumen fluid were obtained over the 6 hour infusion/sampling period (starting with time 0) for VFA analysis and Co determination. A detailed description of the final trial day rumen sampling is depicted in **Figure 5.1**.

Calves were assessed for intestinal and respiratory health throughout the trial. A 4-point scale was used for twice daily fecal scoring (Diaz et al., 2001). In this system, scores were as follows: "1 = firm, well-formed (not hard), 2 = soft, pudding-like, 3 = runny, pancake batter, and 4 = liquid, splatters." Calves were scored before each feeding. Any calf with a fecal score of 3 or greater was monitored closely and if deemed necessary was offered 100 g of electrolyte powder (Diaque; Boehringer Ingelheim, Copenhagen, Denmark) dissolved into hot water for a total of 1.89 kg solution. The oral electrolyte solution was offered at least 2 hours after each feeding until the scours subsided and any signs of dehydration (e.g. eyelid depression score and skin tent score) were normal. As for respiratory health, a 4-point scale was also used for twice daily respiratory scoring. In this system, scores were as follows: 1 = calf breathing at a normal rate, 2 = calf not showing signs of distress, but an observable increase in breathing compared to score 1, 3 = labored calf breathing with other signs of respiratory infection (lethargy, nasal

discharge, etc.), 4 = score of 3 plus rectal temperature greater than or equal to 39.4°C. Calves were scored before each feeding. Any calf with a respiratory score of 3 or greater was flagged for a more extensive examination after feeding. This examination included a rectal temperature measurement, examination of nose and ears for signs of infection, further observation of breathing patterns, and general behavior.

Analytical procedures of sampling/harvest day samples

Tissue Collection and Gross Rumen Measurements

At harvest, samples were taken for rumen morphometric analyses according to our previously described methods (Yohe et al., 2015). Briefly, the full stomach and reticulorumen were weighed and then evacuated for empty reticulorumen weight determination. Rumen samples intended for morphology and gene expression analyses were obtained from 4 locations in the rumen (ventral and dorsal samples from the cranial and caudal regions) using a punch biopsy tool with an internal diameter of 2.54 cm, stored in saline overnight, manually dried with a paper towel the following day, and then weighed. Rumen tissue samples were also stored in 10% neutral buffered formalin overnight and then switched to 70% ethanol before being measured for 2D papillae area. Tissue sections were viewed under a dissecting scope (Olympus SZ40; Olympus Corporation, Tokyo, Japan) and pictures were taken using an Olympus SZ-CTV adaptor (Olympus Corporation). Images were opened with and analyzed in Image-Pro Plus version 7.0 (Media Cybernetics, Inc.; Rockville, MD) so that area of 50 representative papillae were measured per calf.

Real-time qPCR

The ventral sac sample of the rumen was utilized to assess gene abundance of rumen epithelial transporters of interest via real-time quantitative PCR (**qPCR**). Forceps were used to peel the muscle layers away from the epithelium; the muscle-containing portion was discarded and the epithelial portion of each sample was flash frozen in liquid nitrogen and stored on dry ice until permanent storage in a -80°C freezer later that same day. Total RNA was extracted using the Qiagen RNeasy Plus Mini Kit (Qiagen; Valencia, CA). Approximately 30 mg of rumen epithelial tissue was taken and placed into 600 μL of lysis buffer (10 μL of β-mercaptoethanol in every 1 mL of buffer RLT Plus) in a 14 mL Falcon tube (Fisher Scientific, Waltham, MA). Each sample was homogenized using a Bio-Gen PRO200 homogenizer (Pro Scientific, Oxford, CT) at 35,000 rpm for 30 seconds and placed on ice. After centrifugation at 20,817 × *g* for 3 min at 23°C supernatant was then placed in a genomic DNA eliminator column and centrifuged at 10,621 × *g* for 30 s at 23°C. The flow-through was then processed using an RNeasy Plus mini kit. RNA purity and quantity were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies; Rockland, DE). For all 11 calves the 260/280 ratio averaged 2.11 ± 0.00, and the 260/230 ratio averaged 1.89 ± 0.13. Single-stranded cDNA was synthesized from each RNA sample according to the method of Yohe et al. (2016). For each sample, 2.0 μg of RNA was denatured in DNase/RNase free water for 12 min at 70°C and samples were then placed on wet ice. Reverse transcription was then performed, which consisted of a master mix (8.7 μL) containing: 4 μL of M-MLV RT 5X buffer (Promega; Madison, WI), 2 μL of 0.1 *M* dithiothreitol, 1 μL of 10 m*M* dNTP (Promega), 1 μL oligo(dT)20 primer, 0.5 μL M-MLV reverse

transcriptase (Promega), and 0.2 µL of RNase inhibitor (Promega). The 8.7 µL of master mix was added to each well of denatured RNA (11.3 µL) in a 0.2 mL thin-walled PCR 8-strip well tube (World Wide Medical Services Inc.; Tampa, FL) for final reaction volume of 20 µL. Reverse transcription was carried out in a Arktik Thermal Cycler (Thermo Scientific; Waltham, MA) with cycle conditions of 40°C for 1 h, followed by 95°C for 10 min.

Resultant cDNA was then diluted 1:1 with addition of 20 µL of DNase/RNase free water. Each qPCR assay was performed on all samples in triplicate, with each reaction mixture (10 µL) containing 0.25 µL of each forward and reverse primer, 4.75 µL of PowerUp SYBR Green Master Mix (Life Technologies; Grand Island, NY), 3.75 µL of DNase/RNase free water, and 1 µL of cDNA (1:1 stock). The qPCR assays were performed using a QuantStudio 6 Flex Real-Time PCR system (Life Technologies) with the following cycling conditions: 95 °C for 3 min; 45 repeating cycles of 94 °C for 15 s (denaturation), respective temperature (**Table 5.3**) for 30 s (annealing), and 72 °C for 30 s (extension); 95.0 °C for 2 min, 55.0 °C for 30 s, and then a temperature increase at 1.0 °C increment to 95.0 °C (melting curve). Each assay included a no-template control and a no reverse-transcriptase control, with the no template control receiving 1 µL of RNase/DNase free water instead of cDNA, while the no reverse-transcriptase control received a 1 µL sample of the reverse transcription product to which no reverse-transcriptase was added. The qPCR was repeated when the resulting coefficient of variation of the replicates' cycle threshold (**Ct**) values was greater than 10%.

Primers were designed with Primer3 (v. 0.4.0; http://bioinfo.ut.ee/primer3-0.40/) (Rozen and Skaletsky, 2000) for all genes used. Validation of primers consisted of

melting curve analyses that were performed after each qPCR assay to determine whether primer dimers or genomic DNA contamination were present during the assay. Target genes of interest were normalized to the geometric mean of *RPS9*, *RPS15*, and *RPS26* (Yohe et al., 2016) (target gene Ct – reference genes Ct = ΔCt) (Vandesompele et al., 2002).

Immunohistochemistry

At slaughter, a full-thickness section of tissue was cut from the ventral sac region (approx. 5 to 10 cm long and 1 to 1.5 cm wide) from each calf rumen and fixed overnight in 10% neutral buffered formalin. After 24 hours in fixative, the tissues were moved to 70% ethanol. Samples were then routinely processed (Leica TP 1020; Leica Microsystems Inc.; Buffalo Grove, IL), embedded in paraffin, cut to 5-µm thickness on a microtome (Model HM 340 E; Micron International GmbH, Germany), and mounted onto positively charged glass microscope slides (Yohe et al., 2015). Immunohistochemistry for the VFA transporter proteins monocarboxylate transporter 1 and monocarboxylate transporter 4 (**MCT1** and **MCT4**, respectively) was performed using previously described methods (Daniels et al., 2009; Tucker et al., 2016). Briefly, microscope slides were deparaffinized in clear-rite (xylene substitute) (3 × 3 min) and hydrated through a descending graded series of ethanol washes (100%, 2 × 3 min; 95%, 2 × 3 min; 70%, 1 × 3 min) into distilled water (2 × 3 min). Antigens were retrieved by boiling slides in 10-m*M* citrate buffer, pH 6.0 for 30 min. Slides were allowed to cool in citrate buffer (approx. 30 min) and then were washed in phosphate buffered saline (3 × 2 min). Individual tissue sections were divided with a PAP barrier pen (Ted Pella Inc.; Redding, CA) to prevent commingling of antibodies. All tissue sections were then

blocked with CAS Block (Life Technologies Corporation) for 30 min. The CAS Block was aspirated, and 100 μL of the primary antibody solution was added per section. Primary antibody characteristics and dilutions are listed in **Table 5.3**. One microscope slide containing 2 to 3 tissue sections per antibody was used for each calf. One section per slide served as a negative control, which consisted of CAS Block instead of the primary antibody solution. Slides were incubated overnight at 4°C in a humidified chamber with primary antibodies.

The next morning, primary antibody solutions were removed by aspiration and slides were rinsed in phosphate buffered saline (3 × 5 min). All tissue sections received 100 μL of the fluorescent secondary antibody solution (**Table 5.3**) and incubated in the dark for 60 min at room temperature. Excess liquid was removed by aspiration, and coverslips were mounted using Prolong Gold antifade reagent containing the nuclear stain 4',6-diamidino-2-phenylindole (**DAPI**) (Life Technologies) and allowed to cure overnight. Protein abundance and localization of both MCT1 and MCT4 were quantified in the following manner. Fourteen digital images per calf were acquired at 40X magnification using a Nikon Eclipse E600 epi-fluoresence microscope fitted with a Nuance FX Multispectral Imaging System (Perkin-Elmer; Waltham, MA). In order to assess the intensity of the signal without confounding the data, within tissue antigen of interest each picture was taken at the same wavelength and exposure time in the Nuance system (MCT1: 625 nm wavelength at 275 ms exposure time; MCT4: 625 nm wavelength at 375 ms exposure time) to generate monochromatic images from the Texas Red channel. Monochrome images were opened with and analyzed in Image-Pro Plus version 7.0 (Media Cybernetics). The rumen epithelium was outlined using a freehand

drawing tool, designating areas of interest. Within areas of interest, staining intensity sum was calculated by an algorithm in Image-Pro Plus version 7.0 software (Media Cybernetics). The staining intensity sum was then divided by the area of interest for each image to yield measurements expressed as: sum of intensity / μm^2. This approach was selected due to the non-nuclear and diffuse staining patterns of both MCT1 and MCT4 (**Figures 5.3** and **5.4**).

VFA absorption and Co determination

At designated sampling times, approximately 5 to 15 mL of strained rumen fluid were collected and stored at -20°C in glass screwtop tubes for later VFA analysis. Volatile fatty acid derivitization was done by gas chromatography using an adapted method from Kristensen (2000). Samples were run on a Thermo Electron Polaris Q Mass Spec (**MS**; Thermo Fisher Scientific Inc., Waltham, MA) in tandem with a Thermo Electron Focus Gas Chromatograph (**GC**; Thermo Fisher Scientific, Inc.) using XCalibur softare version 1.4 (Thermo Fisher Scientific Inc.). The column used was a Varian FactorFour capillary column VF-170ms (30 m, 0.25 mm, 0.25 μm). One microliter of derivatized sample was loaded with inlet temperature set to 225°C on a split ratio of 80 running a constant flow of Helium carrier gas set to 1.2 mL/min. The GC was initiated at 75°C ramped at 5°C/min to 135°C then at 40°C/min to 225°C. The MS was programmed to run in positive SIM mode collecting three consecutive segments m/z pairs for acetate (43, 47), propionate (57, 59), and butyrate (71, 73) in that elution order. The processing method used to integrate the area under the curves for each m/z utilized the ICIS algorithm.

At designated sampling times, approximately 5 to 15 mL of strained rumen fluid were collected and stored at -20°C in plastic tubes for later Cobalt analysis. Initially, samples underwent nitric acid digestion using method 3030H (EPA, 1998) to remove organic matter. After removal of all organic material, samples were resuspended in 50 mL mineral free water and sent to the VT Soil Testing Lab for Co determination via inductively coupled plasma atomic emission spectroscopy.

Statistical analyses

Intake, growth, gross rumen measurements, qPCR, and IHC data were analyzed using the MIXED procedure of SAS 9.4 (SAS Institute, Cary, NC). All models included the fixed effects of treatment, with repeated measures data (intake and growth) including week, and the interaction of treatment and week. Calf nested within treatment was the random effect in all models. Period was initially included in all models, but was found to be insignificant and thus removed. All analyses included the best-fit covariance structure when appropriate; denominator degrees of freedom were not specified. Best-fit covariance structures were as follows: MR DMI, total DMI, ME intake, ADP intake, and hip height measurements used autoregressive, voluntary water intake and total water intake used unstructured, body weight and withers height used toeplitz, and ADG used heterogeneous autoregressive. Significance was declared when $P \leq 0.05$. Orthogonal polynomial contrasts were performed when significance was found for the interaction of treatment and week or just the effect of week. Data for qPCR was not normally distributed (significant Shapiro-Wilk test) and were then log10 transformed and reanalyzed. Untransformed data are presented. Least squares means ± standard errors of

the mean are reported. Health data were analyzed using the FREQ procedure of SAS 9.4 to compare frequency of fecal and respiratory events between treatments.

Results and Discussion

Intake, growth, and health

The dietary treatments were formulated to be both isocaloric and isonitrogenous (NRC, 2001) to prevent any confounding factors influencing rumen growth, development, and function. Intake data for MRO and MRS calves in **Table 5.4** shows there are differences in DMI for MR ($P = 0.0001$), starter grain (MRO calves were not given starter grain), and total DMI ($P = 0.008$), but metabolizable energy (**ME**) and apparent digestible protein (**ADP**) did not differ for the interaction of treatment by week ($P = 0.778$ and $P = 0.520$, respectively) or by treatment alone ($P = 0.935$ and $P = 0.295$, respectively). This lack of difference in ME and ADP intake shows that the diets were successful in maintaining energy and nitrogen balance between treatments, thus our dietary goals were achieved. The diets provided daily ME intakes between 3.51 Mcal/d to 4.05 Mcal/d, which would not be considered an elevated/higher plane of nutrition (Geiger et al., 2016; Steele et al., 2017), but is within the range of ME requirements for dairy calves gaining between 0.4 kg/d to 0.6 kg/d fed either MR only or MR and starter (approx. 2.76 Mcal/d to 4.31 Mcal/d) (NRC, 2001). Intake of ADP provided 0.15 kg ADP/d to 0.18 kg ADP/d, which is also within the ADP requirements for dairy calves gaining between 0.4 kg/d to 0.6 kg/d fed either MR only or MR and starter diets (approx. 0.13 kg ADP/d to 0.19 kg ADP/d) (NRC, 2001). I noted a lack of increase in ME and ADP intake during week 2, which is most likely due to the rumen cannulation surgery occurring during this time period. Also, it should be noted that calves on the MRS

treatment did not always voluntarily consume the required calf starter, which necessitated placement of refusals into the rumen via the cannula. We recognize that this action may have altered certain aspects of nutrient digestion (e.g. mechanical breakdown of feed reducing particle size, saliva production during chewing), but this ensured the isocaloric and isonitrogenous aspects of these diets.

Voluntary water intake increased as calves aged and was higher in MRS (**Table 5.4**; treatment and week interaction, $P = 0.008$; week, $P = 0.046$; and treatment, $P = 0.006$). Total water intake also increased as calves aged but total water intake was higher in MRO (**Table 5.4**; treatment and week interaction, $P = 0.010$; week, $P = 0.001$; and treatment, $P = 0.001$). MRO calves were fed MR at 10.5% solids whereas MRS calves were fed MR at 14% solids. The difference was planned and the higher solids in MRS was intended to promote both voluntary water and starter feed intake. This seemed to work (**Table 5.4**) and follows previous research that correlated dry feed intake to water intake (Meale et al., 2017). Throughout the experiment, MRO calves were likely less thirsty than MRS calves due to the increased amount of water fed with MR – hence lower voluntary water consumption. As experimental weeks progressed, water delivery to MRS through MR decreased whereas water delivery through MR remained the same for MRO. This explains why total water intake is higher in MRO. When calves drink MR, 97% of the water associated with it bypasses the rumen and enters the abomasum; when calves voluntarily drink water, it goes to the rumen. Ruminal water is required for supporting microbial population growth in the rumen; treatment differences in water consumption and effects on rumen microbes were not studied in this research.

From a growth standpoint (**Figure 5.2**), the isocaloric and isonitrogenous nature of the diets was corroborated by BW not differing for the interaction of treatment by week or treatment alone ($P = 0.257$ and $P = 0.414$, respectively) or ADG throughout the trial for the interaction of treatment by week or treatment alone ($P = 0.902$ and $P = 0.941$, respectively). Based on Dairy NRC (2001) data, both diets provided the ME and ADP necessary to gain somewhere between 0.4 kg/d and 0.6 kg/d throughout the trial, which is reflected in the ADG achieved between the treatments for the duration of the 6 week trial (**Figure 5.2B**). As mentioned for the intake, there was a noticeable dip in ADG around 2 week, which is most likely due to the rumen cannulation surgery that had occurred a few days before weighing the calves. Soberon and Van Amburgh (2013) and Gelsinger et al. (2016) demonstrated the positive relationship between preweaning ADG and first lactation milk yield, which could be achieved regardless of form of calfhood diet (i.e. liquid or liquid and starter). Compared to the ADG from the current 6 week study (MRO: 0.46 ± 0.03 kg/d, MRS: 0.46 ± 0.03 kg/d), the recommendation by Gelsinger et al. (2016) to maintain a minimum ADG of 0.5 kg/d to achieve elevated first lactation milk production shows these diets providing 3.87 ± 0.06 MCal of ME/d and 0.17 ± 0.003 kg/d of ADP were not meeting the nutrient requirements to sustain somatic growth especially after week 4 (**Figure 5.2B**).

