The Effect of Supplemental Butyrate on Passive Transfer of Immunity and on VFA Transporter Abundance During the Weaning Transition

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Authorization to Submit Thesis

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Abstract

The acquisition of passive transfer of immunity and weaning transition are two events in early calfhood that are critical to calf health and growth. Without adequate passive transfer calves are more susceptible to disease for the first 6-8 weeks of life and without a successful weaning transition calves may experience weight loss and nutritional stress. Dietary inclusion of sodium butyrate may improve both passive transfer and the weaning transition. In experiment 1, twenty neonatal calves were assigned to either control (CTRL-C) or sodium butyrate supplementation (BUT-C). Butyrate supplementation reduced IgG absorption in BUT-C calves but did not affect average daily gain. It is not recommended to supplement calves with butyrate before the cessation of intestinal immunoglobulin absorption. In experiment 2, thirty-six Holstein bull calves were assigned to milk-only (PRE-M), calf starter and hay (PRE-S), calf starter and hay with a two-week weaning transition (POST-S), or calf starter and hay with a two-week weaning transition plus supplemental sodium butyrate (POST-B). Calves were harvested and rumen epithelial tissue was examined for VFA transporter abundance (MCT1) plus transporters involved in intracellular pH regulation (NHE3 and NBC1). MCT1 abundance was not affected by the addition of solid feed to the diet, weaning, or butyrate supplementation. NBC1 abundance increased with the dietary inclusion of solid feed, suggesting the developing rumen has the capacity to adapt to dietary changes. NHE3 abundance decreased during weaning, suggesting that the rumen epithelium acidifies itself through the weaning transition, though the physiological relevance of this phenomenon is unknown.

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Chapter 1: Literature Review

1.1 Calving Transition

On a dairy farm, heifer calves are the future of the milking herd. The mammary gland is fully formed but not fully developed at birth, and grows at the same rate as the rest of the body until about three months of age (Lohakare et al., 2012). From late gestation until weaning is a critical time of development during which nutrition and stress need to be managed, as growth of the mammary gland effects milk yield in the first lactation (Sejrsen, 1994). Passive transfer of immunity is also important for young calves, as they do not get immunoglobulin transfer from the dam prior to colostrum ingestion (Borghesi et al., 2014), and may not begin to produce their own antibodies in sufficient quantities until 8-10 weeks of life. Therefore, the time from late gestation to weaning is important for calf health, growth, and ultimately for their future contributions to the herd.

1.1.1 Protein Requirements/Energy Requirements

The transition period for dairy cows, defined as 3 weeks before and three weeks after parturition (Drackley, 1999) is a time of intense metabolic adaptation. The transition from a late-pregnancy non-lactating animal to a non-pregnant lactating animal requires increased energy, protein, and other nutrients. Dietary deficiencies can predispose an animal to a host of metabolic diseases that are commonly associated with parturition, including ketosis, retained placenta, displaced abomasum, dystocia, and milk fever. Thus, meeting dietary demands is essential to an effective transition period and a productive lactation cycle afterwards.

The industry standard management practice for late-pregnancy cows is to increase the energy in the ration approximately three weeks prior to parturition (Overton and Waldron, 2004). Late-pregnancy cattle require 75% more metabolizable energy than a non-pregnant non-lactating animal of the same size (Moe et al., 1971); energy requirements increase further upon the commencement of lactation. Since it is not possible to provide the total amount of post-partum energy needed via the diet with the high lactation volume of modern dairy cattle, cows will begin to

mobilize their triglyceride stores to meet the demands of lactation (Drackley, 1999, Roche et al., 2009). Most cattle enter some state of ketosis post-partum to provide energy for lactation, and thus regulation of energy input should be performed through the diet pre-partum. In addition to instating an increase in dietary energy, monitoring body condition scores (BCS) of cattle through the dry period is important for a successful transition. Cows that are overweight at parturition have more body fat to mobilize and are more susceptible to metabolic diseases associated with transition (Fronk et al., 1980, Roche et al., 2009). Thin cows experience a less dramatic negative energy balance (Holter et al., 1990) and thus, avoiding a dramatic increase in BCS during parturition may be beneficial through the transition period and into lactation. Energy management strategies are important for priming the gastrointestinal tract (GI tract) to successfully process a higher-energy post-partum diet while also reducing the risk of post-partum metabolic disorders.

Like energy requirements, protein requirements increase dramatically during late pregnancy and early lactation. In late gestation protein is required for fetus growth, but in early lactation protein is required to commence and sustain milk production. By early lactation, protein requirements of the mammary gland alone are around 83% of the dietary protein intake. As with energy, lactation requirements are prioritized, leaving little extra protein for maintenance requirements to be met (Drackley, 1999). Insufficient protein allotment during the transition period results in a decrease in microbial protein synthesis, fiber digestibility, and feed intake (Schwab and Broderick, 2017). While overall protein requirements increase during the transition period, lysine, methionine, and histidine are often found to be limiting in the rations of dairy cows no matter what physiological state they are in (Overton and Waldron, 2004; Schwab and Broderick, 2017). To offset negative protein balance, protein can be fed in excess, but industry interest is leaning towards targeted amino acid supplementation rather than increasing dietary CP (Schwab and Broderick, 2017). Protein is an expensive additive, and thus targeted amino acid supplementation has shown benefits for reducing feed costs while improving protein efficiency and increasing production (Schwab and Broderick,

2017). Supplementation of lysine and methionine during early lactation has been shown to have positive effects on milk yield and milk protein yield (Socha et al., 2005), implying an increased need for protein during lactation. In contrast, pre-partum amino acid supplementation has shown little effect on milk yield (Socha et al., 2005). Lysine and methionine supplementation pre-partum may not have an effect on milk yield and components, but can reduce the risk for retained placenta and ketosis (Curtis et al., 1985). The success of amino acid supplementation both pre-partum and during lactation demonstrates a deficiency in dairy cattle diets at many physiological states.

Reduction of negative energy and negative protein balance during the transition period are key to minimize metabolic disorders associated with parturition and increasing milk yield in early lactation. Energy provision in the diet must be regulated starting pre-partum. Protein supplementation focusing on amino acid balance appears to be more efficient and cost effective than increasing total dietary CP to offset negative protein balance. The major challenge with the transition comes with the interaction of protein and energy requirements; both increase with the onset of lactation, and requirements must be met for successful colostrum production and milk production throughout the lactation period.

1.1.2 Colostrum Production

Colostrum is important in bovines (bos taurus) due to a lack of passive Ig transfer through the placental wall (Borghesi et al., 2014). Without pre-partum transfer of Ig, calves rely on post-partum IgG absorption as the sole source of immunity until endogenous production begins. Colostrum components are produced during the last 2- 4 weeks of gestation (Delouis, 1978; Brandon et al., 1971), and colostrum is considered to be of good quality if it has greater than 50 g/L of IgG and <100,000 cfu/mL on a total plate count (McGrath et al., 2016). Despite its importance, up to 60% of colostrum produced in the U.S. does not meet minimum standards for cleanliness and immunoglobulin content (McGrath et al, 2016; Cabral et al., 2013). Quality and quantity can be affected by average temperature and weather (Cabral et al., 2016), milk yield during the previous

lactation (Cabral et al., 2016), parity (Mulder et al., 2017), breed (McGrath et al., 2016), time of colostrum harvest post-partum (McGrath et al., 2016), and energy balance during the dry period (Mulder et al., 2017). Understanding and improving colostrum quality is therefore crucial to ensuring calf health and survival.

Colostrum is milk that has a greater concentration of immunoglobulins; IgM and IgA are produced in the mammary gland, while IgG is produced outside of the mammary gland (Larson et al., 1980). Levels of immunoglobulin production in the mammary gland are unknown, as Ig transport from the blood makes this difficult to quantify (Porter, 1972, Larson et al., 1980, Baumrucker et al., 2010). Transfer of colostral components from the blood to the mammary gland are helped by an increase in mammary gland membrane permeability during late gestation (McGrath et al, 2016). Due to increased permeability, somatic cell count of colostrum is higher than that of milk, due to loosening of tight junctions during colostrum production (McGrath et al, 2016; Nguyen and Neville, 1998). Though IgG is the most abundant immunoglobulin, the actual mechanism of transport is unknown (Mayer et al, 2005, Larson et al., 1980, Baumrucker et al., 2010). IgG is shuttled to and absorbed by the mammary gland, likely via surface receptors, though other methods may exist (Brandon et al., 1971; Barrington et al., 2001; Mayer et al., 2005). Recent literature still has not defined IgG transport to the mammary gland, details of Ig production, or acquisition of other colostral components.

Developing a better understanding of how colostrum is produced in the dam will help to optimize its production. Improved colostrum quality via increased Ig content could be beneficial to calves and may reduce incidence of failure of passive transfer (FPT). Although calf absorption mechanisms also play a role in passive transfer status, increasing the concentration of Ig will be beneficial during the short calf absorption window. Many factors play a role in determining colostrum quality and quantity produced by a dam, and most of those factors can be controlled through proper nutrition, management, and an effective pre-partum vaccine program. More research

into how pre-partum nutrition effects colostrum production would be beneficial for replacement heifer management strategies.

1.1.3 Consequences of Failure of Passive Transfer

The goal of any colostrum management program is to provide enough IgG to ensure passive transfer of immunity from dam to calf, which requires the administration of good quality colostrum. Good quality colostrum should have 50 g/L IgG or greater (Godden, 2008) and should be administered within 4 hours of life. Even if colostrum is of good quality, it can still be a source of bacterial contamination. High concentrations of bacteria in colostrum have been shown to reduce Ig uptake in calves, even with high concentrations of IgG (Godden, 2008; Cummins et al., 2017). The process of pasteurization reduces colostral bacterial counts and may improve the efficiency of absorption (Godden, 2008), but does also reduce IgG concentrations. Poor colostrum management can lead to a high percentage of calves with failure of passive transfer (FPT).

FPT occurs when serum IgG concentration are less than 10 mg/mL at 24 and 48 hours of life (Godden, 2008) or a serum total protein value of less than 6.0 (Donovan et al., 1986). Many factors have an effect on whether or not a calf will have adequate passive transfer, including the timing and method of colostrum feeding (Stott et al., 1979a; Weaver et al., 2000), plus the quantity and quality of colostrum fed (Chapman et al., 1986; Stott et al., 1979b). Despite advances in our understanding of passive transfer and methods to optimize absorption, rates of FPT in the U.S. have not changed significantly over the last twenty years, averaging between 20-40% of heifer calves diagnosed with FPT (Tyler et al., 1999; APHIS, 2007; Windeyer et al, 2014).

Calves diagnosed with FPT have been found to have lower body weights (Windeyer et al., 2014), ADG (Faber et al., 2005), higher veterinary costs (Faber et al., 2005; Raboisson et al., 2016), higher incidence of respiratory disease (Windeyer et al., 2014; Raboisson et al., 2016), and lower milk production for the first two lactations (Faber et al., 2005) than calves that had adequate passive transfer. Increased incidence of disease also increases incidence of mortality, with most disease-

related deaths being related to scours, respiratory problems, and FPT (McGuirk, 2007; Tyler et al., 1999; Raboisson et al., 2016). FPT accounts for 39% of all calf mortality (Tyler et al., 1999). Monitoring serum total protein values to determine if FPT has occurred is helpful to determine the relative levels of disease and mortality that should be expected for a group of calves.

1.2 Calf Management

1.2.1 Environment and Housing

Beyond colostrum, calf health is also affected by management and housing decisions, which will impact growth, health, and immunity of calves. Environmental pathogens increase risk of diarrhea and pneumonia. Diarrhea and other digestive problems cause more than half of pre-weaned heifer deaths, while respiratory problems account for nearly one fourth of pre-weaned heifer deaths and over half of heifer deaths post-weaning (NAHMS 2014). Controlling exposure to pathogens is essential to calf health because calves are immunoimmature for the first several weeks of life. Management of ventilation, temperature, type of bedding, and housing strategy are essential to controlling pathogen load in the environment of neonatal calves.

Traditionally, calves are housed individually until weaning to help prevent the spread of calfhood diseases (NAHMS, 2011). As of 2014, 92.9% and 74.1% of producers in the Western and Eastern U.S., respectively, housed calves individually. Providing solid barriers between calf hutches avoids nose-to-nose contact and is thought to reduce the spread of respiratory diseases (Lago et al., 2006), and thus, group housing may not be the best option for young calves. Another factor that may affect the success of group housing is the age range of calves that are housed together (Lorenz et al., 2011). Housing calves of similar ages together has been shown to improve calf health (Lorenz et al, 2011), while other studies have found negative associations with group housing and calf health (Place et al., 1998), and still other studies have found no consistent relationship the two (Costa et al., 2016). Group housing may be beneficial only when reared in a closed group of similarly aged calves with no individuals entering or leaving (Costa et al., 2016); keeping a closed group helps to prevent

the introduction of new pathogens and diseases. Individually housing calves has been the standard practice on the majority of U.S. farms, but properly managing group-housed calves may be an opportunity to reduce labor while allowing similar growth rates (NAHMS, 2011).

Poor ventilation in calf housing facilities has shown negative effects on respiratory disease rates (Nordlund, 2008; Lago et al., 2006; Lorenz et al., 2011; Costa et al., 2016; Windeyer et al., 2014; Urie et al., 2017) and association with increased airborne bacterial counts (Lago et al., 2006, Nordlund, 2008). Enclosed calf pens and poorly ventilated barns can create microenvironments where air is not properly circulated (Lago et al., 2006; Bates and Anderson, 1979), increasing the airborne bacterial count and increasing the chance that a calf will need to be treated for a respiratory disease (Windeyer et al., 2014; Urie et al., 2017). In addition to airborne bacteria, ammonia levels, high humidity, and dust may increase risk for respiratory disease in improperly ventilated facilities (Lorenz et al., 2011; Callan and Garry, 2002), especially during winter months where airflow is often limited to control temperature (Windeyer et al.,2014). Commonly, disease management for calves focuses on acquisition of immunity and maintenance of immune coverage. These data show that biosecurity measures and cleanliness should also be considered as means of disease prevention (Callan and Garry, 2002). Even with effective ventilation management, other environmental factors exert disease pressure.

Lastly, cleanliness of calf facilities is important for reducing disease pressures in early calfhood. Many of the pathogens that cause calf scours can be found in areas where the adult cows are housed and are potential sources of infection for calves; this particularly applies to maternity areas where cow and calf will inhabit the same space for a period of time (McGuirk, 2008). For this reason, calves are often housed separately from dams (NAHMS, 2011). Calf bedding is one main source of pathogens that the calf is exposed to, especially since calves spend a large amount of time lying down. Straw bedding, while an effective insulator during cold weather, is also associated with increased bacterial counts (Lago et al., 2006). Keeping calf bedding clean is essential to reducing

exposure to pathogens that could cause disease. Group housing can be effective and thus eliminating nose-to-nose contact is not necessary, but keeping calf housing as clean and dry as possible regardless of housing situation is key to disease prevention.

In general, reducing contact with pathogens is the most effective way to reduce disease transmission. Since most calfhood pathogens are environmental, keeping a closed system with optimal ventilation and sanitary procedures while optimizing temperature, bedding type, bedding depth, and housing type will give calves the best chance to avoid common calfhood diseases. Avoiding pathogens and disease is not 100% effective, however, and farmers must utilize both environmental manipulation and also effective passive immunity acquisition for optimum calf health and survival.

1.3 Calf Nutrition

1.3.1 Liquid Feed

Liquid feed makes up the majority of a pre-weaned calf's energy intake. Liquid feed is important because it bypasses the rumen via the esophageal groove, which is activated by the suckling reflex. Directly shuttling liquid feed into the abomasum is important because calves are born as non-ruminants and thus rely on the abomasum to start the digestion process. Management strategies must include decisions about feeding whole milk versus milk replacer, the amount of milk that is offered per day, fresh water intake, and, if feeding milk replacer, what percentage of fat and protein are necessary in the diet.

Plane of nutrition has a large impact on total calf starter consumption during the pre-weaning period. Decreasing milk availability may have negative effects on production later in life due to a decreased plane of nutrition (Khan et al., 2011a; Soberon et al., 2012), but may increase starter intake and thus rumen development during weaning (Kertz et al., 1979; Khan et al., 2011a). Calves that eat a greater amount of starter pre-weaning can have greater weight gains than lower intake

calves (Kertz et al., 1979; Jasper and Weary, 2002; Berends et al., 2012. Significant rumen development is seen when calves are supplemented with solid feed, including increased rumen weight and feed conversion ratio (Bernerds et al., 2012), but calves that are fed a greater allotment of milk replacer may have increased milk production during their first lactation (Soberon et al., 2012). A balance between providing adequate energy through liquid feed and encouraging intake of solid feed is ideal.

The use of whole milk or milk replacer to feed calves varies between facilities and is often influenced by the current milk price. Feeding waste whole milk can be cost effective, but only on farms feeding at least 23 calves per day (Godden et al., 2005; Heinrichs et al., 1995). Either option will provide adequate energy for growing calves; though whole milk generally contains higher levels of fat on a dry matter (DM) basis and milk replacer contains higher levels of lactose. Generally, calves that are fed milk replacer have lower rates of gain (Godden et al., 2005; Lee et al., 2009), lower weaning weights (Godden et al., 2005), and lower feed efficiency (Lee et al., 2009). Despite the potential reduction in growth rates, milk replacer may be a better option for some farms. When milk prices are high, milk replacer can be cost effective, and allows a greater portion of liquid feed to be offered to calves without using saleable milk; again, this is very dependent on size of the farm and number of calves being fed (Godden et al., 2005).

Traditionally, milk replacer contains 20% protein and 20% fat, as compared to a Holstein's whole milk at 27% protein and 26% fat on a DM basis (Kertz and Loften, 2013). In recent literature, feeding a more accelerated replacer (27-30% protein and 10-25% fat) has been shown to increase weight gain pre-weaning (Hill et al., 2007; Geiger et al., 2014; Kmicikewycz et al., 2013), increase frame size components (Geiger et al., 2014; Kmicikewycz et al., 2013), and increased circulating NEFA (Kmicikewycz et al., 2013). Increased milk replacer protein content above 20% promotes bodyweight gains (Hill et al., 2006). Increasing fat content in milk replacer above 20% could have negative effects on overall metabolizable energy intake due to a decrease in calf starter intake (Kertz

and Loften, 2013), though this could be confounded by other factors. A calf's energy, but not protein, requirements increase during winter months, making fat-fortified milk replacers useful to increase energy delivery without affecting protein content (Jaster et al., 1992; Kertz and Loften, 2013). Having both a summer and a winter milk replacer with varying fat levels may not be practical for some farms, and thus adding a fat supplement is also an option (Kertz and Loften, 2013).

While the nutritional value of milk or milk replacer does play a role in calf performance, growth, and solid feed intake, the amount of liquid feed that is offered to a calf is also important. Limit feeding of liquid feed is associated with increased starter intake pre-weaning (Quigley et al., 2006; Silva et al., 2018). Providing additional nutrients via increased milk replacer consumption has been shown to improve both BW gain and feed efficiency, but also to decrease calf starter intake (Quigley et al., 2006; Silva et al., 2018). While higher allotments of liquid feed can result in weight gain advantages pre-weaning (Rosenberger et al., 2016; Haisan et al., 2019), these advantages often disappear as calves approach weaning age (Haisan et al., 2019).

Though milk or milk replacer provides energy and water to a calf in the form of liquid feed, it is also important to discuss the effects of drinking water intake on calf performance and health. On average, producers wait until 17d of age to give free drinking water to dairy calves (Heinrichs et al., 1995; USDA NAMHS 2016; Wickramasinghe et al., 2018;). There is a high correlation between water intake and calf starter intake, with greater increases in both factors observed around weaning (Quigley et al, 2006; Kertz et al., 1984). It is important to note that the positive relationship between drinking water and starter intake increases when less milk, and therefore less metabolizable energy via liquid feed, is offered (Wickramasinghe et al., 2018). Overall, free choice water is beneficial and essential to optimal calf growth.

The type and amount of liquid feed offered to calves pre-weaning has a significant effect on their subsequent growth and production. Limit-feeding liquid feed may increase starter intake and

thus increase rumen development, as long as the limit that is set does not result in malnutrition. Feeding a larger quantity of liquid feed can result in better weight gains pre-weaning, but can cause a decrease in gain through the weaning transition most likely associated with decreased development in the rumen. Decreased development results in decreased metabolizable energy availability for calves during weaning and can subsequently effect post-weaning growth. Producers can combat this effect by utilizing methods to encourage solid feed intake in calves that receive high volumes of liquid feed or increased fat inclusion in their milk replacer.

1.3.2 Solid Feed

Calf starter and forage each play a role in rumen development, playing an important role in weight gain, feed efficiency, and rumen pH regulation in the pre-weaning calf. Solid feed intake is inversely proportional to the amount of liquid feed offered prior to weaning (Khan et al., 2011a), and thus often does not reach appreciable levels of intake until calves are two weeks of age (NRC, 2001; Jasper and Weary, 2002). Several nutritional and management factors have been found to increase starter intake in young calves, including replacing part of the liquid feed with solid feed (Berends et al., 2012), supplementation of soy milk (Ghorbani et al., 2007), and pair-housing calves from an early age (Costa et al., 2015).). Drastic increases in solid feed intake are seen with the initiation of weaning due to the removal of milk replacer meals (Jasper and Weary, 2002; Khan et al., 2011a). The age at weaning can have an effect on starter intake, especially in early weaned calves, likely due to decreased rumen development early on (Khan et al., 2011a).

There are many forms of solid feed that can be fed to calves. Overall, results on physical form of calf starter effect on intake and growth have been inconsistent, with some studies finding no effect on ADG or feed intake (Terré et al., 2015), and others seeing an increase in intake with texturized starter (Bach et al., 2007). Feeding a texturized calf starter instead of non-texturized starter does have a positive effect on rumen development (Terré et al., 2015; Khan et al., 2016) and thus may be more beneficial. If a non-texturized or pelleted starter must be fed, a low-NDF inclusion

may increase ADG over a high-NDF pellet (Terré et al., 2013). Non texturized starter may be more rapidly fermented, causing a drop in rumen pH, where texturized starter has a slower rate of starch fermentation (Suarez-Mena et al., 2016), though low rumen pH does not appear to negatively affect growth during weaning (Laarman et al., 2012).

In addition to calf starter, forage consumption prior to weaning is important for rumen development. Forage consumption has been shown to increase rumen development in calves that are fed a large quantity of milk (Khan et al., 2011b). Forage can have a positive effect on solid feed consumption if a calf is at risk for acidosis (Terré et al., 2013; Suarez-Mena et al., 2016); acidosis in calves can decrease DMI and can result in a particularly low rumen pH for young calves with little rumen development(Suarez et al., 2006). Forage consumption can help to raise the rumen pH via increased fiber digestion (Suarez et al., 2006; Laarman et al., 2012; Terré et al., 2015). When a pelleted starter is fed, forage consumption appears to be beneficial, while feeding a texturized calf starter does not necessitate forage supplementation (Suarez-Mena et al., 2016). Aside from buffering the rumen, forage consumption promotes physical rumen development and rumination behavior (Terré et al., 2013; Khan et al., 2016) and therefore may be beneficial regardless of the rumen pH or physical form of starter fed.

Offering both forage and calf starter pre-weaning may improve weight gain and feed efficiency when compared to calves only receiving starter (Coverdale et al., 2004). Again, this may be related to rumen development, regulation of rumen pH, and an increased capacity to produce and absorb VFAs. The benefits of solid feed intake pre-weaning, whether it be in the form of calf starter or forage, make solid feed supplementation a critical part of any weaning program. Without adequate rumen development pre-weaning, the transition to solid feed can be nutritionally stressful. A balance must be found between liquid feed offered and solid feed consumption to maximize both nutrient intake and fermentable carbohydrates for the developing rumen. While high doses of milk may increase bodyweight gains initially, the effects of weaning on an underdeveloped rumen are not well

understood. Both forage and calf starter supplementation have been recommended for pre-weaning calves due to their positive effects on gastrointestinal development, which is considered to be vital for a successful weaning transition (Gorka et al., 2018).

1.3.3 Weaning Transition

In the U.S. dairy industry weaning is usually carried out between 6-8 weeks of life (Anderson et al., 1987). This usually gives calves enough time to start consuming solid feed before they are weaned off of their liquid feed. Usually, weaning begins when a threshold of either age or solid feed intake is reached. In either case, weaning can cause calves to elicit stress behaviors, requiring careful management to ensure a successful weaning transition.

Calves weaned based on age are typically weaned at 6-8 weeks of age (USDA, 2014). Early weaning, generally at 4-6 weeks of age (Anderson et al., 1987; Kehoe et al., 2007), reduces calf milk costs and overall labor but must be managed to ensure early rumen development for successful weaning (Anderson et al., 1987; Kehoe et al., 2007). Early weaning with a highly palatable starter or increasing the plane of nutrition may increase rumen activity due to increased starter intake and thus rumen development (Anderson et al., 1987; Eckert et al., 2015). Late weaning, after 9 weeks of life, gives calves an increased amount of digestible energy via milk prior to weaning, which allows for increased growth (de Passille et al., 2012), but may not be cost-effective. In addition, keeping calves on a liquid diet for a longer period decreases total calf starter intake and may affect rumen development (Silva et al., 2018).

Some producers chose to wean calves based on their intake of solid feed, which should be appreciable by the second week of life (NRC 2001). Calves that begin weaning upon consumption of 700 g/d calf starter and complete weaning upon consumption of 2000 g/d have increased rumen papillae length compared to calves that were weaned at 12 weeks of age (Roth et al., 2009). Calves that begin weaning earlier using automated systems show greater growth rates and gains due to an overall greater digestible energy intake (de Passillé and Rushen, 2012). Use of an individualized

weaning program appears to reduce negative effects of weaning on energy intake and weight gain by catering to a calf's individual rate of solid feed intake (de Passillé and Rushen, 2016).

Weaning can be performed abruptly or can be performed gradually by decreasing the concentration of milk offered or decreasing the number of feedings. Gradual weaning can take several forms, including dilution of the milk meal to maintain quantity of liquid feed, or initiating a step-down program to remove portions of the milk meal one at a time (Khan et al., 2007). Nutritionally, abrupt weaning and gradual weaning both affect the amount of consumed digestible energy (de Passille et al., 2012). Removing any quantity of liquid feed from a calf's diet will encourage solid feed intake, regardless of weaning strategy (Anderson et al., 1987; Jasper et al., 2007; Khan et al., 2007; Sweeney et al., 2010) Gradual or step-down weaning of calves leads to a decrease in energy intake during the pre-weaning period, but does lead to a greater intake of calf starter (Khan et al., 2007; Sweeney et al, 2010). Abrupt weaning calves have greater weight gains through the pre-weaning period but may lose weight through the weaning transition due to low starter intake at the removal of the milk meal, and insufficient rumen development (Sweeney et el., 2010). Sweeney et al. (2010) suggest a moderate gradual weaning program over 10 days – this gives calves time to increase their starter intake but does not limit DE for an extended period.

Often calves show signs of behavioral stress when a milk meal is removed – they may vocalize more often, stand for longer periods of time, and engage in cross-sucking behavior (de Passille et al., 2012). Weaning calves via dilution of milk with water does not elicit the same behavioral responses as removing the entire milk meal (Jasper et al., 2007; Budzynska and Weary, 2008); this suggests that it is not just the removal of milk that causes stress behaviors, but the removal of the ability to suckle on a bottle. Weaning stress is caused by more than just nutritional factors – behavioral and routine changes, as well as housing and social changes, can induce stress as well.

A successful weaning program is one that reduces both nutritional and behavioral stress before, during, and after the weaning transition. Weaning gradually, providing a bottle to suckle on post-weaning, and limiting changes to housing or social structure may be beneficial to calves that experience behavioral stress. Solid feed intake is critical to a successful weaning transition due to its positive effects on rumen development; having calf starter available early on and weaning calves when they reach an intake threshold appear to be helpful additions to a weaning program, serving to reduce the decrease in energy intake upon the removal of the milk meal. Using threshold-based weaning ensures that the calf is ready to switch to consuming solid feed as the primary energy source, and also ensures that some rumen development has taken place. Regardless of weaning age or method, gastrointestinal development pre-weaning is the key to a successful weaning transition.

1.4 Gastrointestinal Development

1.4.1 Small Intestine and Ig Absorption

Adequate transfer of immunoglobulins from the dam to the calf is important due to the naïve immune system of the neonate and the lack of prenatal immunoglobulin (Ig) transfer (Weaver et al., 2000). For the first 48 hours of life the calf's gut is "leaky" and large molecules are able to pass non-selectively into the intestinal epithelium (Staley et al., 1972). Insufficient absorption of Ig, also termed failure of passive transfer (FPT), is a common problem on U.S. dairy farms, with up to 35% of heifer calves experiencing FPT (Weaver et al., 2000). Out of all of the causes of calf mortality, FPT is responsible for 39% of deaths (Tyler et al., 1999), making proper colostrum administration of importance to dairy producers. Several factors affect the absorption window and thus absorption capacity and efficiency, including the amount and quality of colostrum fed, along with the timing of colostrum ingestion.

The amount of IgG fed affects the rate and efficiency of absorption. Stott et al. (1979a) observed that the amount of colostrum fed did not affect the window of absorption for IgG, however, feeding large amounts of IgG past 12 hours of life still resulted in decreased circulating IgG levels,

indicating that the amount of IgG that can be absorbed decreases with age. Providing calves with good quality colostrum is important for adequate Ig absorption, but too much Ig can exceed a calf's pinocytotic capacity and result in a lower apparent efficiency of absorption (AEA; Besser et al., 1985). If not maximizing efficiency, feeding colostrum with a high concentration of IgG may beneficial and could override some of the negative effects of improper timing and too small of volume of colostrum fed (Jaster, 2005).

Several studies have assessed the absorption capacity of the neonatal small intestine for the first 24 hours of life. The main route of Ig absorption is pinocytosis, which occurs prior to intestinal epithelial cells maturation after 24 hours of life (Jochims et al., 1994). Additionally, it appears that maximal pinocytotic activity occurs at four hours after birth and thus, early feeding will yield the maximal Ig absorption capacity (Stott et al., 1979b; Weaver et al., 2000; Jasper et al., 2005). Closure is accelerated when the first colostrum feeding occurs (Stott et al., 1979a), but may be slowed when colostrum is high in protease inhibitors (Carlson et al, 1980).

The most important factors of Ig absorption are the quality of colostrum administered and the timing of the first colostrum feeding. Maximal pinocytotic activity for neonatal intestinal enterocytes is during the first four hours of life – past that, cells begin to mature and non-selective absorptive capacities are lost. Thus far, there has not been a way to extend the non-selectivity of the neonatal intestine to lengthen the window of absorption. Feeding high quality colostrum with a high concentration of IgG allows for the full pinocytotic capacity to be utilized and provides the best chance for a calf to obtain passive transfer.

1.4.2 Rumen Development

After closure of the small intestine, the next major gastrointestinal development goal is rumen development. At birth, the rumen is non-functional (Warner et al., 1956). The morphological development of the neonatal rumen begins in earnest when the calf starts to produce VFAs from fermentation of solid feed. Typically, young ruminants will not begin to consume significant

quantities of solid feed prior to two weeks of age (Anderson et al., 1987; Lane et al., 2000). Early production of VFA's is the critical window of rumen development (Remond et al., 1995).This development from a non-functioning organ to the main digestive chamber of the forestomach involves drastic changes in tissue, cell size and cell density, transporter abundance and nutrient absorptive capacity, and maturity of tissue barrier integrity to ensure optimal gut health.

Morphological rumen development depends upon physical and nutritional stimulation. The commencement of solid feed intake provides both of these factors and is critical for rumen development (Beharka et al., 1998; Lane et al., 2000; Khan et al., 2011a; Pazoki et al., 2017). Morphological changes do not necessitate increased metabolic capacity; in milk-only fed calves, some morphological changes are seen, but the rumen is not considered to be developed or ready for the weaning transition. With morphological development must come metabolic development for the calf to shift to obtaining energy from liquid feed to solid feed. Development encourages development; metabolic improvements will demand greater morphological changes as the young calf develops into a true ruminant.

In the milk-fed neonatal rumen, glucose is the primary energy source for tissues, mostly coming from absorption in the small intestine and transport through the bloodstream (Baldwin et al., 2004). When comparing a two week old neonatal ruminant to an adult ruminant, oxygen and glucose uptake both decrease significantly in the adult animal (Giesecke et al., 1979); only 3% of total energy absorbed by the rumen wall in an adult ruminant is in the form of glucose (Remond et al., 1995). Metabolic development is necessary to process the shift in energy substrates from glucose to VFA.

Rumen epithelial development leads to an increase in the utilization of butyrate as an energy source, though glucose is still utilized during the developmental transition (Giesecke et al., 1979). While the rumen epithelium is dependent on the consumption of solid feed for some aspects of

development, it appears that the breakdown of butyrate into other products occurs independently of rumen fermentation (Giesecke et al., 1979; Baldwin et al., 2004). Though the breakdown of butyrate occurs independently, it is likely that butyrate's effects on rumen fermentation and gene expression combine to increase development. Butyrate breakdown products, namely acetoacetate and D(-)-3-hydroxybutyrate, are produced by the rumen epithelium and increase in their rate of production between 4 and 10 weeks of age (Giesecke et al., 1979).

The rumen epithelium uses a portion of the nutrients that it transports for its own maintenance. The epithelium uses 18-30% of acetate, 30-70% of propionate and 74-90% of butyrate produced in the rumen (Remond et al., 1995; Bergman, 1990). VFAs likely contribute about 70% of caloric requirements of ruminants (Bergman, 1990); much of the ATP produced powers Na-K-ATPase pumps, which are critical for the transport of nutrients and the regulation of gradients and ion flow in the epithelium itself. Na-K-ATPase activity increased in the ruminal epithelium with a 75% concentrate diet fed in lambs (McLeod and Baldwin, 2000), likely due to an increased need to transport VFAs from fermentation. There is also a link between Na transport abundance and butyrate transport abundance (Sehested et al., 1999). In addition, feeding a diet with increased metabolizable energy increases rumen epithelial concentrations of DNA and RNA, though this is likely a factor of increased rumen weight and cellular hyperplasia (McLeod and Baldwin, 2000).

Increases in butyrate production and subsequent breakdown drives metabolic development in the rumen epithelium. VFAs supply energy to the rumen epithelium itself and to the animal as a whole. The shift from glucose oxidation to VFA oxidation marks a critical point in rumen metabolic development – the onset of solid feed intake, fermentation, VFA production, and then VFA absorption begin the process of transitioning the neonatal non-ruminant into a fully functional ruminant. Though the naïve rumen has some ability to process and transport VFAs, metabolic development likely increases transport capacity and facilitates the switch from glucose to VFA for energy substrates.

1.4.3 Rumen Physiology & Transport

One of the principal functions of the rumen epithelium is the transport of nutrients from the rumen to the bloodstream. Development of papillae increases surface area for absorption, which leads to an increased number of cells and transporters. Perhaps the most commonly discussed is the transport of volatile fatty acids that are used by the ruminant for energy.