Diet affected fecal scores (data not shown); MRO calves had a higher frequency of scores ≥ 3 (i.e. runny consistency like pancake batter) compared to MRS calves for the whole trial (27.6% vs. 16.5% of all observations, respectively; $P < 0.0001$). Interestingly though, MRS calves were offered oral electrolytes more often (29 occasions) compared to MRO calves (18 occasions) throughout the experiment. This seems to indicate that

higher fecal scores in MRO were more likely caused by absence of fecal bulk than due to presence of bacterial or viral pathogens. In support of that, administration of oral electrolytes only commenced after visually inspecting the calf and checking for signs of dehydration; MRO calves were not typically clinically dehydrated. Taken together, these observations point to the MRO calves potentially experiencing something called "nutritional scours" that would be reflecting their exclusively liquid diet rather than an actual sign of illness (Roy, 1964; Leaver and Yarrow, 1972). No calf had a respiratory score ≥ 3 and no calves were treated for respiratory disease.

Rumen morphometric measurements

Results for rumen morphometric measurements are presented in **Table 5.5**. Calves on the MRS treatment had heavier empty rumens compared to the MRO calves (0.68 ± 0.04 kg vs. 0.39 ± 0.04 kg, respectively; $P = 0.0004$), which supports previous findings that calves fed calf starter experience more rumen growth compared to calves fed a liquid only diet (Harrison et al., 1960; Sutton et al., 1963b; Stobo et al., 1966). There was no difference between treatments in empty omasal plus abomasal weights (MRS: 0.63 ± 0.07 kg, MRO: 0.49 ± 0.07 kg; $P = 0.200$), demonstrating that even when calves are fed diets differing in main site of digestion, the liquid diet that bypasses the rumen does not stimulate growth of the abomasum (with omasum).

While there were no differences in the total (MRO: 1.35 ± 0.10 kg; $P = 0.420$, MRS: 1.46 ± 0.10 kg) or the muscularis portion (MRO: 0.78 ± 0.05 g; $P = 0.311$, MRS: 0.70 ± 0.05 g) of the biopsy core samples, there was a trend for an increase in the weight of the epithelial portion for the MRS calves compared to MRO calves (0.77 ± 0.07 g vs. 0.57 ± 0.07 g, respectively; $P = 0.068$). This is generally supportive of enhanced rumen

epithelial growth (i.e. proliferation) when the tissue is exposed to a solid concentrate diet (Tamate et al., 1962; Sutton et al., 1963b; Stobo et al., 1966). Interestingly, even without an obvious difference in the total or epithelial biopsy weights, there was an increase in the 2D area of the rumen papillae of MRS calves compared to MRO calves (0.76 ± 0.08 mm^2 vs. 0.15 ± 0.08 mm^2, respectively; $P = 0.0001$), which supports many previous findings of solid concentrate diets fed to calves enhancing rumen growth by affecting proliferation of the rumen tissue resulting in larger papillae (Harrison et al., 1960; Stobo et al., 1966; Heinrichs, 2005).

Rumen epithelial qPCR

The only difference between treatment for any of the VFA transport-related genes was a trend for an increase in MRS compared to MRO calves for *MCT1* expression (*P*=0.060; **Table 5.6**). This trending increase in expression of *MCT1* is interesting due to the implication of *MCT1* in transporting VFA from the epithelium to the interstitial fluid/portal blood circulation (Graham et al., 2007). Laarman et al. (2012a) noted a decrease in *MCT1* mRNA abundance in calves fed only MR and hay compared to MR, hay, and starter around 50 days of age, but there was no treatment that was only MR and diets were not formulated to be isocaloric or isonitrogenous. The other two VFA transporter genes (*MCT2* and *MCT4*) did not exhibit any differences between the treatments, which may reflect the experimental buffer used causing a uniform increased expression of VFA transporters into the rumen epithelium (MCT4) as well as between the rumen epithelial cell layers (MCT2) based on their cellular localization (Kirat et al., 2007; Kirat et al., 2013). The other genes examined (*NHE1*, *NHE2*, and *NHE3*) are relevant to VFA transport due to their ability to help regulate intracellular pH (Muller et

al., 2000). There were no differences observed for the *NHE* genes, which also may be explained by the experimental buffer that was given to both treatments. The reasoning behind this may be due to exposing each rumen to the same pH resulted in controlling the concentration gradients of H^+, Na^+, K^+, and other ions that may play a role in controlling the convoluted VFA transport/intracellular pH relationship (Connor et al., 2010). Lack of change in expression of the genes noted may have also been due to the isocaloric and isonitrogenous nature of the diets that supplied the rumen epithelium to equal "nutrients" even if they were mainly coming from opposite sides (i.e. rumen lumen vs. blood). How these gene expression results compare to protein abundance as well as activity (i.e. VFA absorption) will be discussed later, but it should be noted that expression of a gene does not necessarily equate to protein abundance or protein activity.

Rumen immunohistochemistry

Enhanced rumen growth of calves fed a solid diet (emphasis on highly fermentable starter diet) compared to calves fed a solely liquid diet was expected. What remained to be seen were the results relative to the function of the rumens of calves fed either a liquid diet of MR or liquid and solid feed diet of MR and calf starter. Assessing VFA absorption involved utilizing immunohistochemistry to measure the abundance of the VFA transporters MCT1 and MCT4 that have previously been identified in the bovine rumen (Kirat et al., 2005; Kirat et al., 2007). Images depicting the staining of both MCT1 and MCT4 in the rumen tissue are shown in **Figures 5.3** and **5.4**. The pattern of the staining of MCT1 is similar to that shown previously by Kirat et al. (2005), Graham et al. (2007), and Flaga et al. (2015) and that hypothesized by Connor et al. (2010) in the basale layer of the stratified rumen epithelium important for movement of VFA from the

epithelial tissue to the extracellular space (i.e. interstitial fluid and blood). There was no difference in the abundance of MCT1 between treatments based on the intensity measures per area of rumen epithelial tissue ($P = 0.188$), which suggests a lack of difference in ability of the tissue to absorb VFA from a facilitated transport mode of action via protein transporter. The staining pattern of MCT4 is similar to that seen by Kirat et al. (2007), but does not seem to fit the hypothesized location by Connor et al. (2010) in the upper layers of the rumen epithelium (i.e. stratum granulosum and stratum corneum). The diffuse staining of MCT4 throughout the rumen epithelial cell layers questions the idea of MCT4 being important for mainly transporting VFA from the ruminal lumen into the rumen epithelial cell layers, but considering the pattern of staining is similar to that seen by Kirat et al. (2007) and comparison to the negative control (**Figure 5.3**) the staining method seems valid. There was no difference in intensity of MCT4 staining between treatments ($P = 0.600$), which suggests a lack of difference in ability of the tissue to absorb VFA from a facilitated transport mode of action via protein transporter from the rumen. Staining results for MCT1 and MCT4 taken together suggest that even though MRO calves were only given a liquid diet of MR their rumens were still just as capable of absorbing VFA when exposed to a defined concentration of VFA when compared to MRS calves. This lack of dietary effect on VFA transporter abundance will be further discussed with the following VFA absorption results to construct a more complete story. That being said, these transporter immunohistochemical results from the present study are not quantitatively assessing VFA absorption, which leaves open the possibility of passive diffusion that could still be playing a role in VFA transport (this will be discussed later).

VFA absorption

To assess VFA absorption the concentration of VFA was taken over a 6 hour period on the last day of the trial, along with measurement of liquid flow rate out of the rumen. Results in **Table 5.7** show there were no differences in fluid exit rate to the omasum ($P = 0.137$), or VFA absorption rates for acetate, propionate, or butyrate ($P = 0.202$, $P = 0.571$, and $P = 0.949$, respectively) between MRO and MRS calves. Also, total 6 hour fluxes of acetate, propionate, or butyrate were not different between MRO and MRS calves ($P = 0.732$, $P = 0.760$, and $P = 0.830$, respectively; **Table 5.7**). All of these results taken together indicate that diet did not alter the ability of rumen absorption when exposed to a high concentration of VFA.

Sutton et al. (1963a) performed a similar study to the current one and showed that calves fed only milk had a decreased maximal absorption rate of acetate from week 1 to 34 (stayed constant around 30 mg acetate/100 mL solution/h) compared to calves fed milk, grain, and hay that increased from week 1 (about 30 mg acetate/100 mL solution/h) to 34 (about 210 mg acetate/100 mL solution/h). The results in the current study go against these earlier findings and instead support the findings of Khouri (1969) who noted no difference in VFA absorption between calves fed only milk or fed milk and hay around 40 to 44 days of age. Rumen VFA absorption via protein-mediated transport may not be able to clarify the differences observed between the current results and those by Sutton et al. (1963a), but the difference may be explained by passive diffusion of VFA. On this note, another difference between our study and that of Sutton et al. (1963a) was the pH of the buffer introduced into the rumen. It has been known for many years that pH has an effect on VFA absorption in the rumen (Danielli et al., 1945; Dijkstra et al., 1993;

Aschenbach and Gabel, 2000; Penner et al., 2011), which is worth pointing out considering Sutton et al. (1963a) utilized a buffer with a pH at 6.5 whereas the current study utilized a buffer with a pH at 5.85. The pH of 5.85 used in the current study was due to previous rumen pH data in calves around 6 week of age (Laarman et al., 2012b; Yohe et al., 2015). Considering a 0.7 difference in pH is rather large, it is conceivable that the lower pH may have facilitated an increase in passive diffusion across the rumen epithelial membrane regardless of dietary treatment, resulting in a lack of effect on VFA absorption rates.

There are other aspects of the manuscript written by Sutton et al. (1963a) that are unclear and would potentially help explain the study differences observed. For example, was the concentration of the buffer used in the first experiment the same used by Sutton et al. (1962)? This is not clear. If so, then a clear dissimilarity may be the difference in concentrations of VFA as well as salts used in the buffer. Another discussion point is that calves fed milk, grain, and hay received rumen inoculations of adult rumen contents whereas the milk-fed calves did not (Sutton et al., 1963a). Sutton et al. (1963a) also did not measure the absorption rates of propionate or butyrate, which does not allow direct comparison between the studies. These questions are not meant to disparage the work by Sutton et al. (1963a) considering their work was thorough and carefully executed, but considering the uncertainties, the gap in time, and the lack of corroborating data it is worth revisiting the question of dietary effect on preweaned calf rumen VFA absorption.

Another aspect of VFA absorption that was not explored by Sutton et al. (1963a) or in the current study is epithelial blood flow. Previous work has demonstrated the importance of epithelial blood flow for VFA absorption (Storm et al., 2011; 2012).

Dietary considerations that may have affected blood flow are CP intake (Storm et al., 2011) and butyrate concentration (Dobson et al., 1971), but considering the diets were isonitrogenous and the buffers introduced into the rumen contained the same amount of butyrate it may be fair to assume these did not have an effect. Storm et al. (2012) demonstrated that rumen epithelial metabolism of VFA may influence blood flow, which may have been altered in the current study due to larger papillae (**Table 5.5**), tendency for heavier epithelial biopsy weight (**Table 5.5**), and thicker epithelial cell layer (data shown in chapter 6) leading to an increased opportunity for VFA metabolism to occur in the rumen epithelium. In the end, blood flow was not measured and there were no apparent differences in VFA absorption; so none of these speculations can be confirmed.

Other factors were considered when assessing rumen VFA absorption, which are shown in **Table 5.8**. There were only a handful of instances when the covariate had an effect. One of these instances was a tendency for the variable "total rumen biopsy weight" to influence acetate and butyrate absorption rate ($P = 0.099$ and $P = 0.057$, respectively). Increased epithelial biopsy weight had a positive effect on acetate, propionate, and butyrate absorption rate ($P = 0.002$, $P = 0.005$, and $P = 0.014$, respectively). And lastly, a denser epithelium increased acetate and propionate absorption rate ($P = 0.004$ and $P = 0.001$, respectively) and a trending influence on butyrate absorption rate ($P = 0.074$). All of these results taken together support the idea that a more developed rumen (i.e. thicker and heavier epithelium) influences VFA absorption rate. This was shown by Sutton et al. (1963a). Interestingly, though, when looking at total VFA absorbed over the 6 hour period, the only trending influence relevant to rumen growth was epithelial density and total butyrate absorbed ($P = 0.065$). And, MCT4

protein abundance had an effect on total acetate absorbed ($P = 0.035$) and a trending influence on total propionate absorbed ($P = 0.102$). These data indicate that butyrate tends to undergo passive diffusion more readily than acetate and propionate, which will instead utilize protein-mediated transport. This supports Bergman's (1990) commentary that rate of ruminal VFA absorption increased with increasing VFA chain length due to passive diffusion being a quicker process.

Conclusions

It appears the hypothesis was not supported and that form of calfhood diet, whether it is solely MR or MR and starter, does not alter VFA absorption when the rumen is exposed to a defined concentration of VFA at 6 week of age. This finding is supported by the lack of difference in abundance of protein transporters (MCT1 and MCT4) as well as rates of VFA absorption and total VFA absorbed during the 6 hour experimental period. Transport of VFA via passive diffusion also does not seem to be altered in preweaning calves under the experimental conditions. It should be noted that total VFA absorption was most likely increased in calves fed MR and starter compared to MR only due to larger rumens and increased surface area, but these results indicate the rumen has the innate ability to absorb VFA regardless of external stimuli.

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Table 5.1. Chemical composition of milk replacer and textured starter fed to calves throughout the trial

Chemical composition, % of DM	Milk Replacer[1]	Textured Calf Starter[2]
Dry matter %	96.2	85.8
CP	22.4	21.5
Fat (ether extract for starter)	21.5	3.95
NDF	--	19.0
ADF	--	9.3
Starch	--	27.7
Ash	9.49	9.36
ME (Mcal/kg)	4.74	3.09

[1]Milk replacer was purchased from Southern States, VA as a 22% CP, 20% fat powder (as-fed) that included milk protein and animal fat sources.
[2]Textured calf starter was purchased from Southern States, VA as an 18% CP (as-fed) feed that contained whole grains from plant protein sources.

Table 5.2. Wash and experimental buffers used throughout the rumen wash, evacuation, and sampling protocol

Chemical composition, mmol/L	Wash Buffer	Experimental Buffer
NaCl	106.01	20.01
$NaHCO_3$	24.00	24.00
NH_4Cl	--	2.50
NaOH[1]	6.50	212.03
KOH	19.99	19.99
K_2HPO_4	2.00	2.00
$CaCl_2$	1.51	1.50
$MgCl_2$	1.50	1.50
Acetic acid	--	143.02
Propionic acid	--	100.01
Butyric acid	--	40.58
HCl[1]	49.72	--
pH[1]	5.85	5.76

[1]pH was adjusted to target 5.8.

Table 5.3. Real-time quantitative PCR (qPCR) and immunohistochemistry info for select genes and proteins

qPCR Gene Symbol[1]	Primer[2]	Primer (5'-3')	Temp[3]	bp[4]	GenBank Accession #
MCT1	Forward 352 Reverse 480	GTCATTGGAGGTCTTGGGCT GGTAGAGAGGAACACAGGGC	62.6	129	NM_001037319.1
MCT2	Forward 300 Reverse 445	TGGTCTCGGCCTCTTACAGT GCCATTCGCTACAGGTCGTT	62.4	146	NM_001076366.2
MCT4	Forward 173 Reverse 322	GTGACACAGCCTGGATCTCC AGAAGGACGCAGACACCATG	64.5	150	NM_001109980.1
NHE1	Forward 1122 Reverse 1253	GTCCCACACGACCATCAAGT AGGGTGCTGATGACAAACGT	62.0	132	NM_174833.2
NHE2	Forward 1602 Reverse 1730	CGAGCAGCTCTACATCCTGG ATGCCAGCAAACACGTCAAC	63.0	129	XM_604493.6
NHE3	Forward 1560 Reverse 1688	CCTCATGAGAAGGTCGGCTC GAACGGATGAAAGCCAGGGA	63.0	129	NM_001192154.1
RPS9 (Reference)	Forward 122 Reverse 229	GTGAGGTCTGGAGGGTCAAA GGGCATTACCTTCGAACAGA	62.4	108	BC148016.1
RPS15 (Reference)	Forward 183 Reverse 329	CAAGGCCAAGAAAGATGCGC TCAGGCTTGATTTCCACCTGG	62.4	147	NM_001024541.2
RPS26 (Reference	Forward 219 Reverse 347	CTGCGTGAGTTGTGCCATTC TACATGGGCTTTGGTGGAGG	62.4	129	NM_001015561.2

Immunohistochemistry Protein antibody[5]	Host Species (Clonicity)	Tested/Target Reactivity	Dilution	Manufacturer	Catalog #
MCT1 (Primary)	Chicken (Polyclonal)	Rat	1:3000	EMD Millipore	AB1286I
MCT4 (Primary)	Rabbit (Polyclonal)	Rat	1:250	EMD Millipore	AB3314P
IgG (Secondary)	Goat	Anti Chicken	1:200	Life Technologies	A11042

IgG (Secondary)	Goat	Anti Rabbit	1:200	Life Technologies	A11037

[1]*MCT* = Monocarboxylate transporter, *NHE* = Sodium-hydrogen exchanger, and *RPS* = Ribosomal protein.
[2]Primer direction and hybridization position on the sequence.
[3]Temp = annealing temperature, °C.
[4]Amplicon size in base pairs (bp).
[5] Protein antibody targets: MCT = Monocarboxylate transporter, and IgG = Immunoglobulin G.

Table 5.4. Intake data for MR, starter, ME and ADP from MR and starter, and water for calves fed two differing forms of diet during the 6 week trial

	Week[2]							Test of Fixed Effects, P-value[3,4]		
Item[1]	1	2	3	4	5	6	SEM[5]	Trt	Wk	Trt*Wk
MR DMI, kg/wk										
MRO	5.44	5.18	5.95	5.95	5.95	6.63	0.20	0.0001	0.0001	0.0001§#¶
MRS	5.38	5.34	5.29	4.64	3.80	3.21	0.18			
Starter DMI, kg/wk										
MRO	--	--	--	--	--	--	--	--	--	--
MRS	0.11	0.19	0.96	1.74	3.08	5.23	0.25			
Total DMI, kg/wk										
MRO	5.44	5.18	5.95	5.95	5.95	6.63	0.20	0.008	0.0001	0.005§#¶
MRS	5.49	5.53	6.25	6.38	6.88	8.44	0.21			
Total ME[6] intake, Mcal/wk										
MRO	25.80	24.56	28.22	28.22	28.22	31.44	1.03	0.935	0.0001†	0.778
MRS	25.82	25.89	28.03	27.38	27.51	31.36	0.94			
Total ADP[7] intake, kg/wk										
MRO	1.13	1.08	1.24	1.24	1.24	1.38	0.05	0.295	0.0001†‡	0.520
MRS	1.14	1.14	1.26	1.25	1.29	1.51	0.04			
Voluntary water intake, kg/wk										
MRO	5.44	5.80	4.17	4.11	3.77	4.52	3.54	0.006	0.046	0.008#¶
MRS	7.37	8.89	7.90	8.93	12.26	23.05	3.23			
Total water intake,[8] kg/wk										
MRO	53.69	52.56	56.94	56.88	56.53	63.31	4.65	0.0001	0.001	0.010§
MRS	41.69	43.00	41.67	38.58	36.52	43.35	4.25			

[1]MRO = MR only fed throughout the trial, MRS = MR with starter fed throughout the trial. Diets were formulated to be isocaloric and isonitrogenous.

[2]Week 6 includes from day 35 up to day 44 of the trial since calves were harvested on days 42, 43, and 44.

[3]Significance declared when $P \leq 0.05$.

[4]Trt = treatment (either MRO or MRS); Wk = week.
[†]Linear effect of week.
[‡]Quadratic effect of week.
[§]Linear effect of MRO treatment.
[#]Linear effect of MRS treatment.
[¶]Quadratic effect of MRS treatment.
[5]Standard error of the mean.
[6]ME was calculated based on equations from the NRC (2001).
[7]ADP = Apparent digestible protein that was calculated based on equations from the NRC (2001).
[8]Total water intake includes both voluntary water intake and any water that was ingested when consuming MR.

Table 5.5. Rumen morphometric measurements of calves fed two differing forms of diet during the 6 week trial

	Treatment[1]			Test of fixed effect, *P*-value[2]
Item	MRO	MRS	SEM[3]	Trt
Empty reticulorumen weight, kg	0.39	0.68	0.04	0.0004
Empty omasal and abomasal weight, kg	0.49	0.63	0.07	0.200
Rumen biopsy samples				
Muscularis, g	0.78	0.70	0.05	0.311
Epithelium, g	0.57	0.77	0.07	0.068
Total, g	1.35	1.46	0.10	0.420
Papillae 2D area, mm^2	0.15	0.76	0.08	0.0001

[1]MRO = calves receiving MR only throughout the 6 week trial; MRS = calves receiving MR and starter throughout the 6 week trial. Both diets were formulated to be isocaloric and isonitrogenous.

[2]Significance declared when $P \leq 0.05$.

[3]Standard error of the mean.

Table 5.6. Relative mRNA abundance (expressed as $2^{\Delta Ct}$ values; higher values equate to more mRNA) of selected genes in rumen epithelial tissue relevant to VFA transport in calves fed two differing forms of diet at 6 week of age

	Treatment[1]			Test of fixed effects, P-value[2]
Gene[3]	MRO	MRS	SEM[4]	Trt
MCT1	0.024	0.054	0.011	0.060
MCT2	0.00098	0.00051	0.00020	0.132
MCT4	0.00029	0.00030	0.00012	0.471
NHE1	0.00031	0.00026	0.00012	0.497
NHE2	0.0016	0.0033	0.0016	0.606
NHE3	0.000091	0.000033	0.000028	0.102

[1]MRO = MR only fed throughout the trial, MRS = MR with starter fed throughout the trial. Diets were formulated to be isocaloric and isonitrogenous.
[2]Significance declared when $P \leq 0.05$.
[3]*MCT* = Monocarboxylate transporters, and *NHE* = Sodium-hydrogen exchangers.
[4]Standard error of the mean.

Table 5.7. Mean fluid exit, VFA absorption rate, and total 6 hour absorption of VFA after being exposed to a defined concentration of VFA in calves fed two differing forms of diet for 6 weeks.

	Treatment[1]			
Item	MRO	MRS	SEM[3]	Test of fixed effects, *P*-value[2]
Fluid exit, %/h	15.70	10.70	2.85	0.137
VFA absorption, %/h				
Acetate	20.90	35.30	7.73	0.202
Propionate	27.40	33.70	7.93	0.571
Butyrate	41.10	420	10.50	0.949
Total 6 hour VFA flux, mmol				
Acetate	176.70	165.70	22.80	0.732
Propionate	74.80	70.50	10.30	0.760
Butyrate	78.80	75.90	11.80	0.830

[1]MRO = MR only fed throughout the trial, MRS = MR with starter fed throughout the trial. Diets were formulated to be isocaloric and isonitrogenous.

[2]Significance declared when $P \leq 0.05$.

[3]Standard error of the mean.

Table 5.8. Mean covariate effects on VFA absorption rate and total VFA absorbed over 6 hours after being exposed to a defined concentration of VFA in calves fed two differing forms of diet for 6 weeks.

	VFA absorption rate, *P*-value[1]			Total VFA absorbed, *P*-value		
Covariate	Acetate	Propionate	Butyrate	Acetate	Propionate	Butyrate
MCT1 abundance	0.808	0.902	0.899	0.599	0.197	0.836
MCT4 abundance	0.281	0.417	0.215	0.035	0.102	0.153
Empty rumen[2] weight	0.728	0.551	0.429	0.420	0.561	0.402
RR 2D area	0.593	0.691	0.606	0.909	0.501	0.646
RR density	0.547	0.501	0.452	0.408	0.708	0.222
Total biopsy weight	0.099	0.227	0.057	0.754	0.700	0.766
Epithelium weight	0.002	0.005	0.014	0.546	0.671	0.187
Epithelium density	0.004	0.001	0.074	0.185	0.283	0.065

[1]Significance declared when $P \leq 0.05$.
[2]reticulorumen.

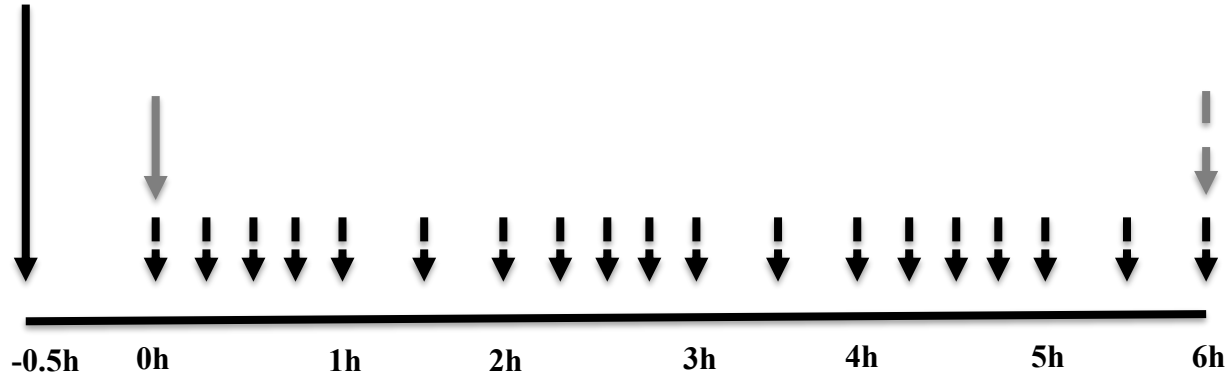

Figure 5.1. Final day of rumen sampling timeline depicted. The solid black arrow shown at -0.5 hours was the beginning of rumen content removal, and subsequent washing of the rumen with warm tap water and then a wash buffer (Table 5.2). Amount of water and wash buffer utilized for MRS calves was based on weight of rumen contents removed whereas MRO calves were administered water and buffer based on the weight of rumen contents/body weight of the first MRS calf used (3.88% of body weight). This was done due to lack of any contents present in the rumen of MRO calves. Starting at 0 hours the solid gray line indicates that experimental buffer was introduced (Table 5.2), the primer dose of LiCoEDTA (40 mL at 15 mg/mL) was given, and the LiCoEDTA infusion (0.158 mg/mL) was started. The dashed black lines starting at 0 hours and ending at 6 hours represent the fluid samples (approximately 5 to 15 mL) taken for VFA analysis and Co determination. The dashed gray line indicates the end of the infusion and sampling portion, which resulted in harvest of the calves for rumen tissue samples.

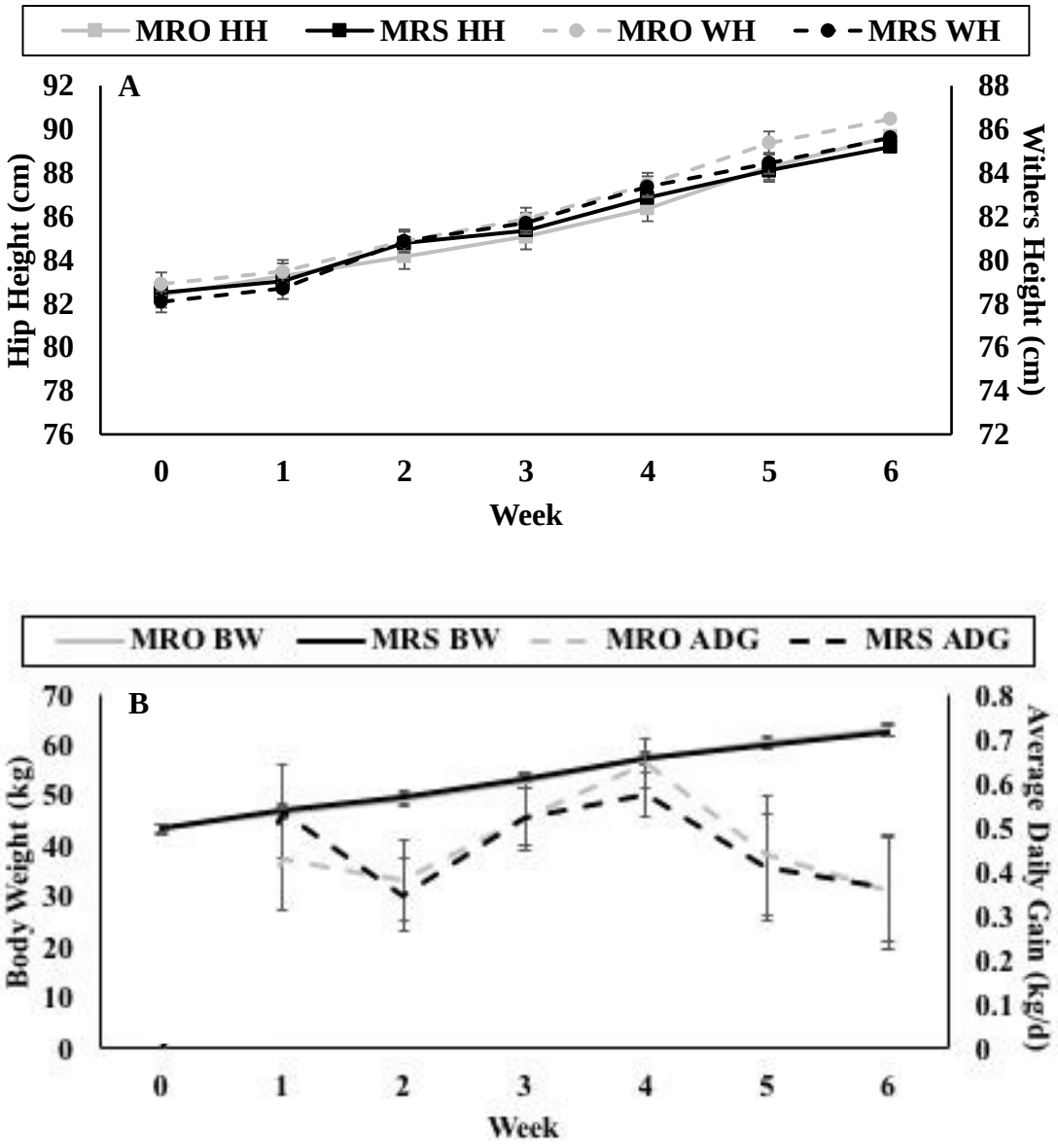

Figure 5.2. Mean (± SEM) growth measurements for calves fed two dietary treatments (MRO: milk replacer only; MRS: milk replacer and starter) during the 6 week trial. Hip height (HH) and withers height (WH) are shown in panel A where there were no differences for the interaction of treatment × week ($P = 0.286$ and $P = 0.247$, respectively) or treatment ($P = 0.900$ and $P = 0.414$, respectively). There was a linear effect of week for both HH and WH ($P < 0.0001$ for both). Body weight (BW) and average daily gain (ADG) are shown in panel B where there were no differences for the interaction of treatment × week ($P = 0.985$ and $P = 0.902$, respectively) or treatment ($P = 0.891$ and $P = 0.941$, respectively). There was a linear effect of week for BW ($P < 0.0001$) with a trending quadratic effect for ADG ($P = 0.075$).

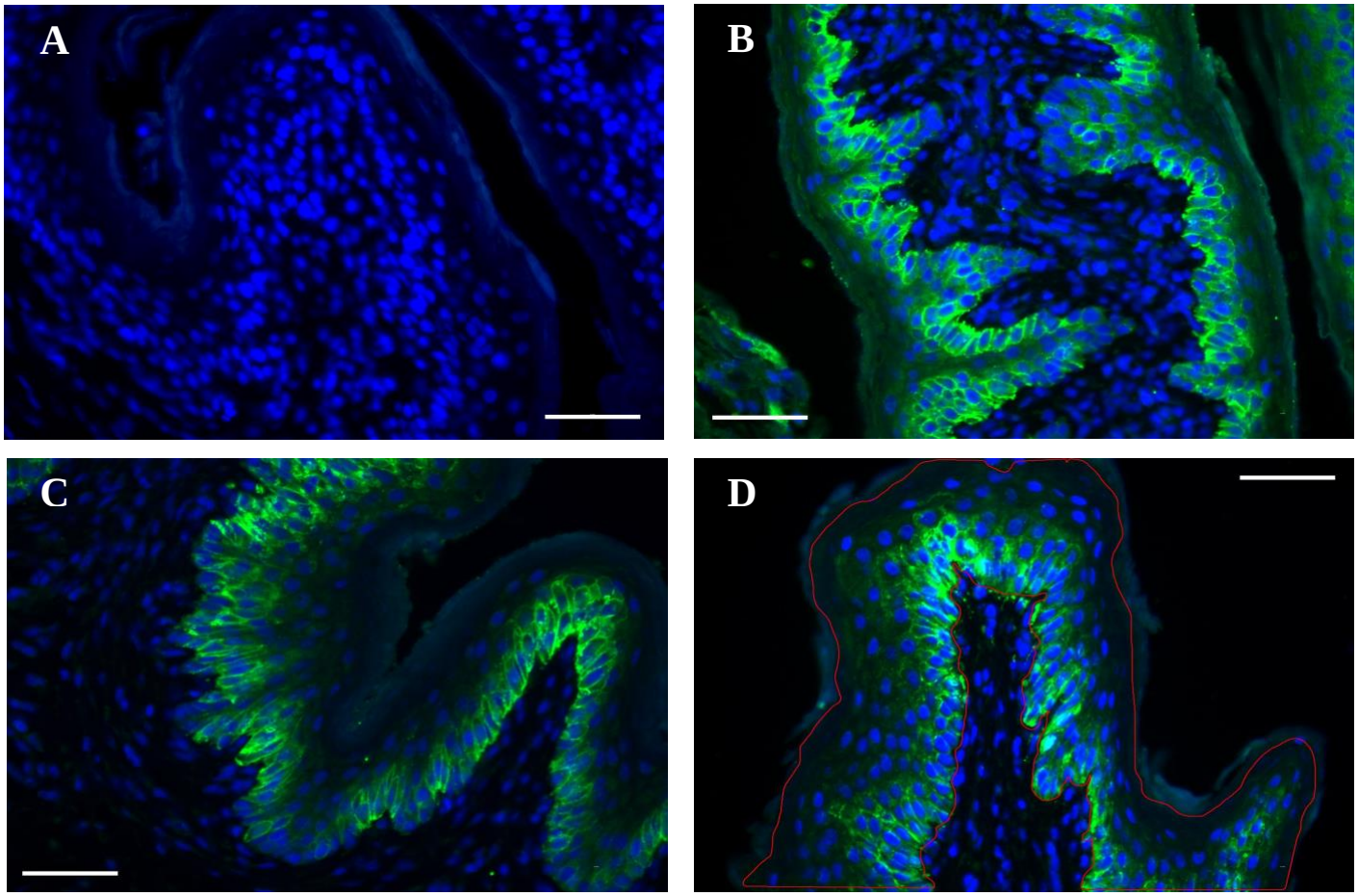

Figure 5.3. Representative immunohistochemical images showing the intensity of staining for MCT1 protein in the rumen of MRO calves (milk replacer only) and MRS calves (milk replacer and starter) during the 6 week trial. Green staining indicates MCT1 protein abundance with blue staining indicating nuclei. A) Negative control of MCT1, B) MRO staining of MCT1 protein, C) MRS staining of MCT1 protein, D) Image showing red outline of epithelial area used to calculate intensity per unit area that was used to assess level of protein abundance of MCT1 present in rumen epithelium. Scale bars represent 50 μm.