Increased transport across the epithelium should allow for increased production of energy metabolites from the breakdown of VFAs. Butyrate transport across the ruminal epithelium is increased during periods of anoxia, but at the same time butyrate metabolism by the epithelium is decreased (Stevens and Stettler, 1966). Though increased transport into the epithelium should result in an increase in the breakdown of VFAs, anoxia may cause a build-up of VFAs within the epithelium (Stevens and Stettler, 1966). VFA transport also appears to increase with a relatively lower luminal pH of 6.4 (Stevens and Stettler, 1966), possibly due to an increase in undissociated VFA's creating a gradient across the epithelium. The gradient is not essential, as passive diffusion of VFAs accounts for a very small percentage of transport, with 90-99% of VFAs in the rumen existing in a dissociated state (AlZahal et al., 2007); dissociated VFAs are eligible for protein mediated transport (Laarman et al., 2013a). Butyrate supplementation can increase monocarboxylate transporter isoform 1 (MCT1) abundance in rumen epithelium, indicating a fluxuating VFA absorptive capacity (Graham et al., 2007; Laarman et al., 2013a). The extent of this fluctuating absorption is unknown and is a potential target for improvement.

Epithelial absorptive capacity is affected highly by both luminal and intracellular pH (Stevens and Stettler, 1966; Schurmann, 2014; Laarman et al., 2015; Dengler et al., 2015). VFA transport into the ruminal epithelium provides substrates for ketone production, and these metabolites are exported from the cytosol by transporters such as MCT1 (Dengler et al., 2015). MCT1 is considered to be a bottleneck for basal VFA transport (Dengler et al., 2015), and so the ketones also serve to acidify the cytosol. Transporters such as sodium/proton exchangers (NHE) and

sodium/bicarbonate co-transporters (NBC) help to expel protons from the cytosol (Laarman et al., 2013), but the regulation and flux of intracellular pH requires further study. A large portion of VFAs absorbed are utilized by the epithelium itself (Bergman, 1990), with a portion of the ATP produced going to power Na/K/ATPase pumps to maintain ion gradients within the epithelium and provide energy for cellular maintenance. Increasing the absorptive capacity may provide additional energy for the epithelium itself as well as additional metabolites to be used elsewhere in the ruminant animal. Long-term adaptation of the rumen epithelium appears to increase VFA absorptive capacity in adult cattle (Laarman et al., 2015), and may be regulated by physiologic conditions including intracellular and intraruminal pH. The effects of intracellular pH on overall gut health have yet to be examined.

Transport of metabolized VFA products out of the cytosol is critical to maintaining intracellular pH. Monocarboxylate transporters serve to acidify the cell when importing VFAs (Graham et al., 2007), and can also put alkalotic pressure on the cytosol when removing metabolized VFA products, plus free protons, to the blood (Muller et al., 2002; Laarman et al., 2013a). Monocarboxylate transporters may also play a role in the intracellular shuttling of VFAs and their metabolites (Dengler et al., 2014). The effects of this shuttling on intracellular pH regulation are unknown, but regulation of intracellular pH is of interest due to its effects on gut health. Acidification of the ruminal epithelium disrupts tight junction proteins which may play a role in epithelial remodeling (Laarman et al., 2015), a process that could result in changes in VFA transport capacity during times of dietary adaptation. Long term effects of dietary transitions and epithelial remodeling, especially in dairy cattle that undergo several dietary transitions per lactation, are unknown.

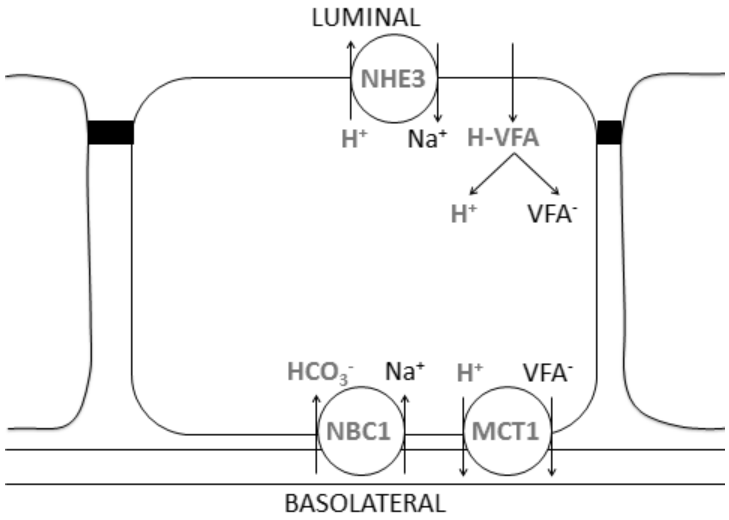

Figure 1.1 VFA transport and intracellular pH regulation in the rumen epithelium (Adapted from Laarman et al., 2013a)

1.4.4 Gut Health/Barrier Integrity

The epithelium of the rumen has many functions, one of which is to act as a barrier between ingested feed, pathogens, particles, and the bloodstream (Steele et al., 2009). Proper barrier function is a sign of a healthy gut – bacteria and other pathogens should not be able to penetrate past the epithelium. Tight junctions are essential for epithelial barrier function (Meissner et al., 2017); the permeability of the paracellular space is regulated by claudin proteins that are found within tight junctions (Günzel and Yu, 2013). The most apical tight junctions are called the zona occludens, and appear to be the site of paracellular permeability determination (Günzel and Yu, 2013.). Many types of stress can lead to dysfunction of the epithelial barrier and disruption of tight junctions and their associated proteins, including acidosis and hyperosmolarity.

Acidosis, a common feed-induced problem on both dairy and beef farms in the U.S., is a primary cause of epithelial barrier dysfunction in the gastrointestinal tract of ruminants. Sub-acute ruminal acidosis is defined as ruminal pH dropping below 5.6 for greater than 3 hours a day, or below 5.8 for more than 5 to 6 hours a day (Humer et al., 2018), which is often induced when producers try to increase the energy content of the diet to support increased milk production in

lactating cows (Abdela, 2016). Acidosis can negatively affect milk yield and milk fat percentage, and can cause a variety of gastrointestinal disorders (Abdela, 2016). It can even interfere with tight junction proteins in the rumen (Steele et al., 2011; Meissner et al., 2017), causing gaps to appear between cells of the epithelium (Steele et al., 2009), and making the rumen "leaky", sometimes in association with the presence of lipopolysaccharide (Emmanuel et al., 2007). A marked decrease in tight junction proteins is observed with low pH and VFA exposure, but not low pH alone (Meissner et al., 2017), indicating that the effects may be due to changes in intracellular pH but not ruminal pH (Laarman et al., 2015). Sloughing of the stratum corneum is seen with acidosis in adult cattle, even with short bouts of low pH, and may allow pathogens to invade the epithelium (Steele et al., 2009). Acidosis may have further reaching effects than impaired barrier function, even requiring an immune response within the rumen epithelium (Steele et al., 2009).

Hyperosmolarity, which can also be induced by acidosis, may be another key mechanism by which barrier function is impaired. Hypertonic solutions cause water to flow out of the cell and therefore decrease cell volume. The decrease in cell volume can increase the paracellular space allow molecules to bypass the tight junctions (Schweigel et el., 2005); osmolarity changes in the rumen epithelium have an effect on nutrient transport. In the abomasum, clotting of whole milk may lead to a decrease in abomasal pH, even though whole milk has a lower osmolarity (Sen et al., 2006. It is unclear how milk entering the rumen via an esophageal tube would affect rumen osmolarity; most of the milk should have been shuttled to the abomasum via the esophageal groove, but it has been shown that some leakage into the rumen does occur, sometimes up to 24% (Labussire et al., 2014). Hyperosmolarity increases passive permeability while also inhibiting Na/H exchangers such as NHE3 (Schweigel et el., 2005). The increase in passive permeability decreases intracellular pH which explains the inhibition of NHE3 (Schweigel et el., 2005). Without proper intracellular pH regulation, rumen epithelial cells can become acidic from the increase in protons when VFA's dissociate in the cytosol. This may create a gradient that would prevent passive diffusion of VFA's

into the epithelium. MCT1 removes protons from the cytosol and NBC1 brings in bicarbonate to help offset the acidic pressure; it has been suggested that observed down-regulation of NBC1 with butyrate supplementation may be beneficial if MCT1 is already removing protons to the bloodstream (Laarman et al., 2013a). Regulation of intracellular pH may play a role in VFA uptake and transporter abundance.

1.5 Strategies to Improve Calf Health and Development

The weaning transition is a time of stress for young calves. In order for calves to thrive during weaning and not experience a drop in ADG or the onset of disease, there must be optimum health and energy intake. Health and immunity can be improved by increasing passive transfer, and energy intake can be improved by increasing the intake of calf starter. Butyrate improves absorption of SCFA (Laarman et al., 2013a; Laarman et al., 2013b) and increases calf starter intake (Gorka et al., 2009; Gorka et al., 2011). Whether increased energy intake improves health, dry matter intake, rumen pH, and growth is unclear.

1.5.1 Passive Transfer of Immunity

Currently, only a limited number of strategies exist for improving passive transfer of immunity. Most discussions on improving passive transfer of immunity focus on increasing colostrum quality through a reduction of the colostrum bacterial load (Armengol and Fraile, 2016), providing adequate nutrition to the dam pre-partum (Kenyon et al., 2011), use of immunostimulants (Yilmaz and Kasikci, 2013), or using vitamin supplementation for immune support. For neonates, supplementation is aimed at improving IgG absorption. Increasing the absorption capacity for colostrum is of particular interest because when the absorptive capacity is met, increasing nutrient and immunoglobulin content does not increase absorption (Kühne et al., 2000). Selenium supplementation may be beneficial as it increases IgG absorption up to 42%, possibly through activation of pinocytotic mechanisms or interaction with the IgG molecule (Kamada et al., 2007). Sodium butyrate supplementation may also be beneficial for Ig absorption. Piglets from sows that

were supplemented with 1,000 ppm of sodium butyrate tended to have increased serum IgA concentrations relative to piglets from non-supplemented sows (Jang et al., 2017). Ig absorption is multifactorial, requiring further development of strategies to improve Ig production, colostrum Ig concentration, and absorptive capacity.

1.5.2 VFA Transport

Though morphological improvements to the rumen epithelium – i.e., increased surface area, papillae length and development, or stimulation of cell proliferation (Baldwin et al., 2004; Malhi et al., 2013; Górka et al., 2018) – may increase the area for VFA absorption, transport of VFAs cannot occur without designated and functional protein transporters.

Increasing the abundance of VFA transporters would allow a greater capacity to transport VFAs out of the lumen of the rumen and also out of the cell into the bloodstream. MCT1 abundance has been described as a "bottleneck" for VFA transport into the blood (Dengler et al., 2015) and presumably MCT4 is similar for transporting VFA's into the epithelium and also for intracellular relocation. Other transporters have been suggested as being involved with VFA transport, including PAT1 and DRA, both which have been described as Cl-/HCO3- exchangers. These can possibly transport VFA's in their anionic form though in the study by Dengler et al. (2015), only PAT1 was up-regulated in the presence of butyrate.

Butyrate infusion increases mRNA expression of MCT4, a VFA transporter in the rumen epithelium (Malhi et al., 2013). Similar results were seen in Ussing chamber experiments with sheep rumen, where MCT1 and MCT4 were up-regulated with a butyrate treatment (Dengler et al., 2015). In addition, increased abundance of MCT1 was observed in mid-lactation cows supplemented with butyrate (Laarman et al., 2013a). Many studies have shown the positive effects of supplemented sodium butyrate on rumen epithelial growth (Gorka et al., 2018). Butyrate supplementation in both young and adult ruminants should be considered for its stimulatory effects on rumen development and its up-regulatory effects on possible VFA transport.

1.6 Knowledge Gap

The absorption of IgG is critical for obtaining passive transfer of immunity in dairy calves (Godden, 2008). Without adequate passive transfer calves are susceptible to disease for the first several weeks of life and have a higher mortality rate pre-weaning (McGuirk, 2007; Tyler et al., 1999; Raboisson et al., 2016). Due to the lack of IgG transfer in utero and the naïve immune system of the calf at birth, any improvement to IgG absorption would be beneficial to the health and longevity of young calves.

Once calves produce antibodies on their own, the next major stressor is the weaning transition. Switching from a liquid diet to a solid feed diet requires physiological, metabolic, and behavioral changes. The challenge is developing a system of weaning that reduces stress while allowing for intake of solid feed, rumen development, adaptation, and continued weight gains. Increasing the intake of solid feed has the potential to promote rumen development, adaptation, and increase weight gains, but the effect of increased starter in the diet on rumen pH is unknown.

In this thesis, the objective is to use butyrate supplementation to improve IgG absorption and colostrum IgG production, along with improving the weaning transition via increased VFA transport capacity. Butyrate supplementation will improve both IgG absorption and colostrum IgG production, while increased calf starter intake from butyrate supplementation will positively affect VFA transport in the developing rumen.

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Chapter 2: Effect of Butyrate on Passive Transfer of Immunity in Dairy Calves

"Effect of Butyrate on Passive Transfer of Immunity in Dairy Calves" 2019. J. Dairy Sci. 102:4190-4197.

2.1 Abstract

The objectives of this study were to determine the effects of supplemental butyrate on: 1) immunoglobulin (Ig) production in dams, and 2) immunoglobulin absorption in their calves. Twenty dry dams fed a close-up TMR were assigned either a control treatment (CTRL-D), or a butyrate treatment where the close-up TMR was supplemented with butyrate at 1% DMI w/w (BUT-D). At calving, calves were assigned one of two treatments: a control group fed colostrum replacer only, and a butyrate group fed colostrum replacer with supplemental butyrate at 2.5% w/v. Serum immunoglobulin G (IgG), glucose, and β-hydroxybutyrate (BHB) were measured weekly in both dams and calves. Additionally, calves were weighed weekly to determine ADG. In dams, serum IgG concentration was not different between CTRL-D and BUT-D (1785 ± 117 vs. 1736 ± 137 mg/dL, respectively; $P = 0.79$), nor was there a change in immunoglobulin levels in the colostrum between control and butyrate. Serum total protein did not differ between CTRL-D and BUT-D dams. Dam DMI did not differ between CTRL-D and BUT-D, but did decrease one week before parturition. Compared with CTRL-C, BUT-C had significantly decreased serum IgG concentration at 24 h (2110 ± 124 vs. 1400 ± 115 mg/dL, $P < 0.01$), week 1 (1397 ± 121 vs. 866 ± 115mg/dL, $P = 0.03$), and week 2 (1310 ± 121 vs. 797 ± 115mg/dL, $P < 0.05$). Additionally, apparent efficiency of absorption (AEA) was lower for the BUT-C compared with the CTRL-C group (35.3 ± 2.1 vs. 25.9 ± 2.0, $P < 0.01$). Differences in serum immunoglobulin concentrations between CTRL-C and BUT-C groups did not affect average daily gain (0.59 ± 0.05 vs. 0.48 ± 0.05 kg/d, respectively; $P = 0.43$), serum glucose concentrations, or serum BHB concentrations. These data demonstrate that, while butyrate inclusion in colostrum negatively affects IgG absorption in newborn calves, calf body weight gains were unaffected.

2.2 Introduction

Managing and improving passive transfer of immunoglobulins (Ig) from dam to calf remains a key determinant in young animal health. Calves are born agammaglobulinemic (no circulating immunoglobulins), due to placental cotyledons that prevent *in utero* passage of maternal antibodies to the fetus (Borghesi et al., 2014). Consequently, calves' only opportunity for passive transfer of immunity occurs post-natally, through maternal colostrum. The Ig obtained via colostrum are the sole source of antibodies until calves start to produce their own Ig in sufficient quantities. Obtaining colostral Ig is, therefore, of utmost importance to calf health.

Without adequate absorption of colostral Ig into the bloodstream, calves experience failure of passive transfer of immunity (FPTI). Between 32-41% of calves experience FPTI, and this incidence has changed little in the last 20 years (Tyler et al., 1999, Beam et al., 2009, Windeyer et al. 2014). Currently, the threshold for FPTI is a blood IgG concentration below 10 mg/mL, or a serum total protein concentration below 5.2 – 5.5 g/dL at 24 - 48 hours of life (Moore, 2012, Windeyer et al., 2014). Promoting passive transfer of immunity, thereby preventing FPTI, is the underlying drive for feeding colostrum.

There are two main factors that determine the success or failure of passive transfer of immunity: the dam's ability to produce sufficient immunoglobulins and transport them into the colostrum, and the calf's ability to uptake those immunoglobulins into circulation (Weaver et al., 2000). Little is known about the actual method of production and transport of immunoglobulins into colostrum (Porter 1972; Jang et al., 2017), though some research points to FcRn receptors as a possible method of transport into the mammary gland (Cervanak and Kacskovics, 2009; Mayer et al., 2005). In calves, absorption of IgG is non-selective (Staley et al., 1972), therefore FcRn receptors are unlikely to play a role in IgG absorption. Furthermore, Ig absorption is time-dependent as absorption capacity declines rapidly after 12 hours of life and gut closure – the inability of the small intestine to absorb macromolecules – is complete around day one of life (Stott et al., 1979a). Increasing maternal

production of Ig and calf Ig absorption rates is key to improving passive transfer of immunity in calves.

Improving production and absorption of Ig may be possible through dietary butyrate supplementation. Supplemental butyrate has been shown to have positive effects on VFA absorption (Laarman et al., 2013a), epithelial energy availability (Bergman, 1990; Laarman et al., 2013b) and IgG concentration in porcine colostrum (Jang et al., 2017), highlighting butyrate's ability to increase transepithelial transport capacity. When pregnant sows are fed supplemental butyrate, piglets born from those sows have higher IgG concentrations (Fang et al., 2014). In young livestock, butyrate supplementation in liquid feed has been shown to have stimulatory effects on small intestine development in calves (Górka et al. 2018) and a tendency to increase circulating IgA concentrations in piglets (Jang et al., 2017).