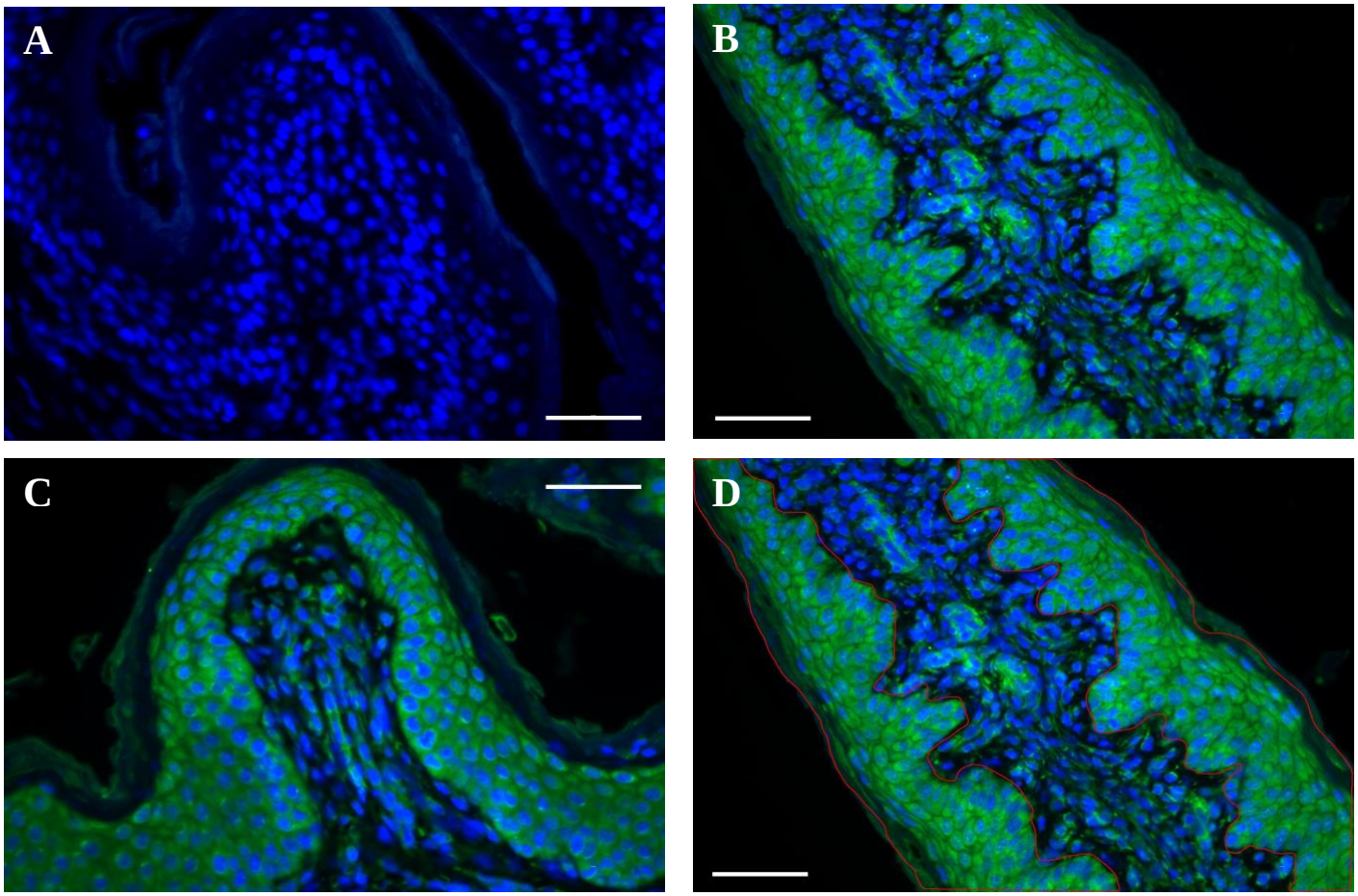

Figure 5.4. Representative immunohistochemical images showing the intensity of staining for MCT4 protein in the rumen of MRO calves (milk replacer only) and MRS calves (milk replacer and starter) during the 6 week trial. Green staining indicates MCT4 protein abundance with blue staining indicating nuclei. A) Negative control of MCT4, B) MRO staining of MCT4 protein, C) MRS staining of MCT4 protein, D) Image showing red outline of epithelial area used to calculate intensity per unit area that was used to assess level of protein abundance of MCT4 present in rumen epithelium. Scale bars represent 50 μm.

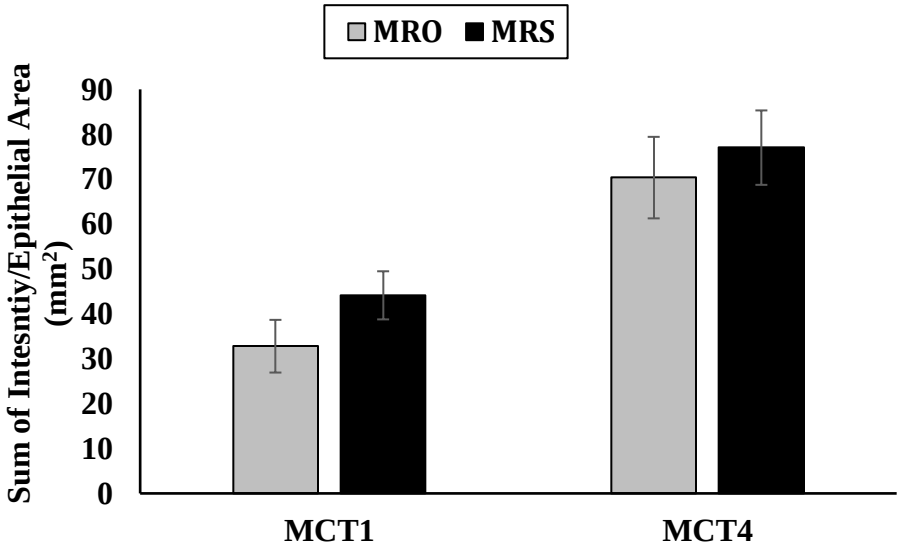

Figure 5.5. Mean (± SEM) immunohistochemical measurements of intensity/epithelial area for VFA transporter proteins MCT1 and MCT4 protein in the rumen of MRO calves (milk replacer only) and MRS calves (milk replacer and starter) during the 6 week trial. No differences were present for MCT1 ($P = 0.188$) or MCT4 ($P = 0.600$).

Chapter 6 : Effects of form of diet on putative stem and progenitor cells and proliferation in the rumen epithelium of preweaned calves

Abstract

Epidermal stem cell function is regulated by external cues from the environment and replenishing the epidermal cell population is initiated in the basale layer of its stratified epithelium. Like the epidermis, ruminal stratified epithelium responds to external stimuli, such as diet. The objectives of this study were to assess the effects of two different feeding programs on abundance of potential stem and progenitor cell populations and proliferation of rumen epidermal cells in preweaned calves. Neonatal Holstein bull calves (n = 12) were individually housed and randomly assigned to one of two diets. Diets were isoenergetic (3.87 ± 0.06 MCal of ME/d) and isonitrogenous (0.17 ± 0.003 kg/d of apparent digestible protein). Diets were: milk replacer only (**MRO**; n = 6), or milk replacer with starter (**MRS**; n = 6). Milk replacer was 22% CP, 21.5% fat (DM basis) while the textured calf starter was 21.5% CP (DM basis). Water was available ad libitum. Feed and water intake were recorded daily. Putative stem and progenitor cells were labeled via administration of a thymidine analog (5-bromo-2`deoxyuridine; **BrdU;** 5 mg/kg BW in sterile saline) for five consecutive days and allowed a 25-d washout period. Calves were euthanized at 43 ± 1 day after a 6 hour exposure to a defined concentration of volatile fatty acids. Rumen tissue was obtained from the ventral sac and processed for fluorescent immunohistochemical analyses of BrdU (putative stem and progenitor cells) and Ki67 (cell proliferation). Data were analyzed using SAS 9.4. Body growth between treatment did not differ, but empty reticulorumens were heavier in MRS calves (MRS: 0.67 ± 0.04 kg; MRO: 0.39 ± 0.04

kg; $P = 0.001$). The percentage of label-retaining BrdU basale cells was higher in MRO compared to MRS calves (2.0 ± 0.3% vs. 0.3 ± 0.2%, respectively; $P = 0.001$). A higher percentage of basale cells undergoing proliferation was observed in MRS than MRO calves (18.4 ± 2.6% vs. 10.8 ± 2.8%, respectively; $P = 0.079$). These results indicate that presumptive stem and progenitor cells within the rumen epidermis are identifiable by their ability to retain labeled DNA long-term and change proliferative status in response to diet.

Key words: dairy, calf nutrition, ruminant physiology

Introduction

Between 63 and 80% of metabolizable energy (**ME**) is derived from volatile fatty acids **(VFA**) in mature ruminants (Siciliano-Jones and Murphy, 1989; Bergman, 1990). Certain VFA, mainly butyrate, are metabolized in the rumen epithelium and presumably contribute to its growth (Sakata and Tamate, 1978; Gorka et al., 2009; Malhi et al., 2013). Clearly, the rumen epithelium plays a crucial role in VFA absorption. What remains to be discovered is how the rumen epithelium grows and replenishes its absorptive surface and how diet affects these processes as the calf begins to consume increasing amounts of dry feed.

In a similarly structured organ, skin, proliferation of cells within that stratified epithelia is initiated in the stratum basale layer and cellular differentiation occurs as cells physically move upward through the spinosum, granulosum, and corneum (Zouboulis et al., 2008). In skin, stem and progenitor cell function are both intrinsically regulated and responsive to external cues from the environment (Rando, 2006). Evidence of the rumen epithelium responding to external dietary stimuli was demonstrated by Goodlad (1981) who showed increased turnover rates of rumen epithelial cells in sheep fed a diet transitioning from forage to concentrate compared to forage based and concentrate based diets. From the results of Goodlad (1981) it can be speculated that diet may influence the ruminal stem and progenitor cell populations and, thus, alter the epithelium to be equipped for absorption and metabolism of available nutrients.

Previous work has investigated whether a population of putative stem cells reside in the rumen by looking at the gene expression of epidermal and small intestinal stem cell markers in the rumen epithelium of calves (Yohe et al., 2016). The genes examined were β1-integrin (***ITGB1***), keratin-14 (***KRT14***), notch-1 (***NOTCH1***), and tumor protein p63 (***TP63***) for their role in epidermal stem and progenitor cell homeostasis (Janes and Watt, 2006; Senoo et al., 2007;

Zhang et al., 2013; 2016) as well as leucine-rich repeat-containing G protein-coupled receptor 5 (***LGR5***) for its role in intestinal stem and progenitor cell homeostasis (Tan and Barker, 2014; 2015). Another potential marker for stem cells that was not examined in Yohe et al. (2016) is the immunophilin FK506-binding protein 51 (***FKBP51***), which has been shown to be present in stem cells due to its role in inhibiting differentiation (Chebotaev et al., 2007a,b).

Successful identification and quantification of rumen epithelial stem and progenitor cells, to our knowledge, has not been achieved. In other organs and tissues, putative stem cells have been identified by their ability to retain labeled DNA for extended periods of time (Gunduz, 1985; Langer et al., 1985). Often the thymidine analog, (5-bromo-2'-deoxyuridine; **BrdU**), is used for this (Bickenbach, 1981; Capuco et al., 2009; Daniels et al., 2009), which is incorporated into cells during the S-phase of the cell cycle (Potten et al., 2002). In this research our objectives were to utilize this label retaining technique with BrdU to investigate the effects of calfhood diets varying in composition (MR only vs. MR and starter) on: 1) putative rumen epithelial stem and progenitor cell abundance, and 2) rumen epithelial cell proliferation in preweaned calves receiving different forms of diet and subsequently challenged with a defined concentration of VFA.

Materials and Methods

Animals, treatments, and experiment

This experiment was approved by the Virginia Tech (**VT**) Institutional Animal Care and Use Committee (protocol #16-165) and conducted from February 2017 to May 2017. A more detailed description of all methods are present in the chapter 5, but relevant details are included herein. A total of 12 healthy Holstein bull calves were purchased from a single farm and transported approximately 240 km to VT. Within 12 hours of birth at the source farm, each calf

was fed a total of two 470 g bags of colostrum replacer (≥ 200 g IgG; Bovine IgG Land O Lakes Colostrum Replacement; Land O Lakes Animal Milk Products Co., Shoreview, MN) mixed in 3.79 L of hot water and the navel of each calf was dipped in an iodine tincture. Where applicable, subsequent feedings were of milk replacer (**MR**; Ledger, 22% CP as-fed, 20% fat as-fed; Southern States Cooperative Inc., Richmond, VA). Calves arrived to VT in one of two arrival periods (n = 6); period 1 calves were on site from February 17, 2017 to April 2, 2017. Period 2 calves were on site from April 11, 2017 to May 25, 2017.

Upon arrival to VT, a single jugular blood sample was collected for serum IgG determination (to assess passive transfer of immunity), calf body weight, hip height, and withers height were measured. After initial measurements were obtained, each calf was moved into an individual pen that contained a rubber mat and frequently laundered cotton towels for bedding. Pens were located in a temperature controlled room in the basement of Litton-Reaves Hall at VT where they remained for the duration of the experiment. Calves had visual and auditory contact with other calves but no physical contact.

Within 24 hours of arrival to VT, calves were balanced by age, body weight, serum IgG, and dam lactation number and split into two dietary treatments. The dietary treatments were: milk replacer only (**MRO**; n = 5) and milk replacer and calf starter (**MRS**; n = 6). A period 2 MRO calf died at 2 week of age from septicemia and was not replaced; its death was not treatment related. Both dietary treatment groups of calves were fed milk replacer (Ledger, 22% CP as-fed, 20% fat as-fed; Southern States Cooperative Inc.) twice daily at 0700 hours and 1900 hours and water was available ad libitum. The calf starter offered to MRS calves was an 18% CP (as-fed) textured feed (Southern States Cooperative Inc.). The MRO and MRS treatment diets were formulated to be isocaloric (based on ME) and isonitrogenous (based on apparent digestible

protein; **ADP**) (NRC, 2001) to negate any confounding factors of nutrition. A more detailed description of the diets is presented in the companion paper, but a brief description of diets follows. MRO calves were offered the same amount of MR throughout the entire duration of the trial whereas MRS calves were fed a diet that had decreasing amounts of MR throughout the trial to accommodate the planned increase in calf starter intake that maintained the necessary energy (formulated to be 4.0 Mcal/d of ME throughout the 6 week trial) and protein (formulated to be 0.198 kg/d of ADP on average throughout the 6 week trial) to meet isocaloric and isonitrogenous constraints. Nutrient composition of the diets is listed in **Table 6.1**.

Putative rumen epithelial stem and progenitor cell labelling

In other organs and tissues, putative stem cells have been identified by their ability to retain labeled DNA for extended periods of time. Often the thymidine analog, BrdU, is used (Bickenbach, 1981; Capuco et al., 2009; Daniels et al., 2009) in order to assess label retaining cells. Here, the technique was employed to label potential rumen stem and progenitor cells. For this, a sterile BrdU solution (20 mg BrdU powder/mL of 0.9% NaCl saline; BrdU powder: cat no. B5002, Sigma-Aldrich, St. Louis, MO) was administered intravenously in the jugular vein (5 mg of BrdU powder/kg of BW) once daily for five consecutive days (Daniels et al., 2009). Right and left jugular veins were alternated. The average infusion volume was 12.5 mL/d and each injection took approximately 1 min to administer. For each calf, the final injection was administered 25 days prior to slaughter, allowing for a 25 day washout period, timed from the final injection. The dose and washout period were determined in a pilot experiment conducted in 2015 (protocol #15-165); data not shown.

Analytical procedures of sampling/harvest day samples

Tissue collection and gross rumen measurements

At harvest, which occurred when calves were 43 ± 1 day old, samples were taken for rumen morphometric analyses according to our previously described methods (Yohe et al., 2015). Briefly, the full stomach and reticulorumen were weighed and then evacuated for empty reticulorumen weight determination. Rumen samples intended for gene expression and histological analyses were obtained from 4 locations in the rumen (ventral and dorsal samples from the cranial and caudal regions) using a punch biopsy tool with an internal diameter of 2.54 cm. Representative full-thickness rumen biopsy samples were also stored in 10% neutral buffered formalin overnight and then switched to 70% ethanol before being processed and readied for hematoxylin and eosin and immunohistochemical staining.

Real-time qPCR

The ventral sac region sample of the rumen was utilized to assess gene abundance of rumen epithelial transporters of interest via real-time quantitative PCR (**qPCR**). Forceps were used to peel the muscle layers away from the epithelium; the muscle-containing portion was discarded and the epithelial portion of each sample was flash frozen in liquid nitrogen and stored on dry ice until permanent storage in a -80°C freezer later that same day. Total RNA was extracted using the Qiagen RNeasy Plus Mini Kit (Qiagen; Valencia, CA). Approximately 30 mg of rumen epithelial tissue was placed into 600 μL of lysis buffer (10 μL of β-mercaptoethanol in every 1 mL of buffer RLT Plus) in a 14 mL Falcon tube (Fisher Scientific, Waltham, MA). Each sample was homogenized using a Bio-Gen PRO200 homogenizer (Pro Scientific, Oxford, CT) at its top speed for 30 s and placed on ice. After centrifugation at 20,817 × *g* for 3 min at 23°C supernatant was then placed in a genomic DNA eliminator column and centrifuged at 10,621 × *g*

for 30 s at 23°C. The flow-through was then processed using an RNeasy Plus mini kit. RNA purity and quantity were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies; Rockland, DE). For all 11 calves the 260/280 ratio averaged 2.11 ± 0.00, and the 260/230 ratio averaged 1.89 ± 0.13. Single-stranded cDNA was synthesized from each RNA sample according to the method of Yohe et al. (2016). For each sample, 2.0 μg of RNA were denatured in DNase/RNase free water for 12 min at 70°C and samples were then placed on wet ice. Reverse transcription was then performed, which consisted of a master mix (8.7 μL) containing: 4 μL of M-MLV RT 5X buffer (Promega; Madison, WI), 2 μL of 0.1 *M* dithiothreitol, 1 μL of 10 m*M* dNTP (Promega), 1 μL oligo(dT)20 primer, 0.5 μL M-MLV reverse transcriptase (Promega), and 0.2 μL of RNase inhibitor (Promega). The 8.7 μL of master mix was added to each well of denatured RNA (11.3 μL) in a 0.2 mL thin walled PCR 8-strip well tube (World Wide Medical Services Inc.; Tampa, FL) for final reaction volume of 20 μL. Reverse transcription was carried out in a Arktik Thermal Cycler (Thermo Scientific; Waltham, MA) with cycle conditions at 40°C for 1 h, followed by 95°C for 10 min.

Resultant cDNA was then diluted 1:1 with addition of 20 μL of DNase/RNase free water. Each qPCR assay was performed on all samples in triplicate, with each reaction mixture (10 μL) containing 0.25 μL of each forward and reverse primer, 4.75 μL of PowerUp SYBR Green Master Mix (Life Technologies; Grand Island, NY), 3.75 μL of DNase/RNase free water, and 1 μL of cDNA (1:1 stock). The qPCR assays were performed using a QuantStudio 6 Flex Real-Time PCR system (Life Technologies) with the following cycling conditions: 95 °C for 3 min; 45 repeating cycles of 94 °C for 15 s (denaturation), respective temperature (**Table 6.2**) for 30 s (annealing), and 72 °C for 30 s (extension); 95.0 °C for 2 min, 55.0 °C for 30 s, and then a temperature increase at 1.0 °C increment to 95.0 °C (melting curve). Each assay included a no-

template control and a no reverse-transcriptase control, with the no template control receiving 1 µL of RNase/DNase free water instead of cDNA, while the no reverse-transcriptase control received a 1 µL sample of the reverse transcription product to which no reverse-transcriptase was added. The qPCR was repeated when the resulting coefficient of variation of the replicates' cycle threshold (**Ct**) values was greater than 10%.

Primers were designed with Primer3 (v. 0.4.0; http://bioinfo.ut.ee/primer3-0.40/) (Rozen and Skaletsky, 2000) for all genes used. Validation of primers consisted of melting curve analyses that were performed after each qPCR assay to determine whether primer dimers or genomic DNA contamination were present during the assay. Target genes of interest were normalized to the geometric mean of *RPS9*, *RPS15*, and *RPS26* (Yohe et al., 2016) (target gene Ct – reference genes Ct = ΔCt) (Vandesompele et al., 2002).

Hematoxylin and eosin staining

Full-thickness rumen biopsy samples from the ventral sac region of each calf that had been formalin fixed and switched over to 70% ethanol for long-term holding underwent routine processing (Leica TP 1020; Leica Microsystems Inc., Buffalo Grove, IL), were embedded in paraffin blocks, cut to 5-µm thickness on a microtome (Model HM 340 E; Micron International GmbH, Germany), and mounted onto positively charged glass microscope slides (Yohe et al., 2015). Hematoxylin and eosin staining was completed using the procedure described by Tucker et al. (2016). Briefly, slides were deparaffinized in clear-rite (xylene substitute) (3 × 3 min) and hydrated through a descending graded series of ethanol washes (100%, 2 × 3 min; 95%, 2 × 3 min; 70%, 1 × 3 min) ending in distilled water (2 × 3 min). Hydrated sections were immersed in Gill's Hematoxylin 2 (2 min), rinsed in tap water, blued in Scott's Tap Water Substitute (30 s), and then rinsed in tap water. Slides were placed in 95% ethanol (30 s) then placed in Eosin Y

working solution (2 min 30 s). Stained slides were dehydrated to clear-rite in a series of graded ethanol solutions (95%, 2 × 10 s; 100%, 30 s and 1 min). Slides were cover slipped using the mounting medium Permount (Thermo Fisher Scientific, Suwanee, GA). Eight digital images were captured from each biopsy sample using an Olympus BX43 microscope (Olympus Corporation of the Americas, Center Valley, PA) fitted with a Retiga R6 camera (QImaging Corporation, Surrey, BC, Canada) with 4 images taken at 40X and 4 images taken at 4X. Rumen tissue Images were subsequently opened in Image-Pro Plus version 7.0 (Media Cybernetics, Inc.; Rockville, MD) where the thickness of the muscularis and submucosa layers were measured at 40X and the epithelium and corneum/keratin layers were measured at 4X using the line tool. Distances were recorded in micrometers (μm). Within each image, 50 linear measurements of each item were recorded by a single observer who was blinded to treatment.

Immunohistochemistry

The same protocol used to process and prepare rumen tissue for hematoxylin and eosin staining was used to prepare microscope slides for the beginning steps of immunohistochemistry protocols. Immunohistochemistry was utilized to quantify cells retaining BrdU label (i.e. putative stem and progenitor cells), and Ki67 label (i.e. proliferating cells). Immunohistochemistry was also used for preliminary screening of tissue antigens that might mark rumen epidermal stem and progenitor cells. Potential stem and progenitor cell markers utilized in this study were FKBP51, ITGB1, and NOTCH1. Immunohistochemical procedures were performed as previously reported (Daniels et al., 2009), with the following modifications. Microscope slides were deparaffinized in clear-rite (3 × 3 min) and hydrated through a descending graded series of ethanol washes (100%, 2 × 3 min; 95%, 2 × 3 min; 70%, 1 × 3 min) ending in distilled water (2 × 3 min). Antigens were retrieved by boiling slides in 10-m*M* citrate buffer, pH 6.0 for 30 min. Slides were

allowed to cool in citrate buffer (approx. 30 min) and then were washed in phosphate buffered saline (3 × 2 min). Individual tissue sections were divided with a PAP barrier pen (Ted Pella Inc., Redding, CA) to prevent commingling of antibodies. All tissue sections were then blocked with CAS Block (Life Technologies Corporation, Grand Island, NY) for 30 min. The CAS Block was aspirated, and 100 μL of the primary antibody solution was added per section. The primary antibody dilutions are listed in **Table 6.2**. For colocalization, BrdU and Ki67 antibodies were administered to tissue sections at the same time (Daniels et al., 2009). Immunohistochemistry for candidate stem and progenitor cell marker screening was done via colocalization of FKBP51 with BrdU; ITGB1 and NOTCH1 antibodies were applied individually to serial sections of tissue from each rumen biopsy mounted onto single microscope slides. In all cases, slides were incubated overnight at 4°C in a humidified chamber with designated primary antibodies. One section per slide served as a negative control, which consisted of CAS Block instead of the primary antibody solution. On day 2 of the protocol, primary antibody solution was removed by aspiration and slides were rinsed in phosphate buffered saline (3 × 5 min). All tissue sections then received 100 μL of the appropriate fluorescent secondary antibody solution (**Table 6.2**) and incubated in the dark for 60 min at room temperature. Excess liquid was removed by aspiration, and coverslips were mounted using Prolong Gold antifade reagent containing the nuclear stain DAPI (Life Technologies) and allowed to cure overnight in the dark. Seven digital images per slide were acquired at 40X magnification using a Nikon Eclipse E600 epi-fluoresence microscope fitted with a Nuance FX Multispectral Imaging System (Perkin-Elmer; Waltham, MA) where excitation light was generated using a mercury lamp light source and standard filter cubes fitted with long pass emission filters. Images were opened with and analyzed in Image-Pro Plus version 7.0 (Media Cybernetics). Within each image, cells positive for only BrdU, only

Ki67, and dual stained cells were manually counted by a single observer blinded to treatment and are reported as % of positive cells in the basale layer of the rumen epithelium (Daniels et al., 2009).

For candidate rumen stem or progenitor cell screening, the main interest was in determining if the candidate tissue antigens (FKBP51, ITGB1, and NOTCH1) were exclusively localized to the stratum basale and, further, colocalized with BrdU. Tissue antigens ITGB1 and NOTCH1 were dispersed through many epidermal strata; marker screening for these two antigens abruptly ended after this finding (see appendix for pictures). Initial screening of FKBP51 in calf earskin led to promising results, so follow-up immunohistochemical testing was performed wherein FKBP51 localization with respect to BrdU staining was examined in representative calf rumen samples (see appendix for pictures). BrdU and FKBP51 were not routinely co-localized in the stratum basale of the calf rumen (see appendix). Thus, for now, screening for potential rumen epidermal stem and progenitor cell markers (besides BrdU retention) has been discontinued in our laboratory. Some discussion will be reported in this chapter.

Statistical analysis

Intake, growth, qPCR, and histology data were analyzed using the MIXED procedure of SAS 9.4 (SAS Institute, Cary, NC). All models included the fixed effects of treatment, with repeated measures data (intake and growth) including week, and the interaction of treatment and week. Calf nested within treatment was the random effect in all models. Period was initially included in all models, but after discovery of insignificance it was discarded. All analyses included the best-fit covariance structure when appropriate; denominator degrees of freedom were not specified. Best-fit covariance structures were as follows: MR DMI, total DMI, ME

intake, ADP intake, and hip height measurements used autoregressive, voluntary water intake and total water intake used unstructured, body weight and withers height used toeplitz, and ADG used heterogeneous autoregressive. Significance was declared when $P \leq 0.05$. Orthogonal polynomial contrasts were performed when significance was found for the interaction of treatment and week or just the effect of week. Data for qPCR was not normally distributed (significant Shapiro-Wilk test) and were then log10 transformed and reanalyzed. Untransformed data are presented. Least squares means ± standard errors of the mean are reported. Significance was declared when $P \leq 0.05$

Results and Discussion

Intake and growth

The dietary treatments were formulated to be both isocaloric and isonitrogenous (NRC, 2001) to prevent any confounding factors influencing rumen growth and development. Intake and growth data are presented in more detail in chapter 5, but important results are discussed in brief. The nature of the diets resulted in differences for MR and starter DMI, but metabolizable energy (**ME**) and apparent digestible protein (**ADP**) did not differ for the interaction of treatment by week ($P = 0.778$ and $P = 0.520$, respectively) or by treatment alone ($P = 0.935$ and $P = 0.295$, respectively). This lack of difference in ME and ADP intake shows that the diets were indeed isocaloric and isonitrogenous, as designed. From a growth standpoint, the balance of energy and nitrogen in the diets was corroborated by BW not differing for the interaction of treatment by week or treatment alone ($P = 0.257$ and 0.414, respectively) and by similar ADG throughout the trial ($P = 0.902$, treatment × week; $P = 0.941$, treatment). It is difficult to find data that exactly replicates the diets fed in the current study, but based off of NRC (2001) data, calves consuming this amount of ME and ADP had BW gains within the expected range of 0.4 to 0.6 kg/d.

Real-time qPCR

MRO calves tended to have higher *LGR5* expression ($P = 0.062$; **Table 6.3**) than MRS. In the intestine, *LGR5* has been shown to be important for maintaining epithelial homeostasis via its role as a coreceptor in the Wnt signaling pathway (Tan and Barker, 2015). Having a trending increase in expression of *LGR5* may indicate an increase in stem cells present in the epithelial tissue sample analyzed with qPCR. Interestingly, *LGR5* was the only intestinal stem cell marker gene examined in the present study whereas the other genes were all relevant to skin epidermis. There was a lack of difference between treatments for the other genes examined in this experiment (*IGTB1*, *KRT14*, *NOTCH1*, and TP*63*). Despite this, in terms of importance for skin epidermis stem cell characteristics, *ITGB1* and *NOTCH1* have been shown to be important for adhesion to either the basement membrane (Jones and Watt, 1993; Janes and Watt, 2006) or to neighboring cells in the basale cell layer (Watt et al., 2008; Zhang et al., 2016). A lack of difference between treatments suggests no effect of nutrients in the rumen altering the adhesive nature of the rumen epithelium, when examined at the gene level on a tissue weight / tissue weight basis.

Both *NOTCH1* and *TP63* have been implicated for their roles in regulating proliferation (Watt et al., 2008; Zhang et al., 2016) and differentiation of progenitor cells (Yang et al., 2006; Senoo et al., 2007), which indicates neither treatment exhibited differences in proliferation or differentiation events in the putative stem and progenitor cell populations in the rumen epithelium, when examined at the gene level on a tissue weight / tissue weight basis.

Something worth consideration is that all results regarding gene expression are not isolated to potential stem and progenitor cells, but rather the entire rumen epithelium that was separated from the muscle. If only the stem cells were to be isolated there may be a different

story for the expression of these genes; one such method could be the use of cryopreserved tissue sections (Choudhary et al., 2010) and subsequent laser capture microdissection that would allow for identification of the putative stem cells (via BrdU labeling) and then subsequent qPCR analysis to look for gene expression of suspected stem cell markers (Steele et al., 2013). From a non-stem cell standpoint, it is apparent that both *ITGB1* and *KRT14* are the most highly expressed genes examined, which makes sense considering ITGB1 is an important protein for adhesion of basale cells to the basement membrane/extracellular matrix (Jones and Watt, 1993; Shen et al., 2017) and KRT14 is known to be one of the major keratin proteins found in the basale layer of skin epidermis important for cytoskeletal structure (Moll et al., 2008; Zhang et al., 2016).

Hematoxylin and eosin staining

Chemically staining the rumen tissue with hematoxylin and eosin allowed for measurement of the differing cellular layers throughout the rumen. Rumen muscle thickness did not differ between treatments ($P = 0.450$; **Figure 6.1**). Two potential explanations for this observation are offered. First, while others have noted that large feed particle sizes stimulate rumen muscle development (Harrison et al., 1960; Harding and Leek, 1972), perhaps the small average particle size of the starter feed we fed did not achieve this effect in MRS compared to MRO calves. The second, and more likely, potential explanation for lack of rumen muscle thickness differences between MRS and MRO is that, despite not being offered starter feed, MRO calves all had at least one matted hairball (bezoar) in their rumen at slaughter (data not shown). These hairballs likely formed through grooming and accumulated mass over time that prevented their passage to the omasum, effectively trapping them in the rumen. Their presence

likely contributed to at least some muscle development in MRO. In our experience, rumen bezoars are common in calves fed all milk diets that are bedded with inorganic bedding.

The other three cell thickness measurements were different between treatments with submucosa being thicker in MRO vs. MRS calves ($P = 0.050$), epithelium being thicker in MRS vs. MRO calves ($P = 0.016$), and corneum/keratin thickness being thicker in MRS vs. MRO calves ($P = 0.052$). The submucosal layer is comprised of connective tissue that includes extracellular components as well as vasculature for nutrient transport (Baldwin, 1998). It is unclear why this layer was thicker in MRO calves that had less nutrients in the rumen, but a potential explanation may involve invagination of the epithelium into the underlying submucosa during growth. In the ruminant forestomach this growth of the epithelial tissue "into" the submucosal tissue has been shown in the growing fetal omasum (Lubis and O'Shea, 1978), which may explain why the undeveloped epithelium in MRO calves had not yet invaded the submucosal layer, whereas the more developed epithelium in MRS calves had already done so.