Currently, little is known about the impact of butyrate on IgG production in cows, and subsequent effects on IgG absorption in calves. Therefore, the objectives of this study were to determine the effect of supplemental butyrate on: 1) IgG production in pregnant cows, and 2) IgG absorption in calves. We hypothesized that butyrate supplementation would increase colostral IgG concentrations in cows and increase Ig absorption in calves.

2.3 Materials and Methods

2.3.1 Animals, Feeding, and Treatments

All animal procedures were approved by the Institutional Animal Care and Use Committee (AUP 2017-33). All cattle were housed in a tie stall facility, and calves were individually housed in pens, at the Palouse Research, Extension, and Education Center (University of Idaho, Moscow, ID, USA). All animals had unlimited access to water.

Twenty multiparous, late-pregnancy Holstein cows were fed a dry, close-up cow TMR ad libitum. Dams were blocked by body weight and assigned to either control (CTRL-D; n=10) or butyrate (BUT-D; n=10) treatments (672 ± 80 vs. 694 ± 71 kg, respectively; P = 0.54). Using ten animals per treatment allows detection of 15% difference between treatments with 80% power when CV within treatment is 10% (Berndtson, 1991). Three weeks before expected calving date, dams in BUT-D received supplemental butyrate at a manufacturer-recommended rate of 1% of their DMI, as rumen-protected butyrate supplement (Ultramix C, Nutriad, Hampshire, IL, USA), while CTRL-D dams received no supplement. Treatments were top-dressed, to ensure all treatment was consumed, during morning feeding at 07h00 until calving. All cows were fed a close-up TMR twice daily (07h00 and 18h00) ad libitum with targeted orts of 5-10%. At calving, cows were removed from the study.

All calves, both heifers, received 200g of IgG in one gallon of colostrum replacer (Sav-a-calf, Milk Products LLC, Chilton, WI, USA) between one half hour to two hours after birth; all births were observed and normal. Calves were allowed to suckle and, if unwilling to finish the full gallon, were given the rest via an esophageal feeder. Only one calf suckled the entire gallon and did not need to be tubed via the esophageal feeder. Calves were assigned to CTRL-C and BUT-C groups, balancing for body weight at birth and dam treatment. Calves were not blocked by sex; distribution included five bulls and four heifers in BUT-C; six bulls and three heifers in CTRL-C. Treatments were assigned such that half of CTRL-C calves came from BUT-D dams and half came from CTRL-D dams, and half of BUT-C calves came from BUT-D dams and half came from CTRL-D dams. In one group, calves received a non-rumen protected butyrate supplement (Ultramix GF, Nutriad, Hampshire, IL, USA) in their colostrum at 2.5% w/v (BUT-C), while control calves did not receive supplement (CTRL-C). Butyrate dosage was based on a previous study (Laarman et al., 2013b), in which butyrate dosed at 2.5% of the DM was effective in increasing SCFA uptake capacity in lactating cows. All calves received whole milk and ad libitum starter until weaning.

2.3.2 Sample Collection and Analysis

Samples of TMR were taken three times per week, composited, and dried in a convection oven at 60°C for 48 hours to obtain dry matter values. Butyrate treatment calculations were then adjusted according to the adjusted DM of the TMR. Weekly, composite TMR samples were analyzed for nutrient composition (Cumberland Valley Analytical Services, Waynesboro, PA, USA).

For the dams, blood serum was collected weekly until calving, and within a half hour of parturition (Vacutainer, Becton, Dickinson and Co, Franklin Lakes, NJ, USA). Samples were centrifuged at 1693 × g for 20 minutes and, when separated, serum was collected and stored at -20°C. Colostrum was collected at first milking (07h00 or 20h00), and was also frozen at -20°C. Both blood and colostrum samples were analyzed for IgG concentration (Bovine IgG Test Kit, Triple J Farms, Bellingham, WA) and also for total protein via refractometer (MISCO Refractometers, Solon, OH, USA).

For calves, BW was measured at birth and then weekly until weaning. Calf serum was collected before colostrum feeding, then daily for the first three days of life, and then weekly until weaning. Samples were collected and centrifuged at 1693 x g for 20 minutes and, when separated, serum was collected and stored at -20°C . Calf serum was examined for IgG content, glucose (Glucose Auto kit, Fujifilm WAKO diagnostics, Mountain View, CA, USA), and BHB concentrations at all time points (3-HB Auto kit, Fujifilm WAKO diagnostics, Mountain View, CA, USA).

Calf apparent efficiency of absorption (AEA) was calculated based on equation by Quigley et al (1998a):

$$AEA = \frac{Plasma\ IgG\ \left(\frac{g}{L}\right) \times\ BW\ (kg)\ \times\ 0.089\ (\frac{L}{kg})}{IgG\ Intake\ (g)} \times 100\%$$

Statistics

Statistical analysis was performed using the MIXED procedure of SAS 9.4 (SAS Institute Inc, Cary, NC, USA). For variables with repeated measures, the statistical model was in the form of:

$$Y = \mu + T_i + D_j + T \times D_{ij} + \varepsilon_{ijk}$$

Where μ is the mean, T_i is the time, D_j is the diet term, TD_{ij} is the interaction of time and diet, and ε_{ijk} is the residual error. Animal was used as the subject of repeated measures, and 5 variance/co-variance structures were tested, and the variance/co-variance structure with the lowest AIC was selected for the analysis, and a Tukey post-hoc test was used to differentiate different time points. For variables without repeated measures, the statistical model used was: $Y = \mu + D_j + \varepsilon_{ijk}$ where μ is the mean, D_j is the diet term, and ε_{ijk} is the residual error. Correlation analysis was done using PROC CORR in SAS v. 9.4 (SAS Institute, Cary, NC).

2.4 Results

2.4.1 Effect of Butyrate Supplementation on Dams

One dam was removed from the study because she was confirmed not pregnant. Another dam had stillborn calves, thus no calves were allocated to the calf portion of the study. There was no difference in serum IgG concentrations for CTRL-D vs. BUT-D (1785 ± 117 vs. 1736 ± 137 mg/dL, respectively; $P = 0.75$, Figure 2.1), but a decrease was observed over time ($P < 0.01$).

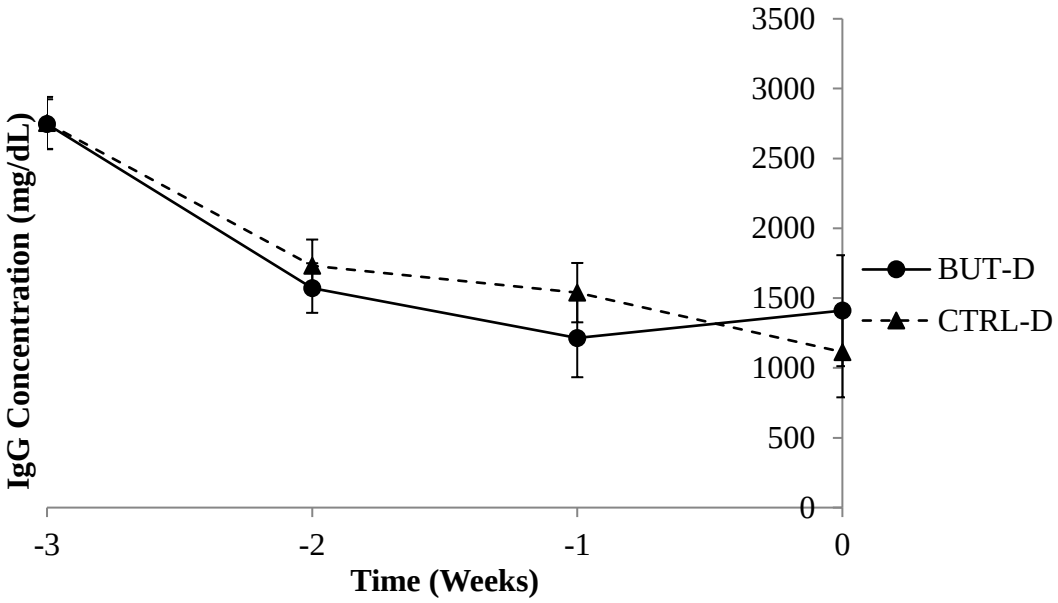

Figure 2.1 Serum IgG concentrations, relative to calving, of cows fed a close-up TMR (CTRL-D) or a close-up TMR with top-dressed butyrate included at 1% DMI (BUT-D). Butyrate supplementation at 1% DMI did not affect serum IgG concentration in dams (P = 0.75) but there was a decrease in serum IgG over time (P < 0.01).

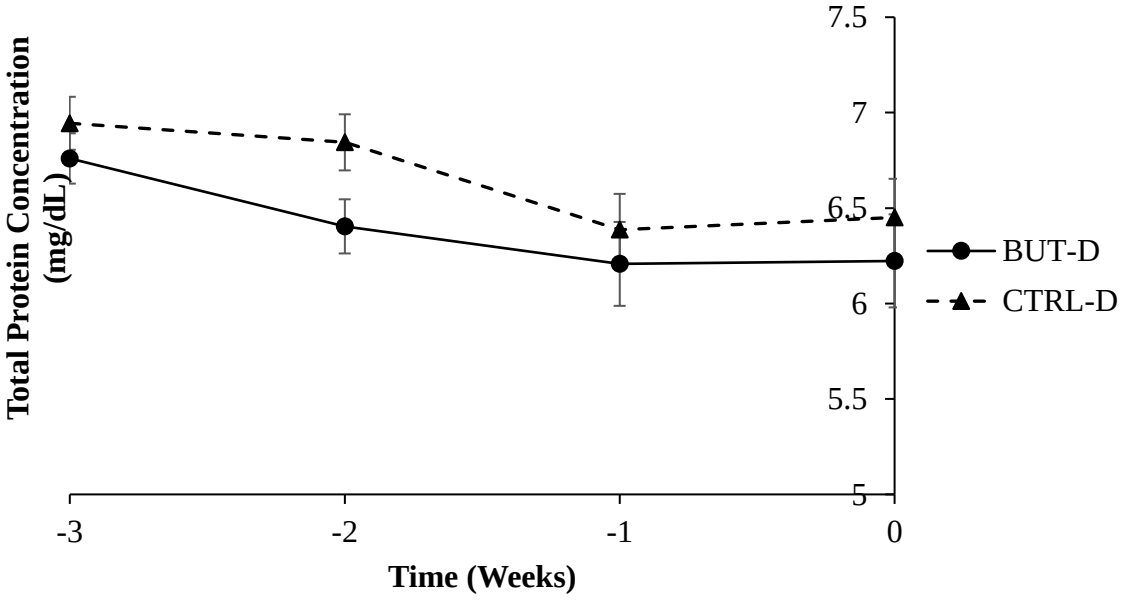

Figure 2.2 Dam serum total protein values, relative to calving, of cows fed a close-up TMR (CTRL-D) or a close-up TMR with top-dressed butyrate included at 1% DMI (BUT-D). Butyrate supplementation at 1% did not affect serum total protein values in dams (P = 0.26) but there was a decrease in serum total protein over time (P < 0.01).

Serum total protein values did not differ between CTRL-D and BUT-D (6.65 ± 0.13 vs. 6.39 ± 0.14 g/dL, *P* = 0.26; Figure 2.2). Colostrum IgG concentrations were also not different between CTRL-D and BUT-D, with a wide range of Ig concentrations obtained overall (160 ± 72.1 vs. 117 ± 35.1 g/L respectively; *P* = 0.46). Similar results were observed with colostral total protein values for CTRL-D and BUT-D (6.65 ± 0.13 vs. 6.39 ± 0.14 g/dL respectively, *P* = 0.20, even though the correlation between Ig concentration and total protein level is not excellent (r = 0.17, *P* = 0.05). Additionally, dam DMI was unaffected by treatment for CTRL-D and BUT-D (14.1 ± 1.34 vs. 14.1 ± 0.93 kg/d respectively, *P* = 0.52, Figure 2.3), but DMI tended to decrease over time across both treatments (P = 0.06). Calving date deviation from expected date of birth did not change in response to treatment (-1.7 ± 1.7 vs. -5.4 ± 1.6 d, CTRL-D and BUT-D, respectively, *P* = 0.26).

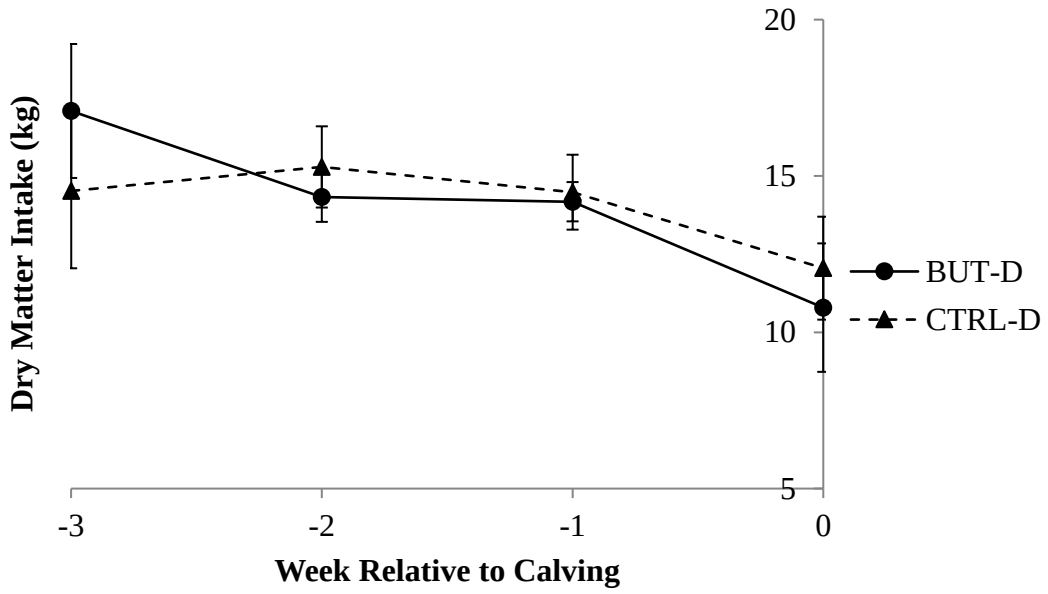

Figure 2.3 Weekly DMI, relative to calving, of cows fed a close-up TMR (CRTL-D) or a close-up TMR with top-dressed butyrate included at 1% DMI (BUT-D). Butyrate supplementation at 1% did not affect DMI in dams (P = 0.52) but there was a trend for decreasing DMI over time (P = 0.06).

2.4.2 Effect of Butyrate Supplementation on Calves

Eighteen calves were born live and were separated into two treatment groups, balancing for dam treatment and body weight (40.0 ± 0.8 vs 39.6 ± 0.8 kg for CTRL-C and BUT-C, respectively; $P = 0.75$; Table 2.1). Between bulls and heifers, body weight at birth differed (43.1 ± 0.7 kg vs. 36.4 ± 0.8 kg; $P < 0.05$; Table 2.1). At 24h, serum IgG concentrations did not differ between bulls and heifers (1906 ± 96 mg/dL vs. 1906 ± 113 mg/dL respectively; $P = 0.99$), but serum IgG values were greater for CTRL-C compared with BUT-C (2110 ± 124 vs. 1400 ± 115 mg/dL, $P < 0.01$; Figure 2.4). The difference in serum IgG concentrations between CTRL-C and BUT-C was sustained through week 1 (1397 ± 121 vs. 866 ± 115 mg/dL, $P = 0.03$), and week 2 (1310 ± 121 vs. 797 ± 115 mg/dL, $P < 0.05$; Figure 2.4). Additionally, serum IgG concentrations decreased over time ($P < 0.01$), and there was a treatment × time interaction ($P = 0.04$). Serum total protein tended to be greater for CTRL-C vs. BUT-C at 4 weeks of age (6.04 ± 0.17 vs. 5.21 ± 0.17 g/dL, respectively; $P = 0.03$; Figure 2.5). In calves, serum total protein tended to be weakly correlated to serum IgG ($r = 0.17$, $P = 0.06$).

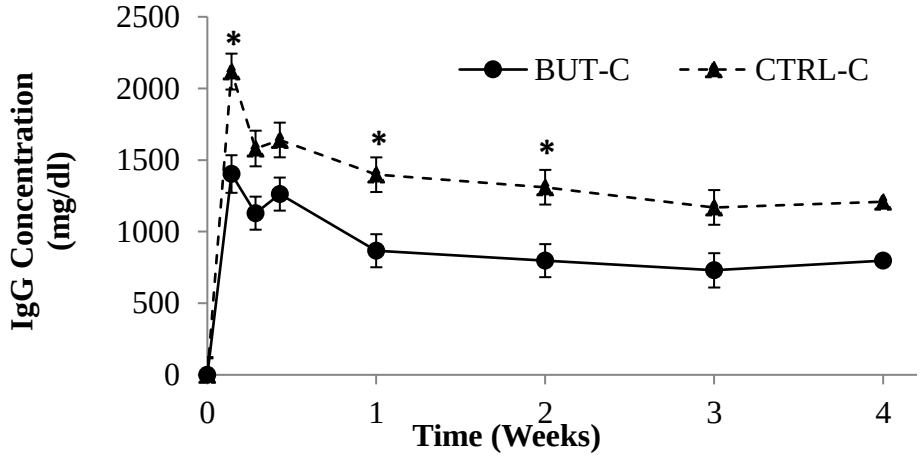

Figure 2.4 Calf serum IgG concentration by week of age for calves receiving colostrum replacer (CTRL-C) and for calves receiving colostrum replacer supplemented with 2.5% butyrate w/v (BUT-C). Butyrate supplementation decreased IgG values in BUT-C at 24 hrs of life (P < 0.01), week 1 (P = 0.03), and week 2 (P = 0.05). There was a diet × time interaction (P = 0.04). Additionally, serum IgG concentrations decreased over time (P < 0.01).