An increase in epithelial layer thickness of MRS calves was expected due to the stimulating nature of feed in the rumen (Tamate et al., 1962; Sutton et al., 1963; Stobo et al., 1966). As opposed to bulk fill items such as hairballs/bezoars, feed that enters the rumen can be fermented to VFA, which can then be absorbed and metabolized by rumen epithelia. Also, this increase in epithelial thickness in MRS calves translated to increased corneum/keratin thickness in the rumen epithelium. Steele et al. (2011) noted no difference in thickness of the rumen corneum/keratin layer when mature cows were switched from a high forage to a high grain diet. Previous work has shown that a decrease in pH due to decreased forage in the diet of calves led to increased corneum thickness, which supports the protective role of the corneum/keratin layer of the rumen (Mirzaei et al., 2015). In this experiment, MRO calves had a higher rumen pH at

content evacuation compared to MRS calves (7.52 ± 0.11 vs. 5.78 ± 0.36, respectively; P = 0.001), which supports this corneum/keratin thickness relationship with rumen pH. Another possibility is that rumen contents of MRO calves (i.e. bezoars) added an abrasive element to the luminal cell layers decreasing keratin thickness that was absent in the starter fed to MRS calves.

Rumen immunohistochemistry

Images of rumen tissue stained for BrdU and Ki67 for both MRO and MRS treatments are shown in **Figures 6.2** and **6.3**, respectively. Also, **Figure 6.4** is a composite image depicting the method of counting and identifying the basale epithelial cells and the positively stained basale cells for BrdU and Ki67.

MRO calves had an increased proportion of BrdU labeled stratum basale cells per unit area compared to MRS calves (P = 0.001; **Table 6.5A**). This increase in proportion of basale cells that retained BrdU label indicates that, per unit area, there may be more putative stem or progenitor cells present in the rumens of calves with undeveloped rumens. To our knowledge, we have no other data to compare our findings to, however similar studies examining BrdU labeled cells in the bovine will be discussed. Previous results in calves showed no difference in proportion of BrdU label-retaining cells (30 day washout) in the mammary gland at 65 day of age when heifer calves were fed differing levels of energy and protein (Daniels et al., 2009). Soberon and Van Amburgh (2017) also compared diets differing in energy and protein fed to heifer calves and observed no difference in BrdU labeled cells in the mammary gland. Another explanation for this observation, which is similar to that posited by Soberon and Van Amburgh (2017), may be that there is no difference in percentage of BrdU labeled cells counted per unit area, but rather the larger MRS rumens (empty reticulorumen weight: MRO = 0.39 ± 0.04 kg and MRS = 0.68 ± 0.04 kg; P = 0.0004) would have essentially diluted the labeled cells in the MRS

calves, thus leading to total BrdU labeled potentially being the same between treatments. Alternatively, even though the washout period should have allowed for removal of these cells, it could be that BrdU label-retaining cells are just growth arrested cells and not true stem or progenitor cells (Daniels et al., 2009).

Proliferation status of the rumen epithelium, which was measured by Ki67 positive basale cells, indicated that MRS calves had a trending increase in proportion of basale cells labeled with Ki67 compared to MRO calves ($P = 0.080$; **Table 6.5B**). These Ki67 data are reflective of the tissue's proliferative status at slaughter, whereas the BrdU data reflect the 25 day washout effects. Goodlad (1981) demonstrated an increased turnover rate in the rumen epithelium of sheep fed a diet transitioning from forage to concentrate (4.3 ± 2.8 day turnover time) compared to forage based (16.5 ± 0.7 d) and concentrate based (10.9 ± 2.0 d) diets. Ohwada et al. (1984) utilized a metaphase-arresting agent in adult ewes fed a diet of orchard grass hay and concentrate to determine rumen epithelial time spent in mitosis, which was found to be 2.8 h. The data from Ohwada et al. (1984) did not allow for calculation of proliferation rate, but assuming a 24 hour cell cycle, provided a growth fraction (i.e. proliferating basale cells) of approximately 9%. This growth fraction agrees with the values obtained by Goodlad (1981) for forage fed ewes (8.95 ± 0.46 %) while being drastically lower than ewes transitioned from forage to concentrate diet (32.65 ± 9.79 %) and about half of that when ewes were fed just concentrate (17.25 ± 1.31 %). These results, paired with the present study, show that calves fed a diet containing starter (MRS) had a similar proliferative rate (18.37 ± 2.57 %) to ewes fed only concentrate (Goodlad, 1981). Interestingly, calves fed MR only had a similar proliferative rate (10.84 ± 2.81 %) to that of ewes fed only forage in the studies by Goodlad (1981) and Ohwada et al. (1984).

Attempting to identify a stem cell marker via immunohistochemistry did not prove successful for FKBP51, ITGB1, or NOTCH1 (**Appendix**). Gene expression data has shown FKBP51 to be upregulated in epidermal stem cells (Chebotaev et al., 2007a,b) while other studies have demonstrated FKBP51 immunostaining in the cytoplasm of certain cancer cells (e.g. oral squamous carcinoma) (Staibano et al., 2011; Russo et al., 2017). The cytoplasmic staining in the current study's images supports this pattern shown previously. The importance of FKBP51 in stem cells is believed to be due to its binding of glucocorticoid receptors (Chebotaev et al., 2007b), which is important due to the role of glucocorticoids in promoting differentiation while inhibiting proliferation (Rogatsky et al., 1999; Budunova et al., 2003); thus, allowing FKBP51 to effectively prevent glucocorticoids from initiating differentiation in the cell and maintain the cells stem-like status. The importance of ITGB1 and NOTCH1 are discussed earlier in the gene expression section, but immunostaining of each protein suggests similar relationships to the rumen epithelium. ITGB1 was present across all epithelial strata (data not shown), which suggests more than a basement membrane adhering role (Jones and Watt, 1993; Janes and Watt, 2006) or potentially some binding that is not strictly ITGB1. Hakkinen et al. (1998) demonstrated immunostaining of ITGB1 in the basal layer of human epidermis. The ITGB1 immunostaining in the current study did not follow this pattern, which may present a difference between skin epidermis and rumen epithelium, or potentially a methodological issue. Immunostaining of NOTCH1 also presented the same ubiquitous, cytoplasmic pattern as ITGB1, which had previously been shown in human and mouse skin (Abdou et al., 2012; Wang et al., 2017). Considering NOTCH1 is important in cell signaling pathways it is not surprising that it was present throughout the epithelium. Further investigation into how these proteins had been proposed as stem cell markers revealed while some localization has occurred via

immunohistochemistry, some results are based on flow cytometry (Jones and Watt, 1993; Fox et al., 2008), which does not always translate well to immunohistochemistry. A lack of colocalization of any of these proteins with BrdU suggests that they are not useful stem cell markers in the rumen epithelium.

Conclusions

The results from this study indicate that form of calfhood diet may play a role in the potential stem and progenitor cell populations present in the rumen epithelium. A trending increase in *LGR5* mRNA expression in MRO calves as well as an increased proportion of BrdU labeled basale cells, per unit area, compared to MRS calves, indicates a lack dietary response in the stem and progenitor cell populations when calves are fed starter along with MR. Also, MRS calves tended to have more rumen epidermal cell proliferation than MRO; this trend appeared to be reflected in the increased thickness and weight of the epithelial tissue layer. How these differences in the epithelial (i.e. absorptive) tissue layer affected function of the tissue are examined in chapter 5.

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Table 6.1. Chemical composition of milk replacer and textured starter fed to calves throughout trial

Chemical composition, % of DM	Milk Replacer[1]	Textured Calf Starter[2]
Dry matter %	96.2	85.8
CP	22.4	21.5
Fat (ether extract for starter)	21.5	3.95
NDF	--	19.0
ADF	--	9.3
Starch	--	27.7
Ash	9.49	9.36
ME (Mcal/kg)	4.74	3.09

[1]Milk replacer was purchased from Southern States, VA as a 22% CP, 20% fat powder (as-fed) that included milk protein and animal fat sources.
[2]Textured calf starter was purchased from Southern States, VA as an 18% CP (as-fed) feed that contained processed grains from plant protein sources.

Table 6.2. Real-time quantitative PCR (qPCR) and immunohistochemistry info for select genes and proteins

qPCR					
Gene Symbol[1]	Primer[2]	Primer (5'-3')	Temp[3]	bp[4]	GenBank Accession #
LGR5	Forward 813 Reverse 943	GATACCGGAGAAGGCCTTTGTA CGCCATTCAAAGTCAGTGTTCTT	62.3	131	NM_001192520.1
ITGB1	Forward 1057 Reverse 1204	TTCAGCCTGTTTACAAGGAACTGA GGCAATTTGCTGTTTTCCAAAATGA	60.5	148	NM_174368.3
KRT14	Forward 662 Reverse 806	ATCAAGGACTACAGCCCCTACTT CCGTCTCGTACTTGGTGCG	64.1	145	NM_001166575.1
NOTCH1	Forward 6183 Reverse 6306	GACCTGGGCAAGTCCGC CAGGAACAAGGGCGTCTCC	64.1	126	XM_010819254.1
TP63	Forward 1122 Reverse 1261	GAAGCGCCCTTTCCGTCA GGGATTCTTTGATCTTCAGCAGC	62.3	140	NM_001191337.1
RPS9 (Reference)	Forward 122 Reverse 229	GTGAGGTCTGGAGGGTCAAA GGGCATTACCTTCGAACAGA	62.4	108	BC148016.1
RPS15 (Reference)	Forward 183 Reverse 329	CAAGGCCAAGAAAGATGCGC TCAGGCTTGATTTCCACCTGG	62.4	147	NM_001024541.2
RPS26 (Reference)	Forward 219 Reverse 347	CTGCGTGAGTTGTGCCATTC TACATGGGCTTTGGTGGAGG	62.4	129	NM_001015561.2

Immunohistochemistry					
Protein antibody[5]	Host Species (Clonicity)	Tested/Target Reactivity	Dilution	Manufacturer	Catalog #
BrdU (Primary)	Chicken (Polyclonal)	Rat	1:3000	EMD Millipore	AB1286I
Ki67 (Primary)	Rabbit (Polyclonal)	Rat	1:250	EMD Millipore	AB3314P
FKBP51	Rabbit (Polyclonal)	Mouse, Rat, Hamster, Dog, Human	1:100	Abcam	Ab2901
ITGB1	Rabbit (Monoclonal)	Cow, Pig, Sheep, Horse, Human	1:100	EMD Millipore	MABT409

NOTCH1	Rabbit (Polyclonal)	Mouse, Rat, Human	1:100	Santa Cruz Biotechnology	Sc-9170
IgG (Secondary)	Goat	Anti Chicken	1:200	Life Technologies	A11042
IgG (Secondary)	Goat	Anti Rabbit	1:200	Life Technologies	A11037

[1]*LGR5* = leucine-rich repeat-containing G protein-coupled receptor 5, *ITGB1* = β1-integrin, *KRT14* = Keratin-14, *NOTCH1* = Notch-1, *TP63* = Tumor protein p63, and *RPS* = Ribosomal protein.
[2]Primer direction and hybridization position on the sequence.
[3]Annealing temperature, °C.
[4]Amplicon size in base pairs (bp).
[5] Protein antibody targets: BrdU = 5-bromo-2'-deoxyuridine, FKBP51 = FK506 binding protein 51 , and IgG = Immunoglobulin G.

Table 6.3. Relative mRNA abundance (expressed as $2^{-\Delta Ct}$ values; higher values equate to more mRNA) of selected genes in rumen epithelial tissue relevant to potential stem cell function in the rumen epithelium of calves fed two differing diets at 6 week of age

	Treatment[1]			Test of fixed effects, P-value[2]
Gene[3]	MRO	MRS	SEM[4]	Trt
LGR5	$9.49x10^{-6}$	$2.78x10^{-6}$	$2.05x10^{-6}$	0.062
ITGB1	0.021	0.017	0.0047	0.454
KRT14	0.15	0.22	0.05	0.325
NOTCH1	$1.5x10^{-5}$	$2.8x10^{-5}$	$1.2x10^{-5}$	0.927
TP63	0.0028	0.0029	0.0014	0.925

[1]MRO = MR only fed throughout the trial, MRS = MR with starter fed throughout the trial. Diets were formulated to be isocaloric and isonitrogenous.
[2]Significance declared when $P \leq 0.05$.
[3]*LGR5* = leucine-rich repeat-containing G protein-coupled receptor 5, *ITGB1* = β1-integrin, *KRT14* = Keratin-14, *NOTCH1* = Notch-1, and *TP63* = Tumor protein p63
[4]Standard error of the mean.

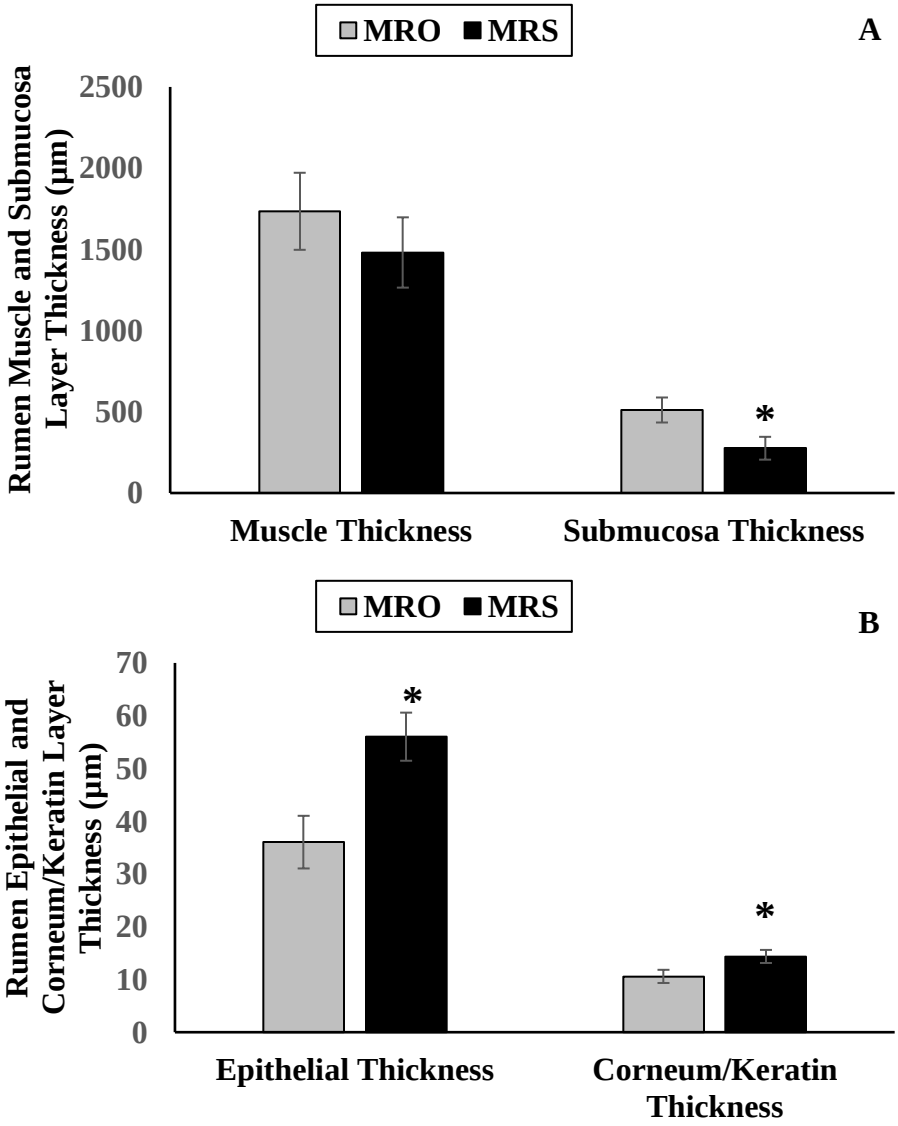

Figure 6.1. Mean (± SEM) hematoxylin and eosin staining measurements for rumen cell layers for calves fed two dietary treatments (MRO: milk replacer only; MRS: milk replacer and starter) during the 6 week trial. A) There was no difference between treatments for muscle layer thickness ($P = 0.450$), but there was an increase in submucosa layer thickness of MRO calves compared to MRS calves ($P = 0.050$). B) Epithelial layer and corneum/keratin layer thickness were increased in MRS calves compared to MRO calves ($P = 0.016$ and $P = 0.052$, respectively).