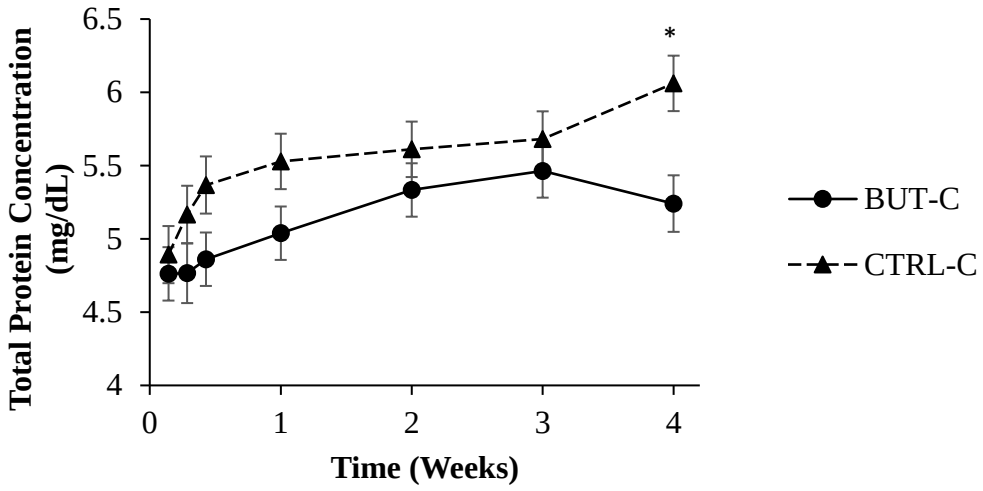

Figure 2.5 Calf serum total protein concentrations by week of life for calves receiving colostrum replacer (CTRL-C) and for calves receiving colostrum replacer supplemented with 2.5% butyrate w/v (BUT-C). Butyrate supplementation decreased total protein values for BUT-C at week 4 of life (P = 0.03). There was no diet × time interaction (P = 0.39).

Calf serum glucose concentrations were unaffected by treatment (104 ± 2.33 vs. 108 ± 2.37 mg/dL, $P = 0.23$; Figure 2.6). Serum BHB concentrations also remained unchanged between CTRL-C and BUT-C (1.03 ± 0.051 vs. 0.872 ± 0.052 mg/dL, respectively; $P = 0.98$; Figure 2.7), but increased steadily by week ($P < 0.01$). Calf average daily gain was not different between CTRL-C and BUT-C (0.59 ± 0.5 vs. 0.48 ± 0.5 kg/d, $P = 0.43$; Figure 2.8).

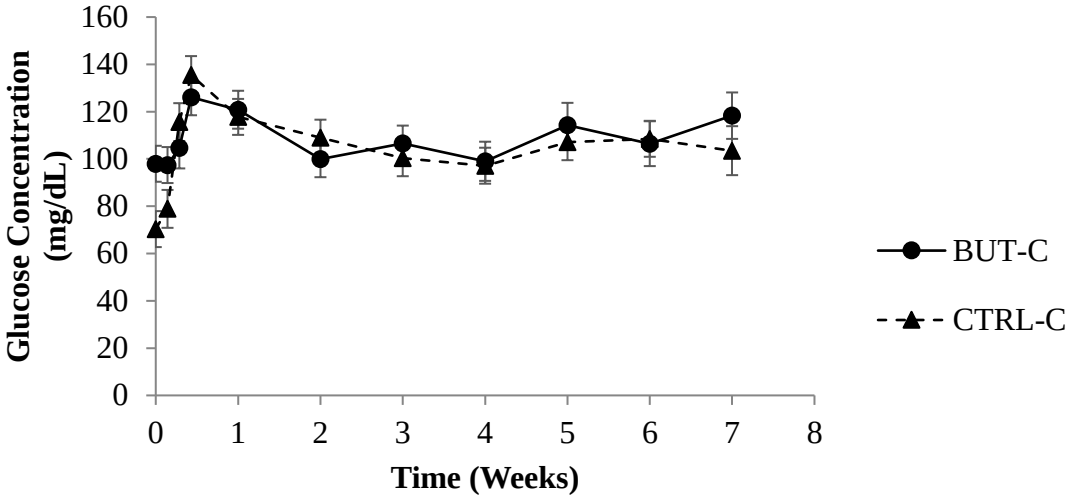

Figure 2.6 Calf serum glucose concentrations by week of life for calves receiving colostrum replacer (CTRL-C) and for calves receiving colostrum replacer supplemented with 2.5% butyrate w/v (BUT-C). Butyrate supplementation did not affect glucose concentration (P = 0.23).

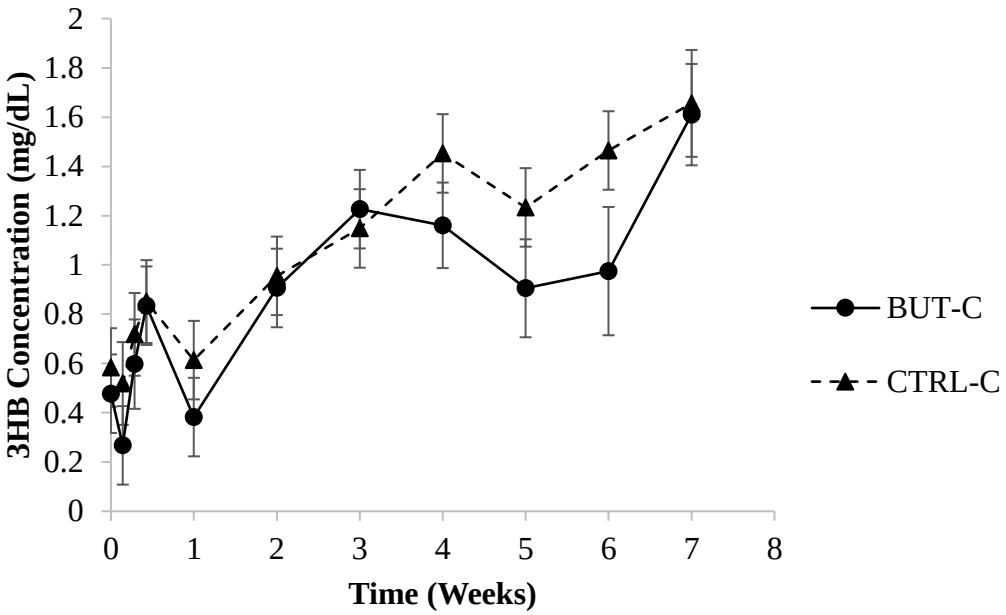

Figure 2.7 Calf serum 3-hydroxybutyrate concentrations by week of life for calves receiving colostrum replacer (CTRL-C) and for calves receiving colostrum replacer supplemented with 2.5% butyrate w/v (BUT-C). Butyrate supplementation did not have an effect on 3-HB concentration (P = 0.98).

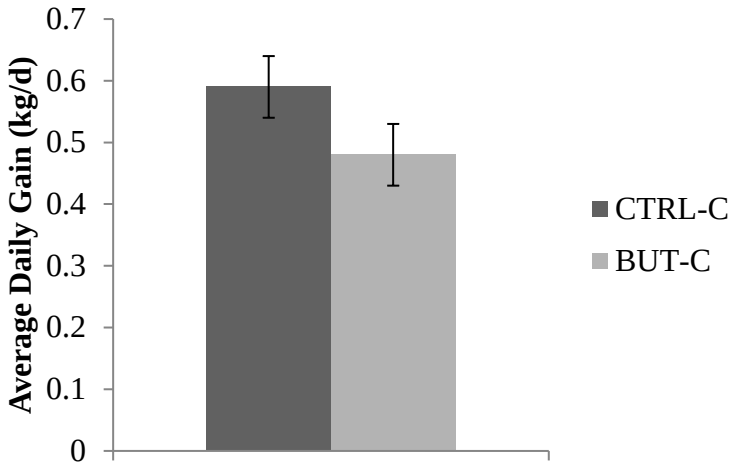

Figure 2.8 Calf average daily gain (ADG) for calves receiving colostrum replacer (CTRL-C) and for calves receiving colostrum replacer supplemented with 2.5% butyrate w/v (BUT-C). Butyrate supplementation did not affect ADG (P = 0.43).

Calf AEA at 24 hours of life was greater for the CTRL-C compared with the BUT-C group (35.3 ± 2.1 vs. 25.9 ± 2.0, $P < 0.01$, Table 2.1) and also differed between bulls and heifers (33.8 ± 1.8 vs. 27.4 ± 2.2, respectively; $P = 0.05$; Table 2.1). AEA was correlated with serum IgG concentration ($r = 0.86$; $P < 0.01$) but not with body weight at birth.

	CTRL-C	BUT-C	*P* value	Heifer	Bull	*P* value
Heifers, Bulls	3 , 6	4 , 5	N/A	N/A	N/A	N/A
Birth weight (kg)	39.6 ± 0.84	39.9 ± 0.81	0.74	36.4 ± 0.90	43.1 ± 0.74	< 0.01
AEA %	35.3 ± 2.08	25.9 ± 1.99	< 0.01	27.3 ± 2.22	33.8 ± 1.83	0.04
ADG (kg/d)	0.59 ± 0.50	0.48 ± 0.50	0.43	0.52 ± 0.05	0.49 ± 0.04	0.64

Table 2.1 Calf sex distribution by treatment, calf birth weights by treatment (P = 0.74) and by sex (P > 0.01), apparent efficiency of absorption (AEA) at 24 hours by treatment (P < 0.01) and by sex (P = 0.04), and average daily gain (ADG) by treatment (P = 0.43) and by sex (P = 0.64). Butyrate supplementation and sex had an effect on AEA but there was no interaction of treatment and sex (P = 0.30).

2.5 Discussion

In this experiment, the effects of supplemental butyrate on Ig production in dams and on Ig absorption in calves were investigated. The evidence suggests that dams showed no response to butyrate in serum or in colostral IgG concentrations. The dose used for dams was based on manufacturer recommendation, and was coated to ensure palatability. At the inclusion rate of 1%, the butyrate-supplemented TMR had 0.6% inclusion of palm oil; because the supplement was top-dressed, the TMR was not separately analyzed. The butyrate dose for calves was based on a previous study where ruminal dosing with butyrate at 2.5% DMI increased absorption capacity of short chain fatty acids (Laarman et al., 2013b).

2.5.1 Effect of butyrate supplementation on dams

In the current study, no differences were detected between BUT-D and CTRL-D for IgG concentrations in colostrum, which may be related to the variability in colostrum collection times. Colostrum was collected at the milking time immediately following calving; milking times were 07h00, 10h00, 20h00, and 23h00; thus, the range of time to first milking was between 1.5h to 10.5h postpartum. Colostrum collected at 6, 10, and 14h postpartum has been observed to have lower IgG concentration as opposed to colostrum collected 2h postpartum (Moore et al., 2005). The variability in time from calving to colostrum harvest likely explains much of the variation in colostrum quality and the lack of significant differences between treatments.

In addition to colostrum harvest times, butyrate dosage may partly explain the lack of differences in colostral IgG. In sows, butyrate increased colostral IgG production at 500 ppm, but not 1000 ppm (Jang et al., 2017); it is possible that in the current study, levels of butyrate were too high to see a response. The supplement fed in the current study was rumen-protected, hence the butyrate was absorbed in the small intestine, like monogastric species. It is possible that butyrate may only impact IgG concentrations in colostrum at very low concentrations, and may act differently if rumen

protected versus non rumen protected. More research is needed on the dose response of the bovine small intestine to dietary butyrate supplementation.

Limited information is available as to the production and transport of Ig into colostrum for both monogastrics and ruminants (Jang et al., 2017; Baumrucker et al., 2010). Butyrate released into the small intestine is absorbed into the peripheral blood supply and pass through the liver. More than 80% of butyrate is metabolized in one pass through the liver (Hocquette and Bauchart, 1999), thus there may be limited potential for dietary supplementation of butyrate to impact mammary transport of IgG into colostrum.

2.5.2 Effects of butyrate on IgG absorption in calves

This study showed that butyrate supplementation of colostrum decreased serum IgG concentrations. Despite lower serum IgG concentrations, average daily gain was not affected by butyrate supplementation in colostrum. In calves, IgG absorption is non-selective in the small intestine (Staley et al., 1972), ruling out an impact of butyrate on FcRn receptors. Additionally, paracellular transport is unlikely to be a significant transport route since no degradation or morphological changes in tight junctions between cells is observed in the first 24h of life (Jochims et al., 1994). In calves, therefore, pinocytosis is likely the principal IgG absorption route (Jochims et al., 1994), and the likeliest target of butyrate action in this study.

Butyrate actively promotes cell differentiation and proliferation (Górka et al., 2014; Hamer et al., 2009) through inhibition of histone deacetylase complex 1 (Davie, 2003). In calves fed sodium butyrate, mid-jejunum epithelial cells had greater mitotis:apoptosis ratios, indicating a faster maturation rate in those epithelial cells (Górka et al., 2014). Calf intestinal pinocytotic activity is lost in the first 24h of life as epithelial cells mature (Jochims et al., 1994). Faster maturation rates would lead to a decreased window for IgG absorption. In this study, butyrate may be reducing IgG absorption by increasing cell differentiation rates, causing epithelial cells to mature earlier, thus

reducing the amount of time that the epithelial cells have full pinocytic activity needed for IgG absorption.

It is possible, but unlikely, that AEA was reduced because of IgG-butyrate binding. While evidence exists of IgG binding to strains of *Streptococcus* spp. and *Staphylococcus* spp. (Björck and Kronvall, 1984; Moks et al., 1986), we are unaware of any evidence for IgG binding to organic acids. Additionally, colostrum and whole milk naturally contain low concentrations of butyrate and recent reviews of physiological impacts and mechanisms of butyrate show no evidence of butyrate binding to organic acids (Górka, et al, 2018).

Other factors affect AEA, including breed, age at first feeding, sex of the calf, and volume of colostrum administered (Quigley et al., 1998b), which were controlled by the experimental design of this study. Additionally, abomasal emptying rate should be considered as it explains 22% of AEA variation (Mokhber-Dezfooli et al., 2012) and may affect AEA if abomasal emptying occurs after 12h of life. When colostrum is fed in the first 12h of life, AEA declines hourly by2.4% to 3.3% (Mokhber-Dezfooli et al., 2012; Osaka et al., 2014). Consequently, some exploration of variance in abomasal emptying rates is warranted.

Factors impacting abomasal clearance are the physicochemical properties of colostrum, such as pH, osmolarity, volume administered, and caloric density. In the current study, post-hoc analysis showed butyrate increased the pH of the colostrum replacer from 5.28 ± 0.02 to 5.90 ± 0.02. Between colostral pH 5.0 and 7.5, however, there is no difference in AEA in calves (Quigley et al., 2000). Therefore, it is unlikely the pH change by butyrate supplementation would affect AEA. Additionally, impact of osmolarity on abomasal emptying of colostrum is mixed. Osmolarity of 600 mOsm in milk replacer slows abomasal emptying (Sen et al., 2006), while addition of 300 m*M* sodium bicarbonate may cause AEA to increase (Morrill et al., 2010) or decrease (Cabral et al., 2014). Osmolarity increased from 276 ± 21 to 921 ± 3 mOsm when the supplement was added, so

may impact abomasal emptying rates. Creating isotonic solutions between treatments was impractical because the supplemented colostrum would require an additional 2.5 L of water, which exceeds abomasal capacity. Therefore, it is possible abomasal emptying rate was impacted by butyrate supplementation, but unclear in which direction, given previous results from bicarbonate supplementation.

There were several limitations to this study. One limitation was the use of colostrum replacer as opposed to maternal colostrum, which may include biological factors that impact absorption. Another limitation is that calf health may impact growth; because of low calf number and resulting statistical power, presence/absence of morbidity was not measured. Although all calves were raised in the same environment (individual housing, feeding, ambient temperature), examination of length of morbidity could give additional insight into the effect of decreased IgG levels on growth and health during early calfhood. Lastly, the relatively short length of the calf component of this study did not allow for a full examination of the effect on calf productivity; following calves for a longer period of time is a useful target for future studies.

2.6 Conclusions

In cows, butyrate supplementation in the dry period did not impact colostrum IgG concentrations or DMI. In calves, however, butyrate supplementation of colostrum decreased serum IgG concentrations. Future research should investigate mechanisms of IgG production in dams, to understand why an increase in colostral IgG was seen in monogastrics but not in ruminants. Additionally, future research should examine the role of butyrate on enterocyte maturation in calves, and its resulting impact on Ig absorption.

2.7 References

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Chapter 3: Effect of Weaning and Supplemental Butyrate on Nutrient Transporter Expression in Holstein Calves

"Effects of Supplemental Butyrate and Weaning on Rumen Fermentation in Holstein Calves" 2019. J. Dairy Sci. 102: 8874-8882.