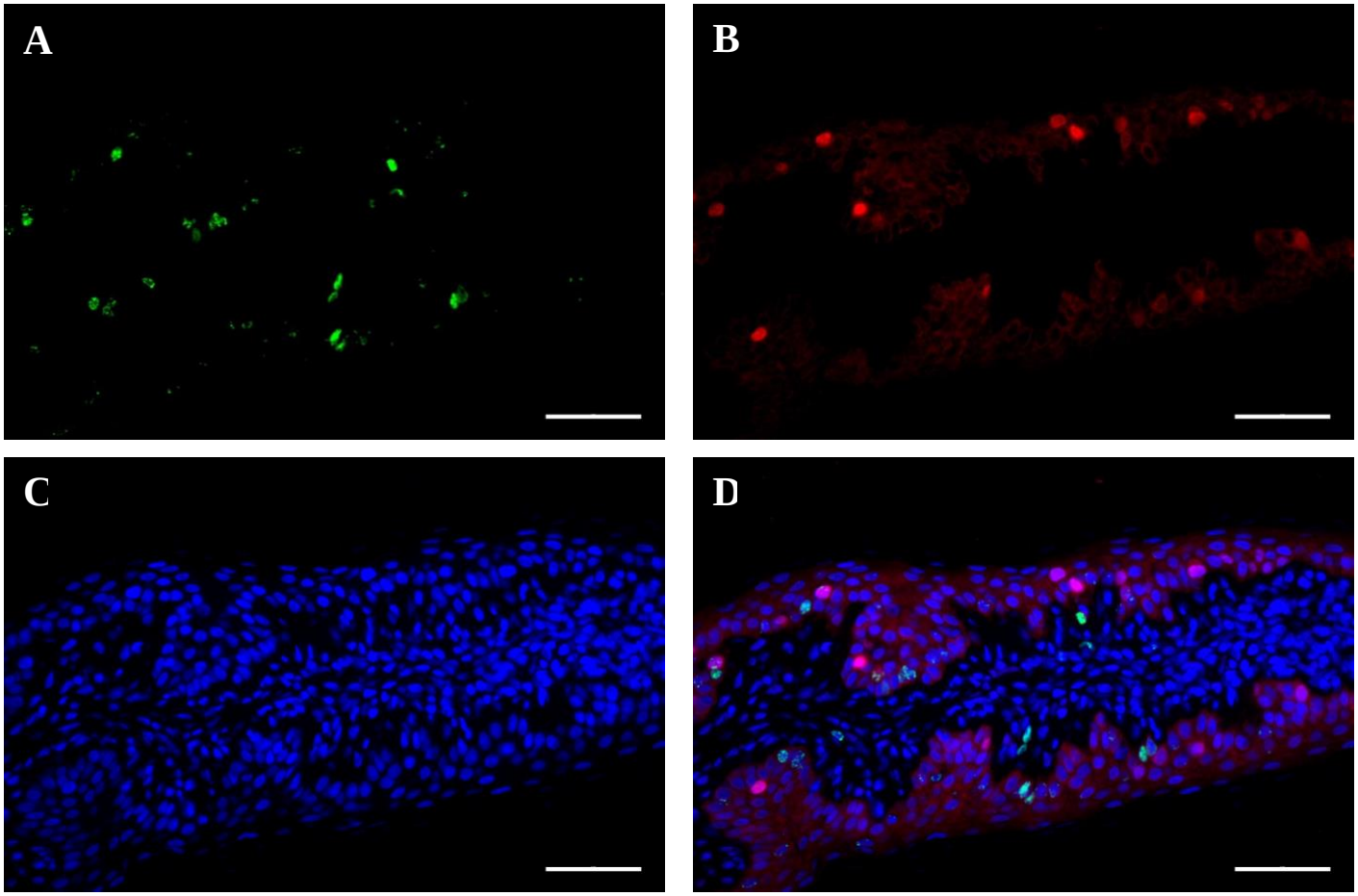

Figure 6.2. Representative immunohistochemical images of BrdU and Ki67 in the rumen of an MRO calf (MR only) during the 6 week trial. A) Green staining indicates a BrdU-positive cell, B) Red staining indicates a Ki67-positive cell, C) Blue staining indicates nuclei (DAPI), and D) Composite image of BrdU, Ki67, and DAPI showing location of staining within the rumen tissue. Images were taken at 40X and scale bars represent 50 μm.

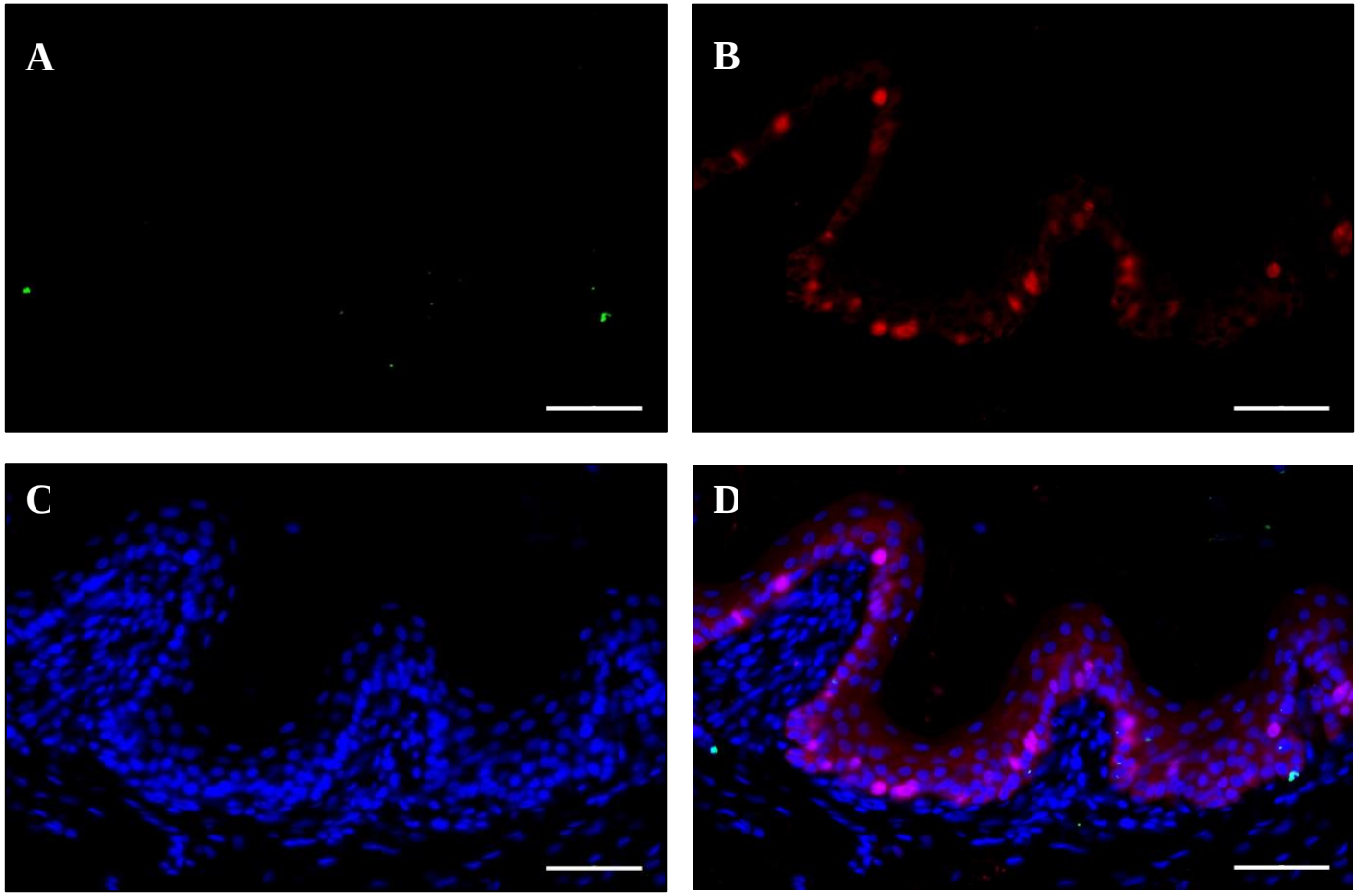

Figure 6.3. Representative immunohistochemical images of BrdU and Ki67 in the rumen of an MRS calf (MR and starter) during the 6 week trial. A) Green staining indicates a BrdU-positive cell, B) Red staining indicates a Ki67-positive cell, C) Blue staining indicates nuclei (DAPI), and D) Composite image of BrdU, Ki67, and DAPI showing location of staining within the rumen tissue. Images were taken at 40X and scale bars represent 50 μm.

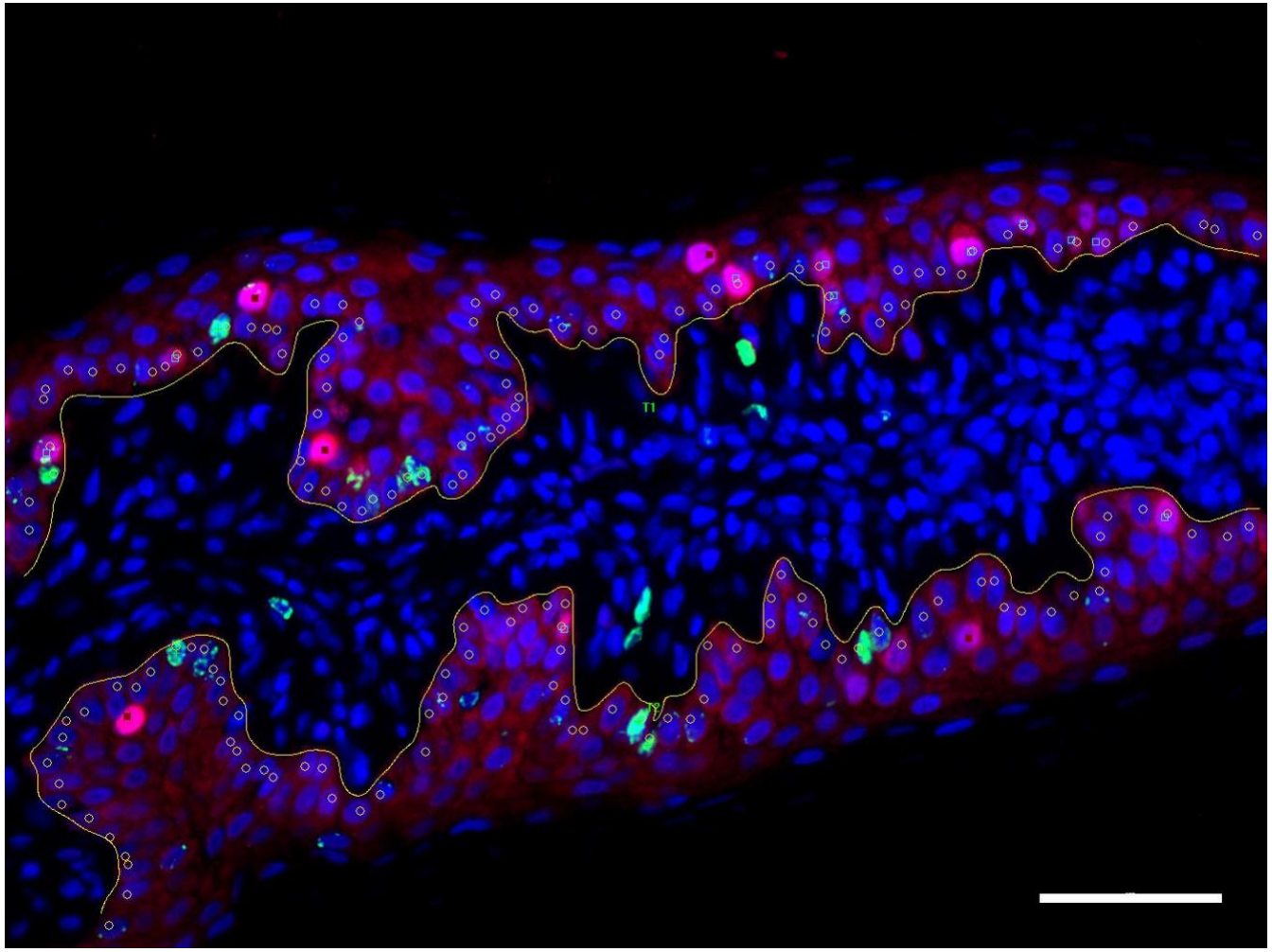

Figure 6.4. Representative immunohistochemical composite image of rumen tissue stained with BrdU, Ki67 and DAPI that has been analyzed for proportion of basale cells labeled with BrdU and Ki67. Image was taken at 40X and scale bar represents 50 μm.

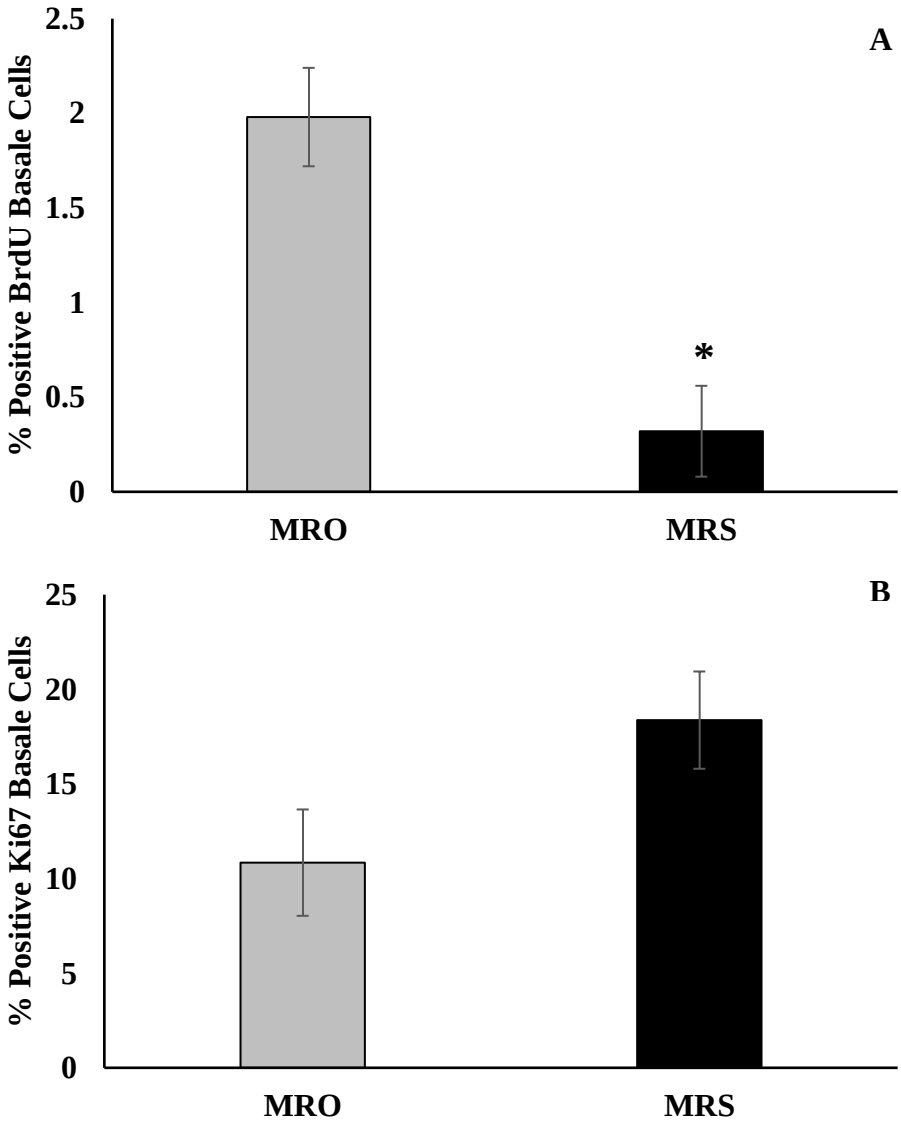

Figure 6.5. Mean (± SEM) immunohistochemistry measurements of BrdU and Ki67 positive basale cells in the rumen epithelium of calves fed two dietary treatments (MRO: milk replacer only; MRS: milk replacer and starter) during the 6 week trial. There was an increase in proportion of BrdU labeled basale cells in MRO calves compared with MRS calves ($P = 0.001$). For Ki67 there was a trending increase in Ki67 labeled basale cells in MRS calves compared to MRO calves ($P = 0.080$).

Chapter 7 : Final Remarks

There were many different areas explored during this body of work, but ultimately the main goal was to add to our ever-growing body of knowledge surrounding calf nutrition. Main takeaways of the dissertation research are briefly discussed.

It appears that when feeding calves a pelleted calf starter there is little effect of nutrient composition (namely starch and NDF) on rumen growth and development. It is fair to say that there may not have been large enough differences between the ratio of starch:NDF in the two diets tested, but it still seems that form of diet (e.g. textured starter vs. pelleted starter) may have a larger impact on rumen growth and development. This may be due in part to the similar processing that occurs to make pelleted diets, which essentially makes all nutrients readily available for microbial fermentation. Also, there is the possibility that digestion of starch may have a limit in the rumen of young calves considering the rate of starch digestion was the same in both diets fed even though there should have been a much higher rate of starch digestion in the diets containing high-starch.

A portion of this work focused on constructing and testing devices that can be used to evacuate, infuse, and sample the rumen of young preweaned calves fitted with rumen cannulae. This procedure was successful and has been reported so that future work concerning calf nutrition may utilize these devices.

The final calf trial had a major goal of assessing the ability of form of diet (MR only vs. MR and starter) to affect VFA absorption. The calves tested were exposed to a defined dose of VFA after a 6 week period, which resulted in no differences between either diet in terms of rate of absorption for acetate, propionate, or butyrate as well as total acetate, propionate, or butyrate absorbed throughout the 6 hour sampling period. Also, to support these data, there were no differences in abundance of VFA transporter protein (MCT1 and MCT4) between diets, which taken together suggests that diet does not necessarily play a critical role in preparing the rumen to absorb VFA. Put in another way, the rumen may have the innate ability to absorb VFA from a young age. That being said, it is important to consider that dry feed does stimulate growth of the rumen tissue, which will increase surface area of the rumen that may be important as the animal gets older and requires increasing amounts of energy for purposes like pregnancy and lactation.

The final calf trial also attempted to identify potential stem and progenitor cells in the rumen epithelium as well as assess proliferation of the rumen tissue when calves are fed either MR only or MR and starter. Interestingly, there was a difference with MR only calves having an increased number of cells that retained the putative stem cell marker (BrdU). On the flip side, calves fed MR and starter had a trending increase of cells that were actively proliferating, which supports the relationship between dry feed and rumen growth and development.

In conclusion, some of the data presented are fairly new to the area of calf nutrition and can be of use in future studies assessing how to more efficiently feed our calves.

Chapter 8 : Appendix

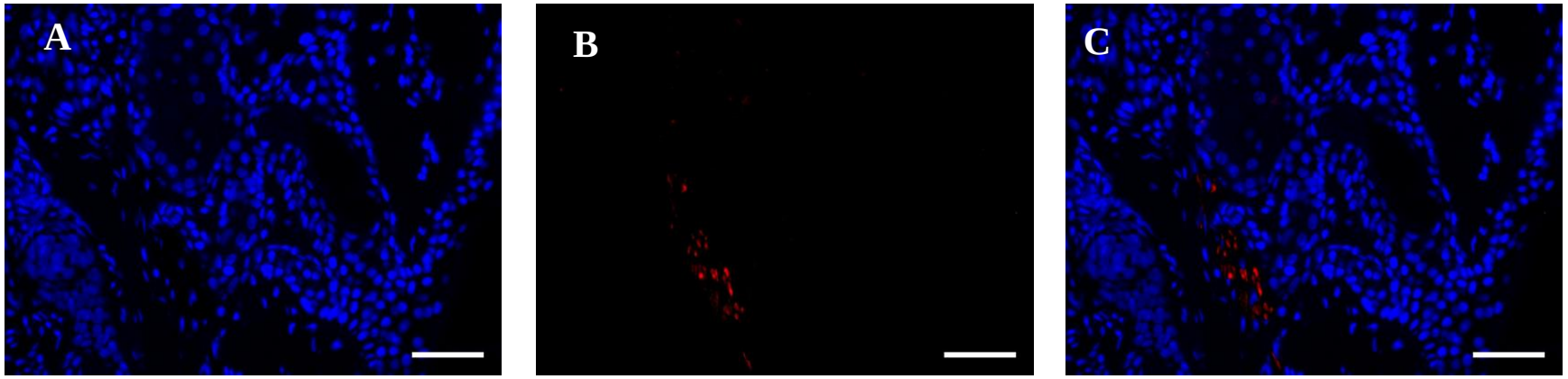

Figure 8.1. Calf ear skin stained with A) DAPI, B) FKBP51, and C) combined DAPI and FKBP51. Images were taken at 40X and scale bars represent 50 μm.

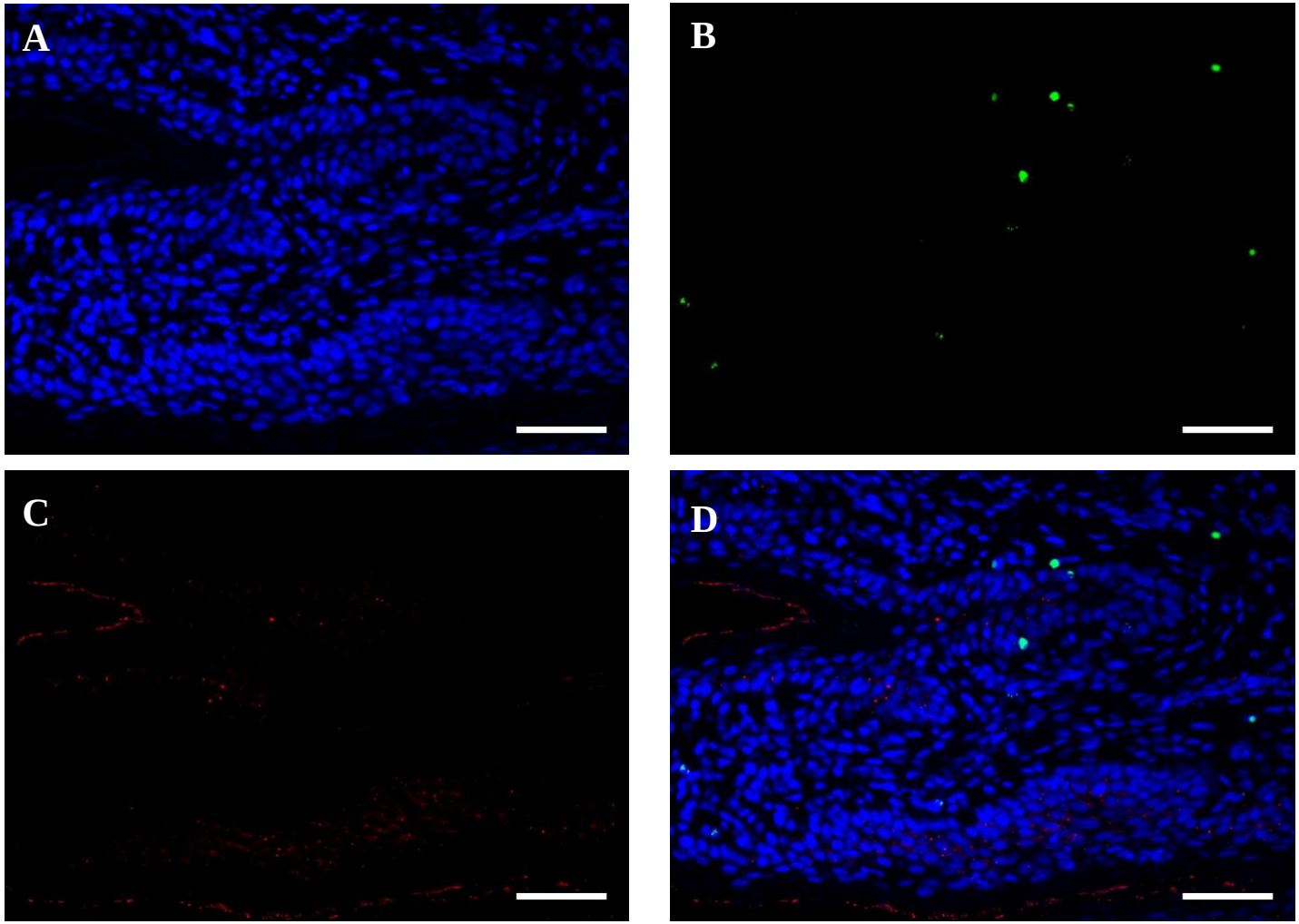

Figure 8.2. Immunohistochemical images of FKBP51 and BrdU immunostaining in an MRO calf. A) DAPI, B) BrdU, C) FKBP51, and D) composite image of DAPI, BrdU, and FKBP51. Images were taken at 40X and scale bars represent 50 μm.

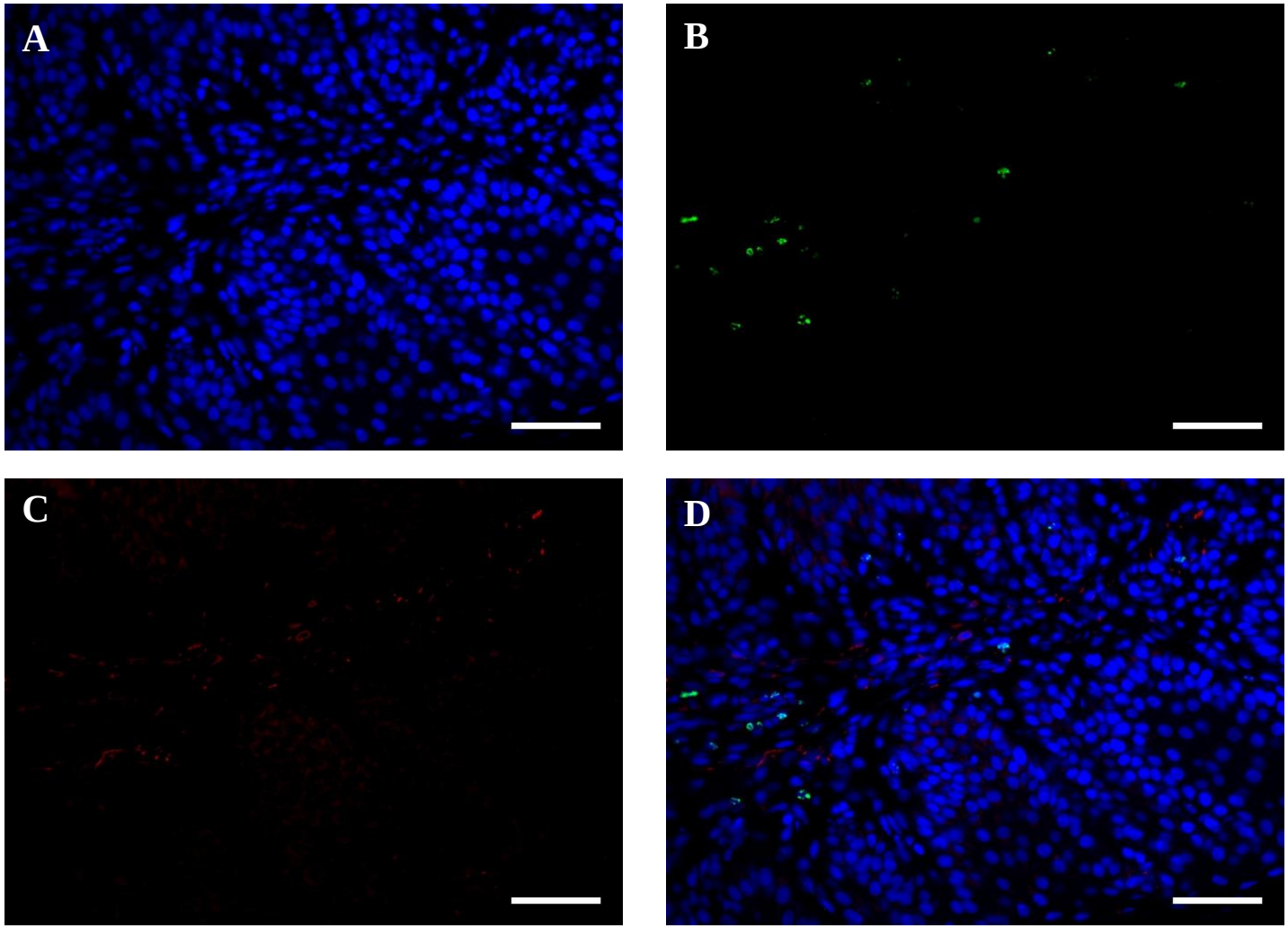

Figure 8.3. Immunohistochemical images of FKBP51 and BrdU immunostaining in an MRS calf. A) DAPI, B) BrdU, C) FKBP51, and D) composite image of DAPI, BrdU, and FKBP51. Images were taken at 40X and scale bars represent 50 μm.

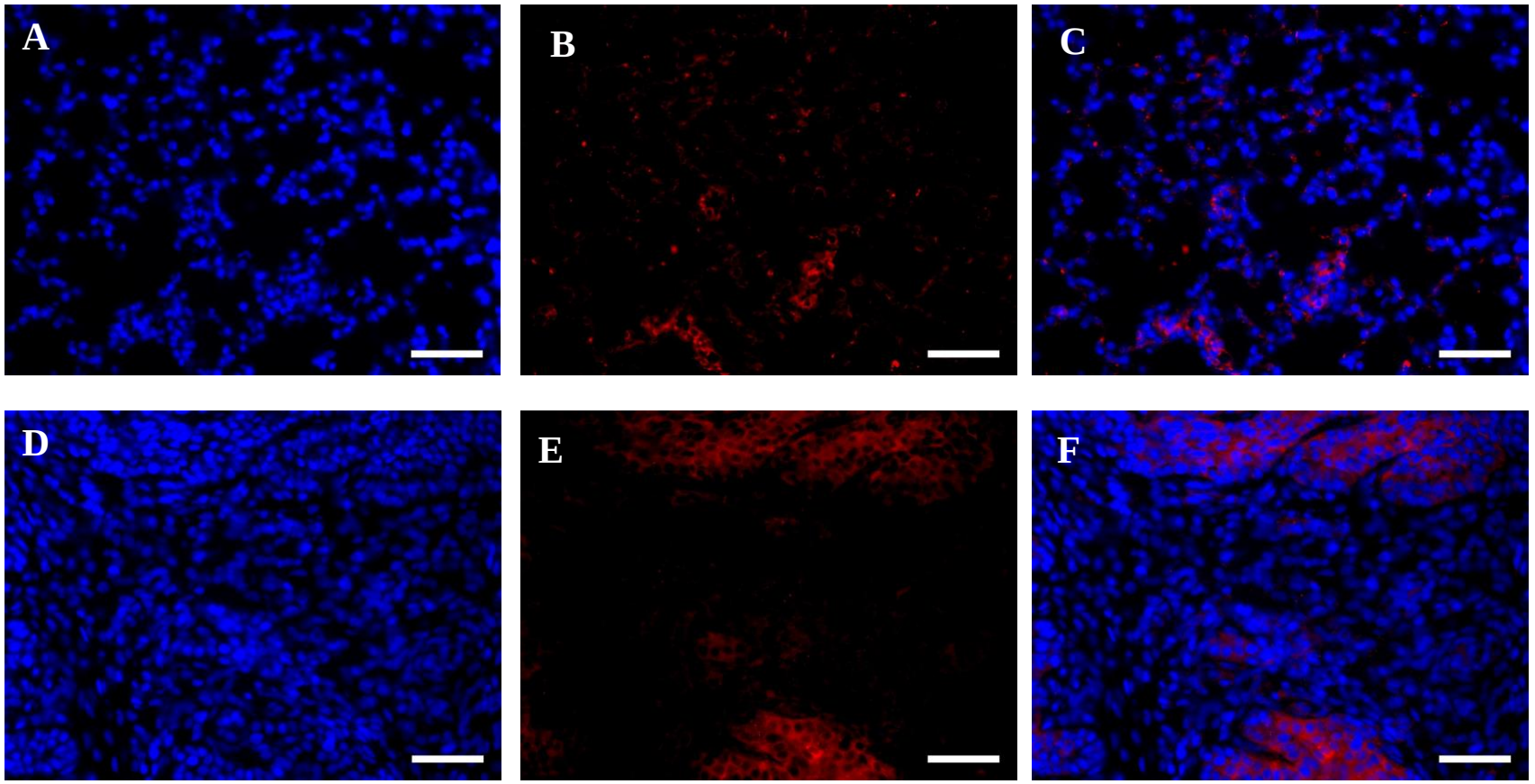

Figure 8.4. Immunohistochemical images of NOTCH1 in control tissue (lung; A-C) and rumen (D-F). Images were taken at 40X and scale bars represent 50 μm.

Table 8.1. Rumen epithelial gene symbol and name

Gene Symbol	Gene Name
MCT1	Monocarboxylate transporter 1
MCT2	Monocarboxylate transporter 2
MCT4	Monocarboxylate transporter 4
NHE1	Sodium-hydrogen exchanger member 1
NHE2	Sodium-hydrogen exchanger member 2
NHE3	Sodium-hydrogen exchanger member 3
ITGB1	Beta 1-integrin
KRT14	Keratin-14
LGR5	Leucine-rich repeat-containing G protein-coupled receptor 5
NOTCH1	Notch-1
TP63	Tumor protein p63
RPS9[1]	Ribosomal protein S9
RPS15[1]	Ribosomal protein S15
RPS26[1]	Ribosomal protein S26

[1]*RPS9*, *RPS15*, and *RPS26* were used as reference genes in qPCR analysis

Table 8.2. Real-time qPCR performance of genes measured in rumen epithelial tissue

Gene	Median Ct[1]	Median ΔCt[2]	Slope[3]	(R^2)[4]	Efficiency[5]
MCT1	20.52	4.29	-2.96	0.99	2.18
MCT2	26.51	10.72	-2.68	0.99	2.36
MCT4	31.10	15.15	-2.88	0.89	2.22
NHE1	28.16	11.73	-2.95	0.99	2.18
NHE2	23.57	7.65	-2.91	0.99	2.21
NHE3	29.17	13.05	-2.55	0.99	2.47
ITGB1	21.67	5.87	-3.08	0.99	2.11
KRT14	16.45	0.99	-3.38	0.99	1.97
LGR5	33.72	18.12	-2.11	0.99	2.97
NOTCH1	31.95	16.07	-2.82	0.99	2.27
TP63	23.65	7.97	-3.13	0.99	2.09
RPS9	16.20	--	-3.04	0.99	2.13
RPS15	15.11	--	-3.14	0.99	2.08
RPS26	15.11	--	-3.22	0.99	2.04

[1]The median Ct was calculated using all samples and all calves

[2]The median ΔCt was calculated as [Ct gene – geometric mean of *RPS9*, *RPS15*, and *RPS26*] for all samples and all calves.

[3]Slope of the standard curve; dilutions used were 1:1, 1:10, 1:100, and 1:1000.

[4]R^2 = coefficient of determination of the standard curve.

[5]Efficiency is calculated as [$10^{(-1/slope)}$].