3.1 Abstract

This study examined the effect of the weaning transition and supplemental sodium butyrate – a primary stimulator of rumen development – on rumen fermentation and volatile fatty acid transporter (VFA) abundance. The transporters examined were monocarboxylate transporter isoform 1 (MCT1), sodium bicarbonate co-transporter 1 (NBC1), and sodium proton exchanger 3 (NHE3). Holstein bull calves (n=36; age= 10.7 ± 4.1d) were assigned to one of four treatment groups: two pre-weaning groups, animals fed either milk only (PRE-M) or milk, calf starter, and hay (PRE-S); and two post-weaning groups: animals fed milk, starter, and hay either without supplementation (POST-S) or with 1% w/w supplemental butyrate during the weaning transition (POST-B). Milk was provided at 1200 g/d; starter, water, and hay were provided *ad libitum*. Weaning in POST-S and POST-B occurred by reducing milk replacer to 900 g/d in week 7, 600 g/d in week 8, 0 g/d at week 9 and tissue harvest at the end of week 9. Rumen pH was measured continuously for seven days before harvest. At harvest, calves were euthanized via captive bolt and exsanguination, after which rumen epithelium and rumen fluid samples were collected. Rumen fluid was analyzed for VFA and rumen tissue was analyzed for VFA transporters. Data were analyzed in SAS with fixed effect of treatment and, where appropriate, repeated effect of week. Between PRE-M and PRE-S, the pre-weaning comparison, total VFA concentrations tended to increase (11.9 ± 11.8 vs. 35.6 ± 11.4 m*M*, respectively; $P = 0.08$), mean rumen pH was unaffected (6.17 ± 0.21 vs. 6.25 ± 0.22, respectively; $P = 0.78$), and NBC1 abundance increased (578 ± 845 vs. 2499 ± 427, respectively, $P = 0.05$). Between PRE-S and POST-S, the weaning comparison, total VFA concentrations increased (35.6 ± 11.4 vs. 154.4 ± 11.8 m*M*, $P < 0.01$), mean rumen pH was unaffected (6.25 ± 0.22 vs. 6.40 ± 0.22, respectively; $P = 0.66$), and NHE3 abundance decreased (3673 ± 532 vs. 2017 ± 563, respectively, *P*

= 0.04)). Between POST-S and POST-B, the post-weaning comparison, total VFA concentrations tended to decrease (154 ± 11.8 vs. 131 ± 11.8 m*M*, P = 0.09), and mean rumen pH decreased (6.40 ± 0.22 vs. 5.83 ± 0.21, P = 0.05), while transporter abundance was unaffected for MCT1, NHE3, and NBC1. The weaning transition induces many physiological and metabolic changes in the rumen, some of which appear to affect transporter abundance.

3.2 Introduction

Rumen development is important to a successful weaning transition in calves as the gastrointestinal tract of young ruminants is not ready for solid feed digestion at birth (Górka et al., 2018). Solid feed intake is the primary driver of increased physiological and anatomical development pre-weaning. The removal of milk feeding is the main cause of solid feed intake increase during weaning (Sweeney et al., 2010), though often the increase in solid feed intake is not enough to offset the loss of metabolizable energy from the removal of milk from the diet, leading to reduced weight gains, or even weight losses immediately post-weaning (de Passille et al., 2011). To meet energy requirements, rumen development and increased solid feed fermentation contribute to a successful weaning transition.

The increase in solid feed fermentation results in increased VFA concentrations and may require an increase in VFA transport capacity. Transport of VFA through the rumen epithelium and into the blood stream becomes more important as the calf is switching from a glucose-dependent system to a VFA-dependent system (Lane et al., 2000). Despite increased fermentation between milk fed calves and calves with access to solid feed (Laarman et al., 2012), no difference has been found in rumen epithelial VFA transporters and ruminal disappearance of VFA pre-weaning (Yohe et al., 2018). It is possible that the developing rumen increases VFA transport capacity in response to increased fermentation. The effect of the weaning transition on VFA transporter abundance has yet to be examined.

In addition to VFA transport, pH in the developing rumen and pH_i regulation in the developing epithelium are of interest. Increased VFA production can lower the pH of the rumen because of the increase in acidic metabolites; intracellularly, the uptake of VFAs can lower pH_i. Some pH_i regulation is performed via membrane-bound transporters, such as Na^+/H^+ Exchanger (NHE3) and $Na^+/HCO3^-$ Transporter (NBC1). These transporters have mostly been studied in adult cattle (Laarman et al., 2013a; Laarman et al., 2013b) and their abundance during the weaning transition is unknown. Rumen pH during pre-weaning does not change between milk fed calves and calves with access to solid feed (Laarman and Oba, 2011; Yohe et al., 2018), even with the increase in solid feed intake. This phenomenon may be explained by metabolic adaptations in the developing rumen. The addition of calf starter to the diet increases the molar proportion of butyrate, while decreasing the molar proportion of acetate (Laarman et al., 2012). Butyrate is one of the primary drivers of rumen development and may help in physiological and metabolic adaptation to grain consumption (Connor et al., 2013). Additionally, the buffering capacity of forage consumption may contribute to the stability of rumen pH. Whether metabolic adaptations and forage consumption through the weaning transition are enough to buffer the rumen while starter intake is increasing is unknown.

Solid feed intake naturally increases upon the commencement of weaning, but often is not enough to offset the loss of metabolizable energy from the removal of the milk meal. Supplemental butyrate increases calf starter intake (Górka et al., 2011), which has the potential to offset the loss energy during weaning and to encourage additional GI tract development (Górka et al., 2018). When calves are weaned, the decrease in energy intake often causes stagnation or decrease in weight gain (Sweeney et al., 2010), which is undesirable to producers and may affect performance later in life. Supplemental butyrate promotes rumen papillae development, increasing the surface area for nutrient absorption (Górka et al., 2018). Nutrient transport capacity during the weaning transition is

unknown, but butyrate's positive effects on rumen development may facilitate improvement of nutrient uptake.

The objective of this study was to examine the effect of the weaning transition on rumen development, VFA transporter abundance, and fermentation parameters. In addition, the effect of butyrate supplementation during the weaning transition was examined. The increase in calf starter intake with butyrate supplementation may be able to prime the GI tract for increased VFA absorption. We hypothesized that the weaning transition would increase fermentation, decrease rumen pH, and thus increase VFA transport proteins and pH_i regulatory transporters. In addition, butyrate supplementation should increase calf starter intake and thus increase VFA transporter abundance in rumen epithelium.

3.3 Materials and Methods

All animal procedures were approved by the Institutional Animal Care and Use Committee (AUP # 2016-32). Full growth and production parameters are presented in a previously published work (McCurdy et al., 2019). Thirty-six Holstein bull calves were obtained from a single commercial farm. All calves were fed 4 L of colostrum at birth and were transported to the Palouse Research, Extension, and Education Center in Moscow, Idaho. Calves were transported in two groups, two weeks apart in April 2017. Upon arrival, all calves were treated with 0.90 mg/kg of bodyweight ampicillin at the recommendation of the attending veterinarian. Throughout the study, calves that showed reduced milk intake, dehydration, or scours were given electrolytes (RE-SORB, Zoetis Services LLC., Parsippany, NJ) in addition to their milk replacer feeding.

3.3.1 Animals and Treatments

At arrival, all calves were either 5 days (n=12), 10 days (n=12), or 16 days of age (n=12). All calves were housed individually on sand and had access to water ad libitum. At 16 days of age,

calves were blocked by initial age and sorted into one of four treatment groups. Pre-weaning treatments included calves were fed either milk replacer only (PRE-M) or milk replacer with access to calf starter and hay ad libitum (PRE-S). Post-weaning treatments were fed milk replacer until weaning and had access to calf starter and hay ad libitum. During the two-week weaning transition at seven to eight weeks of age, post-weaning treatment calves either had no starter supplementation (POST-S), or were given supplemental rumen-protected butyrate in their calf starter (Ultramix-C, Nutriad, Hampshire, IL) at an inclusion rate of 1% w/w (POST-B). Pre-planned comparisons were PRE-M vs. PRE-S, PRE-S vs. POST-S, and POST-S vs. POST-B to examine results pre-weaning, weaning, and post-weaning, respectively.

3.3.2 Tissue Harvest Procedures

For collecting rumen tissue samples, calves were euthanized via captive bolt and exsanguination, after which rumen epithelium samples were taken. The samples were taken from the ventral sac of the rumen, stripped of any fat and musculature, rinsed with PBS, and were stored in 4% formalin for 24 hours. After 24 hours, the samples were transferred to a 70% ethanol solution until they were processed, encased in paraffin, and mounted on slides (Washington Animal Disease Diagnostics Laboratory, Pullman, WA). Tissue and rumen fluid were harvested from PRE-M and PRE-S at 6 weeks of age, before the onset of weaning; tissue and rumen fluid were harvested from POST-S and POST-B calves post-weaning at 9 weeks of age, one week after complete removal of milk replacer. Calves were given a ruminal pH logger (Dascor Inc., Escondido, CA) during week 6 (PRE-M and PRE-S) or week 9 (POST-S and POST-B) that recorded rumen pH continuously every two minutes. After 7 days of pH readings, calves were harvested. During tissue harvest the pH logger was retrieved from the rumen; rumen fluid samples were also collected, strained through four layers of cheesecloth, and snap-frozen in liquid nitrogen for later VFA analysis.

3.3.3 Weaning Procedures

All calves were fed milk replacer at 1200 g/d (28% CP, 18% fat; Calva Advantage, Calva Products LLC. Acampo, CA) at 0630 h and 1700 h until 6 weeks of age. Milk refusals were recorded, and any refusal over 400g/d was fed through an esophageal tube. For POST-S and POST-B, milk replacer feeding was reduced to 900g/d in week 7, further reduced to 600g/d in week 8, and was removed completely at the beginning of week 9. During weeks 7 and 8, POST-B calves were given supplemental rumen-protected butyrate at a rate of 1% w/w mixed into their calf starter. In week 9, when no milk was provided, POST-B calves were returned to non-supplemented calf starter until harvest one week later. Starter (22.1% CP, 36.5% starch; AMPLI-CALF Starter 20, Land O' Lakes LLC., Tulare, CA) and medium chopped alfalfa hay (19.8% CP, 42.2% NDF) were fed daily at 0700 h.

3.3.4 Immunofluorescence Tissue Preparation

Mounted rumen tissues were de-paraffinized with washes of xylene, 100% ethanol, and 70 % ethanol. The tissues were then re-hydrated in a 3% sodium citrate solution for 15 at 95^{o}C for antigen retrieval. Antigens of interest were monocarboxylate transporter 1 (MCT1), sodium proton exchanger 3 (NHE3), and sodium bicarbonate co-transporter 1 (NBC1). Subsequently, the tissues were blocked and permeabilized with a 10% goat serum and 0.3% Triton-X100 blocking buffer for 30 min. Primary antibodies for MCT1 (anti-MCT1/SLC16A1, polyclonal, host species rabbit, confirmed in human and mouse, 1:200 dilution, Novus Biologicals, Oakville, ON, Canada), NHE3 (anti-NHE3/SLC9A3, polyclonal, host species rabbit, confirmed for human and mouse, 1:100 dilution, Novus Biologicals, Oakville, ON, Canada), or NBC1 (anti-SLC4A4, polyclonal, host species rabbit, confirmed for human, 1:200 dilution, Novus Biologicals, Oakville, ON, Canada) were

added and were incubated at room temperature for 90 min. Next, slides were washed5 times with PBS and the secondary antibody (IgG cross-adsorbed, polyclonal, goat anti-rabbit, DyLight 488, 1:200 dilution, Invitrogen) was applied for MCT1, NHE3, or NBC1. Samples were then incubated in the dark for 40 min. Slides were again rinsed 5 times with PBS and then coverslips were placed using a mounting medium that contained DAPI nuclear stain (Prolong Gold Anti-fade, Cell Signaling Technolgies, Danvers, MA). A negative control without any primary antibody was stained for each of the three antibodies tested. Stained slides were stored at -20^{o} until analysis.

3.3.5 Sample Analysis

Rumen pH was analyzed for minimum, mean, and maximum pH, duration of ruminal pH less than 5.8, standard deviation of pH measurements, and area under the curve of ruminal pH less than 5.8. For VFA analysis, rumen fluid was acidified with 25% metaphosphoric acid, centrifuged at 24,750 × g for 20 minutes, and the supernatant frozen at -20^{o}C for 12 hours. The supernatant was then thawed and centrifuged at 13,000 × g for 10 minutes, after which it was analyzed on an Agilent 6890 series gas chromatographer with a 7673 series injector (Agilent, Wilmington, DE), with a DB-FFAP column using hexane and acetone solvents and 2-ethylbutyric acid 99%, as previously described (Hall et al., 2018). Analysis provided concentration of individual VFAs in the collected rumen fluid.

Immunofluorescence was detected using a confocal spinning disk microscope (Nikon TiE inverted microscope; Yokogawa X1 Spinning Disk). Gain, offset, and LUT settings were adjusted to minimize oversaturation and were kept consistent for each antibody. Images of individual papillae were taken from each blinded slide, three images of three different papillae per slide. Quantification of transporter abundance was achieved by drawing around the perimeter of individual cells to obtain the whole cell signal values for each antibody per cell. The measurement of each cell perimeter was defined as “relative cell circumference”, whereas “cell area” was the area within the cell perimeter.

Five cells were quantified per image (Laarman et al., 2016). If abundance CV values were greater than 10%, an additional five cells were quantified. A section of the background of each image was used to calculate corrected whole cell signal values using the following formula (Gavet and Pines, 2010):

$$WCS = ID_{cell} - (A_{cell} \times M_{background})$$

Where WCS = Whole Cell Signal; ID_{cell} = integrated cell density; A_{cell} = cell surface area; and $M_{background}$ = mean background signal. All quantifications were executed using ImageJ software.

3.3.6 Statistical Analysis

Statistical analysis for transporter abundance, relative cell circumference, rumen pH, and the VFA profile was performed using the MIXED procedure of SAS 9.4 (SAS Institute Inc., Cary, NC, USA). The statistical model used was

$$Y = \mu + D_j + \varepsilon_{ijk}$$

Where Y is the dependent variable, μ is the mean, D_j is the diet effect, and ε_{ijk} is the residual error. Relative cell circumference was used as a covariate for transporter abundance analysis.

Statistical analysis for ADG was performed using recorded bodyweights and PROC REG. Feed intake and blood metabolite data were analyzed using PROC MIXED in SAS for a randomized complete block design. The model used was

$$Y = \mu + D_i + w_j + t \times w_{ij} + \varepsilon_{ijk}$$

Where Y is the dependent variable, μ is the mean, D_j is the diet effect, w_j is the time effect, $t \times w_{ij}$ is the interaction between time and diet, and ε_{ijk} is the residual term. The variance-covariance structure of the repeated measures was modeled separately with the appropriate structure selected using the

lowest values of the fit statistics based on the Akaike information criterion. Significance was declared at $P \leq 0.05$ and tendencies were declared at $0.05 < P \leq 0.10$.

Pre-planned contrasts were set up to compare PRE-M vs. PRE-S (effect of starter pre-weaning), PRE-S vs. POST-S (effect of weaning), and POST-S vs. POST-B (effect of butyrate supplementation).

3.4 Results

Production data and fermentation parameters are presented in detail in McCurdy et al., (2019), though relevant data and graphs have been included here. Calves were housed outside in individual hutches. During week 6 of the study, a heat wave began to negatively affect calf health, despite attempts to keep calves hydrated and cool. At this point, POST-S and POST-B calves were moved to an indoor facility, where the temperature was similar to the outside temperatures before the heat wave. Calves continued to be housed separately, on sand, and received the same treatment and feed. The stress of relocating the calves indoors is likely less than that of continued heat stress, but could have impacted the results.

3.4.1 Production Factors

Average daily gain did not differ between PRE-M and PRE-S (0.87 ± 0.03 vs. 0.82 ± 0.03 kg/d, respectively, $P = 0.33$; Figure 3.1; McCurdy et al., 2019). Serum concentrations of BHB were lesser in PRE-M calves compared with PRE-S calves only during week 4 (0.258 ± 0.042 vs. 0.372 ± 0.040, $P = 0.03$; Figure 3.2; McCurdy et al., 2019). There was no effect of age on BHB concentrations ($P = 0.69$), and there was no time × treatment interaction ($P = 0.78$). Serum glucose concentrations did not differ between groups ($P = 0.45$) but did increase with age ($P = 0.04$; Figure 3.3; McCurdy et al., 2019). Starter intake increased between PRE-M and PRE-S in the week prior to harvest (0 ± 0.00 g/d vs. 76 ± 165 g/d; $P < 0.01$; Figure 3.4; McCurdy et al., 2019).

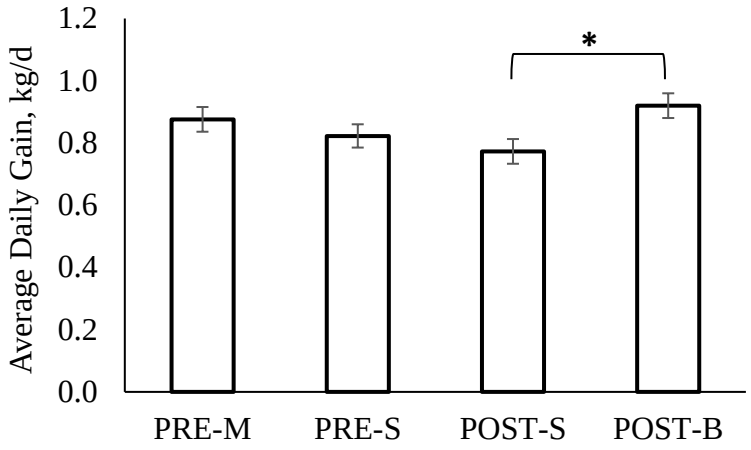

Figure 3.1 Average daily gain (kg/d) of dairy calves at 6 weeks of age, before weaning (PRE), or 9 weeks of age, post-weaning (POST). PRE-M: Milk only; harvested pre-weaning (6 weeks). PRE-S: Milk, starter, hay; harvested pre-weaning (6 weeks). POST-S: Milk, starter, hay, no supplementation during weeks 7-8; harvested post-weaning (9 weeks). POST-B: Milk, starter, hay, 1% w/w butyrate supplementation during weeks 7-8; harvested post-weaning (9 weeks). From McCurdy et al. (2019).

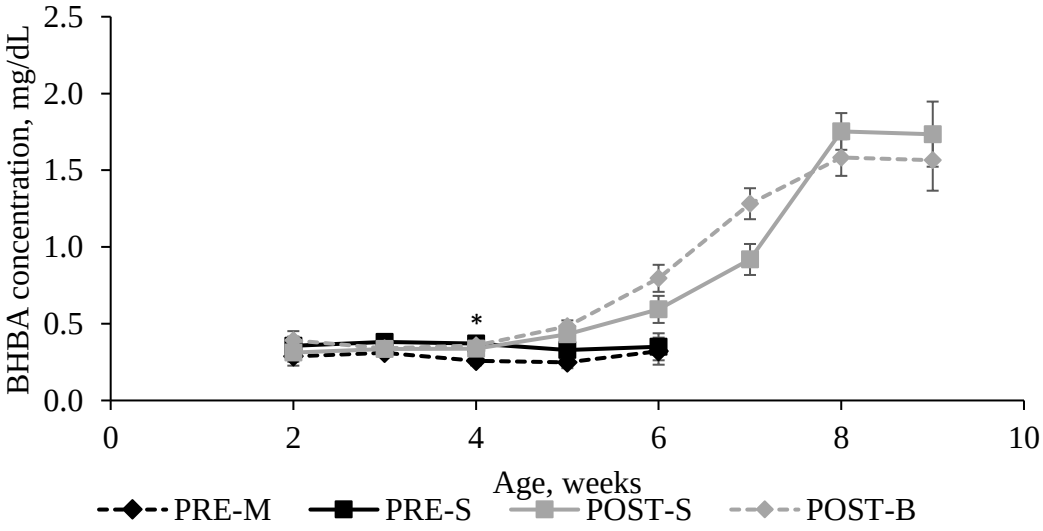

Figure 3.2 Plasma BHB concentration of dairy calves at 6 weeks of age, before weaning (PRE), or 9 weeks of age, post-weaning (POST). PRE-M: Milk only; harvested pre-weaning (6 weeks). PRE-S: Milk, starter, hay; harvested pre-weaning (6 weeks). POST-S: Milk, starter, hay, no supplementation during weeks 7-8; harvested post-weaning (9 weeks). POST-B: Milk, starter, hay, 1% w/w butyrate supplementation during weeks 7-8; harvested post-weaning (9 weeks). From McCurdy et al. (2019).

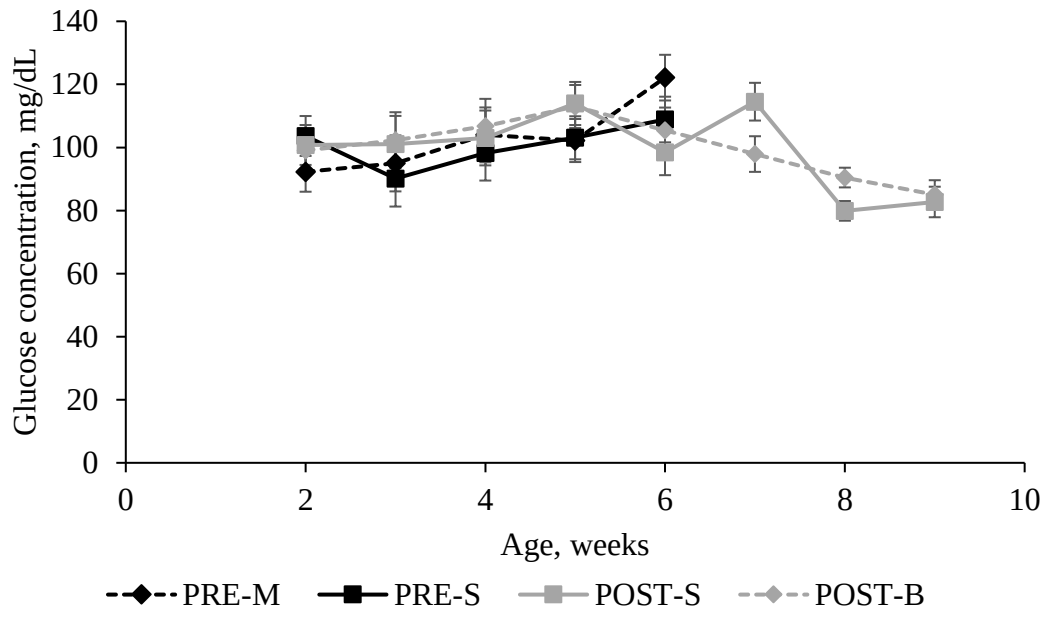

Figure 3.3 Plasma glucose concentration of dairy calves at 6 weeks of age, before weaning (PRE), or 9 weeks of age, post-weaning (POST). PRE-M: Milk only; harvested pre-weaning (6 weeks). PRE-S: Milk, starter, hay; harvested pre-weaning (6 weeks). POST-S: Milk, starter, hay, no supplementation during weeks 7-8; harvested post-weaning (9 weeks). POST-B: Milk, starter, hay, 1% w/w butyrate supplementation during weeks 7-8; harvested post-weaning (9 weeks). From McCurdy et al. (2019)

For PRE-S versus POST-S calves, average daily gain did not differ (0.82 ± 0.03 vs. 0.77 ± 0.03 kg/d, respectively, $P = 0.37$; Figure 3.1), though POST-S had an increased calf starter intake for the week prior to tissue harvest (76 ± 165 vs. 2,247 ± 171 g/d, week 6 vs. week 9, $P < 0.01$; Figure 3.4). Hay intake was greater for POST-S compared to PRE-S in the week before weaning (784 ± 98 vs. 144 ± 95 g/d, $P < 0.01$; Figure 3.5; McCurdy et al., 2019)

Starter intake was greater for POST-B compared with POST-S beginning in week 7 (706 ± 218 vs. 1,151 ± 218 g/d, $P = 0.04$) and continuing in week 8 (1489 ± 219 vs. 2071 ± 219 g/d, $P = 0.01$) and week 9 (2,247 ± 171 vs. 3,102 ± 171 g/d, $P < 0.01$; Figure 3.4), but with no time × treatment interaction ($P = 0.28$). Hay intake did not differ between POST-S and POST-B calves during butyrate supplementation in week 7 (507 ± 98 vs. 442 ± 98 g/d, respectively, $P= 0.64$) or week 8 (689 ± 98 vs. 633 ± 102 g/d, $P = 0.64$; Figure 3.5) Plasma glucose concentrations did not

differ between POST-S and POST-B (Figure 3.3). Along with increased starter intake, plasma BHB concentrations were greater for POST-B than POST-S calves during week 7 (1.28 ± 0.10 vs. 0.92 ± 0.10 mg/dL, respectively, *P* = 0.01) but did not differ in week 8 (1.58 ± 0.12 vs. 1.75 ± 0.12 mg/dL, *P* = 0.31) or week 9 (1.73 ± 0.21 vs. 1.56 ± 0.19 mg/dL, P = 0.55; Figure 3.2). Average daily gain was greater in POST-B calves than in POST-S calves (0.91 ± 0.04 vs. 0.77 ± 0.04 kg/d, *P* = 0.01, Figure 3.1).

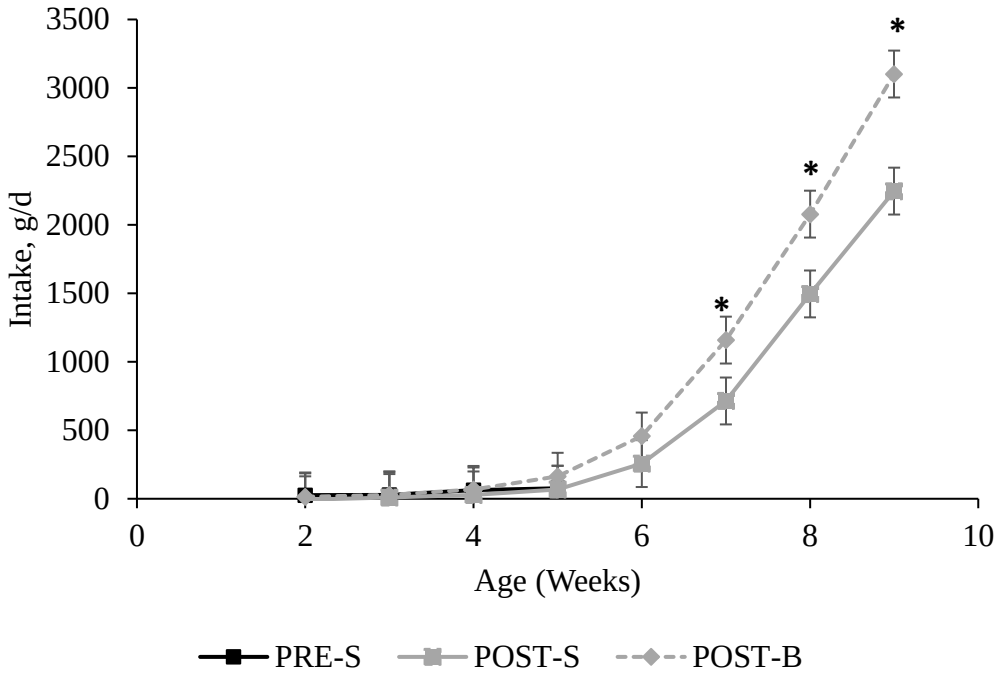

Figure 3.4 Calf starter intake for dairy calves at 6 weeks of age, before weaning (PRE), or 9 weeks of age, post-weaning (POST). PRE-S: Milk, starter, hay; harvested pre-weaning (6 weeks). POST-S: Milk, starter, hay, no supplementation during weeks 7-8; harvested post-weaning (9 weeks). POST-B: Milk, starter, hay, 1% w/w butyrate supplementation during weeks 7-8; harvested post-weaning (9 weeks).From McCurdy et al. (2019)

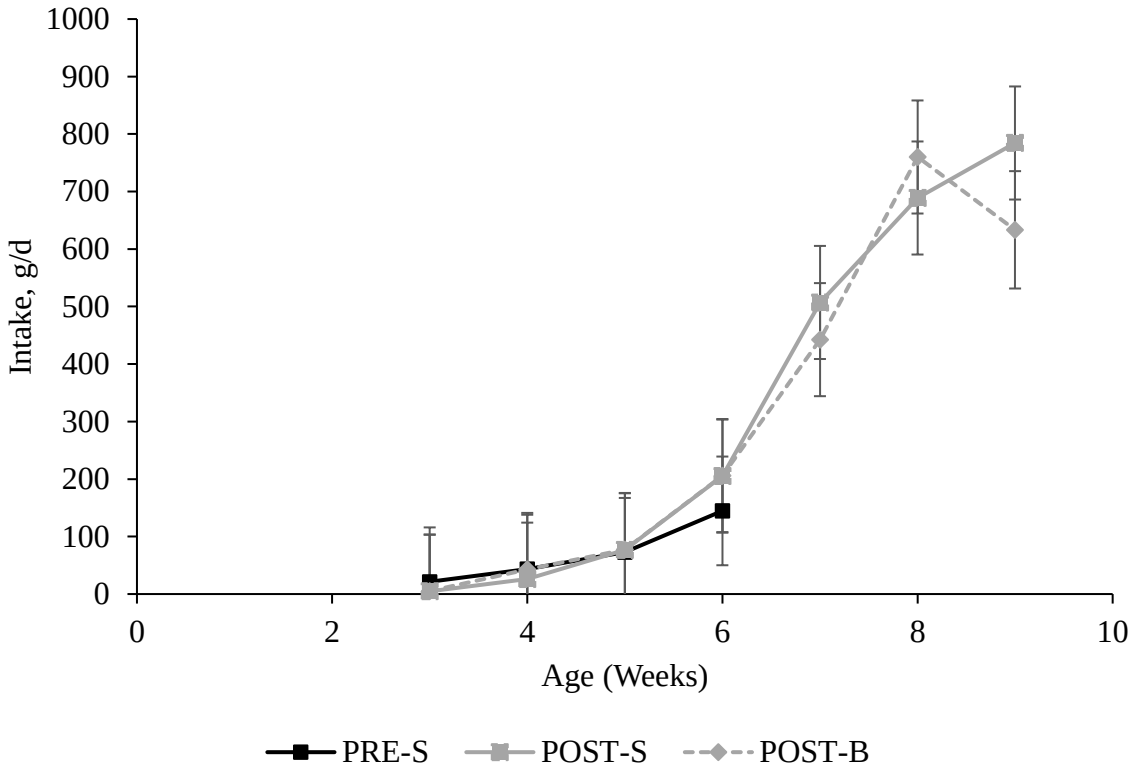

Figure 3.5 Hay intake for dairy calves at 6 weeks of age, before weaning (PRE), or 9 weeks of age, post-weaning (POST). PRE-S: Milk, starter, hay; harvested pre-weaning (6 weeks). POST-S: Milk, starter, hay, no supplementation during weeks 7-8; harvested post-weaning (9 weeks). POST-B: Milk, starter, hay, 1% w/w butyrate supplementation during weeks 7-8; harvested post-weaning (9 weeks).From McCurdy et al. (2019).

3.4.2 Fermentation Parameters

Total VFA concentration tended to increase in PRE-S compared with PRE-M calves (11.9 ± 11.8 vs. 35.6 ± 11.4 m*M*, respectively, $P = 0.08$; Table 3.1; McCurdy et al., 2019). In PRE-M versus PRE-S calves, the percentage of propionate greater for PRE-S calves (15.3 ± 2.7 vs. 23.1 ± 2.6%, $P < 0.01$; Table 3.1), and thus the acetate to propionate ratio decreased for PRE-S calves (4.7 ± 0.5 vs. 3.0 ± 0.5, $P < 0.01$; Table 3.1). Mean rumen pH was unchanged between PRE-M and PRE-S (6.17 ± 0.21 vs. 6.25 ± 0.22, $P = 0.78$; Table 3.2; McCurdy et al., 2019) as were min pH, max pH, and duration of ruminal acidosis. Duration of ruminal acidosis is defined as time where the pH was less than 5.8 (Table 3.2).

Total VFA concentration was greater for POST-S than for PRE-S (154.4 ± 11.8 vs. 35.6 ± 11.4 m*M*, respectively, $P < 0.01$; Table 3.1). The percentage of acetate, propionate, and butyrate in the rumen fluid, plus the A:P ratio did not change between PRE-S and POST-S (Table 3.1). Also, mean pH, min pH, max pH, duration of ruminal acidosis, and area under the curve of pH 5.8 were not different between PRE-S and POST-S (Table 3.2).

There was a tendency for POST-S to have greater VFA concentrations than POST-B calves (154 ± 11.8 vs. 131 ± 11.8 m*M*, respectively, $P = 0.09$ Table 3.1). An increase in propionate percentage was also present between POST-S and POST-B (27.8 ± 2.7 vs. 34.8 ± 2.7%, $P = 0.02$Table 3.1), and difference was also found in the A:P ratio (2.4 ± 0.5 vs. 1.6 ± 0.5, $P = 0.20$;Table 3.1). Mean pH decreased for POST-S versus POST-B calves (6.4 ± 0.22 vs. 5.83 ± 0.21, P = 0.05,Table 3.2) and there was a tendency for the pH to remain lower for longer, as the duration of pH < 5.8 increased (209 ± 201 vs. 730 ± 188 min, $P = 0.07$; Table 3.2).

Table 3.1 Fermentation parameters for pre-planned comparisons of PRE-M vs PRE-S, PRE-S vs. POST-S, and POST-S vs. POST-B. Total mM tended to increase for PRE-M versus PRE-S, and propionate percentage increased. For PRE-S vs. POST-S, total mM increased but no changes were seen in VFA percentages or A:P ratio. Total mM tended to increase between POST-S vs. POST-B and propionate percentage increased. From McCurdy et al., (2019)

					P value		
VFA	PRE-M	PRE-S	POST-S	POST-B	PRE-M vs. PRE-S	PRE-S vs. POST-S	POST-S vs. POST-B
Total, m*M*	11.9 ± 1.8	35.6 ± 1.4	154.4 ± 11.8	131.0 ± 11.8	0.08	< 0.01	0.09
Acetate, %	64.5 ± 2.5	62.5 ± 2.4	58.4 ± 2.5	53.1 ± 2.5	0.60	0.25	0.14
Propionate, %	15.3 ± 2.7	23.1 ± 2.6	27.8 ± 2.7	34.8 ± 2.7	< 0.01	0.22	0.02
Butyrate, %	8.4 ± 1.5	8.4 ± 1.4	9.1 ± 1.5	7.5 ± 1.5	0.97	0.72	0.45
A:P Ratio	4.7 ± 0.5	3.0 ± 0.5	2.4 ± 0.5	1.6 ± 0.5	< 0.01	0.42	0.20

Table 3.2 Ruminal pH measurements for pre-planned comparisons of PRE-M vs. PRE-S, PRE-S vs. POST-S, and POST-S vs. POST-B. The pH measurements were unchanged for PRE-M vs. PRE-S and PRE-S vs. POST-S. Mean pH was lower for POST-S vs. POST-B and the duration of subacute ruminal acidosis tended to be longer for POST-B calves. From McCurdy et al. (2019).

					P value		
	PRE-M	PRE-S	POST-S	POST-B	PRE-M vs. PRE-S	PRE-S vs. POST-S	POST-S vs. POST-B
Min pH	5.07 ± 0.37	5.82 ± 0.35	5.28 ± 0.40	4.99 ± 0.37	0.15	0.32	0.60
Mean pH	6.17 ± 0.21	6.25 ± 0.22	6.40 ± 0.22	5.83 ± 0.21	0.78	0.66	0.05
Max pH	6.72 ± 0.21	7.15 ± 0.22	7.23 ± 0.24	6.86 ± 0.21	0.17	0.82	0.25
Standard Deviation	0.21 ± 0.03	0.29 ± 0.04	0.24 ± 0.04	0.21 ± 0.03	0.01	0.27	0.45
Duration pH <5.8, min/d	485 ± 188	280 ± 178	209 ± 201	730 ± 188	0.44	0.79	0.07
Area under curve pH <5.8, pH × min/d	360 ± 130	90 ± 122	77 ± 130	353 ± 121	0.12	0.94	0.13

3.4.3 Transporter Abundance

MCT1 abundance did not differ between PRE-M and PRE-S when relative cell circumference was included in the model (81,680 ± 13,540 vs. 70,560 ± 8,553 A.U., respectively, *P* = 0.47; Figure 3.6). Similarly, NHE3 abundance was not different between PRE-M and PRE-S (1,693 ± 737 vs. 3,156 ± 563 A.U., *P* = 0.50; Figure 3.7). NBC1 abundance did differ, increasing from PRE-M to PRE-S (578 ± 845 vs. 2,499 ± 427, respectively, *P* = 0.05; Figure 3.8). It should be noted that negative numbers could not be measured for relative abundance; here where the error is larger than the average value, only positive values are biologically relevant. Additionally, relative cell circumference did not differ between PRE-M and PRE-S (336 ± 6.7 vs. 347 ± 6.3 A.U., respectively, *P* = 0.22; Figure 3.9).

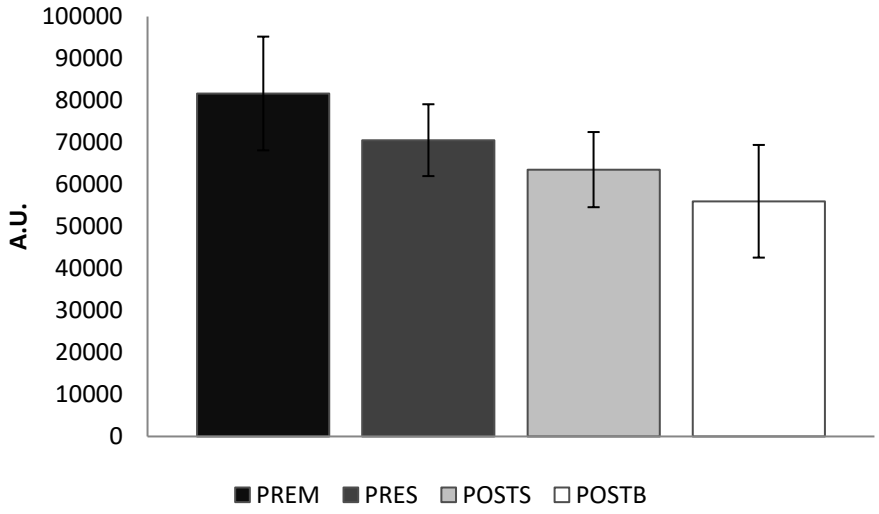

Figure 3.6 MCT1 abundance for dairy calves at 6 weeks of age, before weaning (PRE), or 9 weeks of age, post-weaning (POST). PRE-S: Milk, starter, hay; harvested pre-weaning (6 weeks). POST-S: Milk, starter, hay, no supplementation during weeks 7-8; harvested post-weaning (9 weeks). POST-B: Milk, starter, hay, 1% w/w butyrate supplementation during weeks 7-8; harvested post-weaning (9 weeks).From McCurdy et al. (2019). No differences were found in MCT1 abundance between PRE-M vs. PRE-S, PRE-S vs. POST-S, and POST-S vs. POST-B.

For PRE-S versus POST-S, MCT1 abundance did not differ (70560 ± 8553 vs. 63546 ± 8950 A.U., respectively, *P* = 0.57; Figure 3.6), but NHE3 abundance decreased (3156 ± 563 vs. 2017 ± 759, respectively, *P* = 0.04; Figure 3.7). There were no changes for NBC1 abundance between PRE-S and POST-S (2499 ± 427 vs. 2151 ± 422, respectively, *P* = 0.56; Figure 3.8) but relative cell circumference increased (347 ± 6.3 vs. 370 ± 6.7 A.U., *P* = 0.02; Figure 3.9).

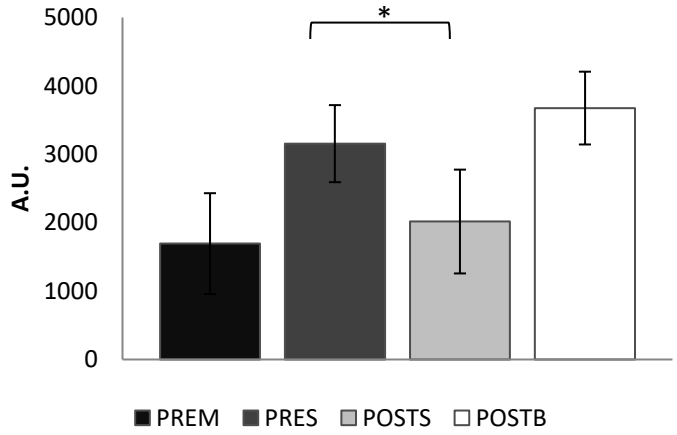

Figure 3.7 NHE3 abundance for dairy calves at 6 weeks of age, before weaning (PRE), or 9 weeks of age, post-weaning (POST). PRE-S: Milk, starter, hay; harvested pre-weaning (6 weeks). POST-S: Milk, starter, hay, no supplementation during weeks 7-8; harvested post-weaning (9 weeks). POST-B: Milk, starter, hay, 1% w/w butyrate supplementation during weeks 7-8; harvested post-weaning (9 weeks).From McCurdy et al. (2019). No difference was found between PRE-M and PRE-S, but NHE3 abundance decreased between PRE-S and POST-S (P = 0.04), and no differences were found between POST-S and POST-B.

POST-S and POST-B calves did not differ in mean MCT1 abundance (63546 ± 8950 vs. 56007 ± 13416 A.U., respectively, *P* = 0.62; Figure 3.6), NHE3 abundance (2017 ± 759 vs. 3676 ± 532 A.U., *P* = 0.67; Figure 3.7), NBC1 abundance (2151 ± 422 vs. 1870 ± 530 A.U., *P* = 0.68; Figure 3.8), and relative cell circumference (370 ± 6.7 vs. 380 ± 6.7 A.U., *P* = 0.31; Figure 3.9).

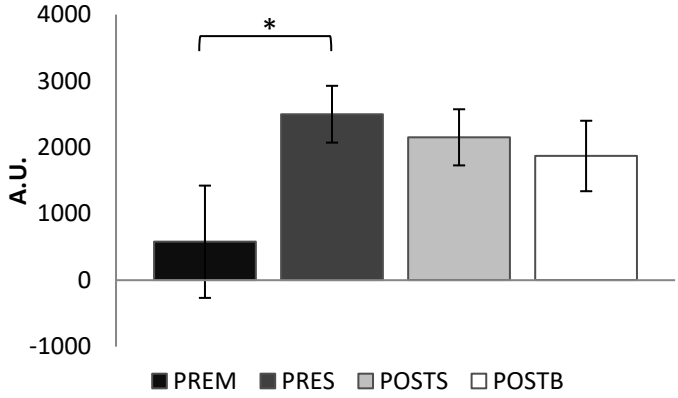

Figure 3.8 Abundance of NBC1 for dairy calves at 6 weeks of age, before weaning (PRE), or 9 weeks of age, post-weaning (POST). PRE-S: Milk, starter, hay; harvested pre-weaning (6 weeks). POST-S: Milk, starter, hay, no supplementation during weeks 7-8; harvested post-weaning (9 weeks). POST-B: Milk, starter, hay, 1% w/w butyrate supplementation during weeks 7-8; harvested post-weaning (9 weeks).From McCurdy et al. (2019). Abundance increased between PRE-M vs. PRE-S (P = 0.05) but did not change between PRE-S vs. POST-S or POST-S vs. POST-B.

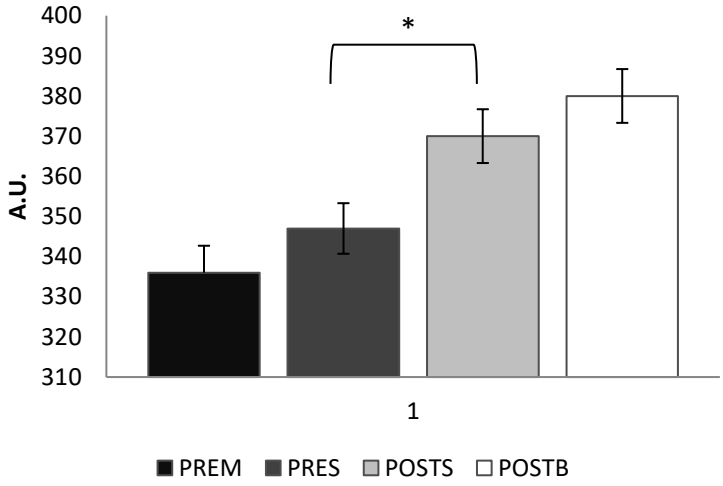

Figure 3.9 Relative cell circumference for dairy calves at 6 weeks of age, before weaning (PRE), or 9 weeks of age, post-weaning (POST). PRE-S: Milk, starter, hay; harvested pre-weaning (6 weeks). POST-S: Milk, starter, hay, no supplementation during weeks 7-8; harvested post-weaning (9 weeks). POST-B: Milk, starter, hay, 1% w/w butyrate supplementation during weeks 7-8; harvested post-weaning (9 weeks).From McCurdy et al. (2019). Relative cell circumference increased between PRE-S vs. POST-S (P = 0.02) but did not change between PRE-M vs. PRE-S or POST-S vs. POST-B.

3.5 Discussion

3.5.1 Effect of weaning and starter intake on VFA concentrations and rumen pH

The weaning transition and the intake of solid feed are mostly driven by the removal of milk feeding (Sweeney et al., 2010), which often results in a decrease in ADG through the weaning transition. This experiment was designed to encourage an increase in starter intake between PRE-M vs. PRE-S (pre-weaning), PRE-S vs. POST-S (weaning), and POST-S vs. POST-B (post-weaning, butyrate supplementation); these increases were observed and significant. Pre-weaning, increases in calf starter intake did not affect mean pH but did increase the percentage of propionate in the rumen (Appendix 3 and Appendix 4). Similar results were observed through the weaning transition despite a 2000g/d increase in starter intake and a four-fold increase in total VFA concentrations. Mean pH, VFA profile, and duration of subacute ruminal acidosis (SARA) were unaffected by treatments. An additional 800g/d of calf starter was consumed by POST-B calves during the week after weaning, and the calves then experienced a decrease in rumen pH and increased duration of SARA; despite the drop in rumen pH, POST-B calves had increased ADG during the weaning transition. Why a 2000g/d increase in starter was well tolerated by the rumen, but an additional 800g/d induced SARA is unknown. It is possible that the difference in physical size and age between PRE-S and POST-S calves negated the decrease in pH because of a larger, more developed rumen. Additionally, forage intake may have a buffering effect on the developing rumen (Laarman and Oba, 2011; Yohe et al., 2018) as is observed in adult cattle (Erdman, 1988). In lactating cows, decreasing diet ADF by one percent results in a 0.0564 drop in rumen pH (Erdman, 1988), and similar results have been observed in calves with low forage intakes (Laarman and Oba, 2011; McCurdy and Laarman, unpublished). Forage intake did not change for butyrate supplemented calves, indicating a greater percentage of NDF with the increase in calf starter intake. Although the developing rumen has the ability to adapt to large amounts of fermentation, it appears that this adaptation has limitations. Adaptation to large

amounts of fermentation may be connected to forage intake and, unlike in the adult rumen, a decrease in rumen pH does not appear to affect ADG or performance.

3.5.2 Impact of weaning transition on VFA transporter abundance

VFAs from ruminal fermentation provide energy to the rumen epithelium as well as the ruminant itself. Due to the neutral pH of the rumen and the status of most VFAs as weak acids, VFAs are often found in their dissociated form (Bergman, 1990). When a dissociated weak acid is transported into the epithelium, the proton associated with the acid will induce a slight drop in pH_i (Bergman, 1990) Methods to compensate for the additional protons include proton extrusion via transporters like NHE3, and also the import of bicarbonate via transporters like NBC1 (Sejrsen et al., 2006). MCT1 also serves as a proton extruder and may reduce the need for additional pH_i regulatory methods (Aschenbach, 2011).

In this study, solid feed intake increased between PRE-M and PRE-S but did not have an effect on rumen pH. Fermentation did occur in the PRE-M calves despite the lack of solid feed, likely because of milk leaking into the rumen via incomplete closure of the esophageal groove or during esophageal tubing events (Tamate et al., 1962). Solid feed intake in PRE-S calves resulted in a greater level of fermentation and thus VFA absorption by the rumen epithelium. An increase in NBC1 abundance from PRE-M to PRE-S calves was observed and this may be explained by pH_i homeostasis mechanisms. An increased amount of incoming VFAs, but no change in VFA export via MCT1, likely lowered the pH_i because of an increase in dissociated protons. The decrease in pH_i could require additional bicarbonate influx to regain pH_i homeostasis (Müller et al, 2000), in this case via NBC1.

During the weaning transition, VFA concentrations increased, but there was no change in NBC1 abundance. Volatile fatty acid absorption was not measured in this experiment, but the assumption that increased VFA concentrations equal increased absorption into the rumen epithelium

do not fully explain the reported changes in transporter abundance. An increase of VFA absorption would equal an increase in H^+ ions in the rumen epithelial cytosol. Besides NBC1, other transporters exist to expel protons from the cytosol and thus regulate pH_i, including MCT1 and NHE3, which may reduce the need for NBC1 activity (Aschenbach et al., 2011). Despite this claim, NHE3 abundance decreased in this study during weaning, and MCT1 abundance did not change. It is possible that other regulatory methods exists that are stimulated by the weaning transition. The only difference between the two treatment groups in the weaning comparison is that POST-S calves went through a two week weaning transition and thus tissues were harvested at the beginning of week 10 of life as opposed to PRE-S calves in which the tissues were e harvested in week 6. Thus, it is not possible to determine if the difference in NHE3 abundance was related to age or to an effect of the weaning transition. The decrease of NHE3 during the weaning transition indicates disrupted pH_i homeostasis. Schweigel et al. (2005) observed an inhibition of NHE3 with increased osmolarity in sheep epithelium, in conjunction with a pH_i decrease and ruminal acidosis. This is different from a study by Laarman et al. (2013), which observed an increase in NHE3 abundance in acidosis-challenged mid-lactation cows. While species differences exist, the rumen functions much in the same way, and the exact mechanism of the difference in pH_i regulation are unknown. Similarly, the difference in pH_i regulation between weaning calves and adult cows is unknown. In this study, there was no observed difference in duration of subacute ruminal acidosis between PRE-S and POST-S calves (Appendix 4), indicating that transporter abundance in weaning calves may respond differently than adult cows to an acidotic challenge. The POST-S calves did have an increased starter intake and a greater concentration of total VFA at harvest, which could cause a pH_i decrease because of VFA absorption, and subsequent inhibition of pH_i homeostasis due to NHE3 inhibition (Schweigel et al., 2005). The method of NHE3 inhibition is unknown though decrease in abundance seen in this study suggests a breakdown of NHE3 during weaning, or possibly sequestration of NHE3. The NHE3 cycles between the apical plasma membrane and intracellular vesicles, which is thought to be a form of regulation via sequestration (Kurashima et al., 1998). This could explain the reduction in

abundance observed during weaning, though the benefit of potentially acidifying the rumen epithelial cytosol during weaning is unknown. It is unlikely that inhibition of NHE3 abundance is due to breakdown of the transporter because the osmolarity induced changes were found to be reversible (Schweigel et al, 2005). It has been suggested that the inhibition pathway may involve protein kinase A dependent phosphorylation of NHE3 (Kurashima et al., 1998; Schweigel et al, 2005).

The expression of NHE3 is up-regulated by VFAs, especially butyrate (Kiela et al., 2007), but no change in NHE3 abundance was observed between POST-S and POST-B calves despite butyrate supplementation. It is unknown why NHE3 abundance was affected by the weaning transition but not by the increase in severity and length of SARA with butyrate supplementation. Sub-acute ruminal acidosis in the POST-B calves did not affect their growth rate; POST-B calves increased their ADG during the weaning transition despite effects in the rumen that would be considered negative in adult cows (Appendix 5). Despite the increase in calf starter intake, VFA concentrations in the rumen tended to decrease with butyrate supplementation, and yet no change in MCT1 abundance was observed (Yohe et al., 2018). Pre-weaning, solid feed consumption does not change the VFA absorption potential of the rumen epithelium (Yohe et al., 2018), and, from this study, we can conclude that the same is true of the weaning transition. Increasing calf starter intake with butyrate supplementation was beneficial for weight gain, and the drop in rumen pH with sustained SARA did not appear to affect transporter abundance, pH_i regulation, or growth in weaning calves.

3.5.3 Impact of diet on relative cell size

Relative cell circumference increased between PRE-S and POST-S but did not change pre-weaning or with butyrate supplementation. The observed change in circumference and the localization of the transporters examined in this study led to relative cell circumference being included as a covariate in the statistical model.

The weaning transition appears to induce drastic developmental changes in the rumen, including an increase in relative cell circumference and thus cell size. Despite the addition of solid feed to the diet and an increase in fermentation, relative cell circumference was not affected pre-weaning. Similarly, relative cell circumference was not affected with butyrate supplementation despite the changes in rumen fermentation characteristics and pH. It is possible that the difference observed in relative cell circumference is related to age; PRE-S calves were slaughtered at 6 weeks of age, whereas POST-S calves were slaughtered at 9 weeks of age. Whether an increase in relative cell circumference at weaning is a sign of maturation is unknown. Maturation of developing tissue requires cell proliferation, though cells will only divide when they reach a critical size (Montagne et al., 1999). The mechanism of cell size regulation is unknown, though appears to be related to the S6 kinase gene in *Drosophila*, which has been shown in knock-in mice (Ruvinsky and Meyuhas, 2006). The regulation of cell size in rumen tissue has yet to be determined, but this data suggest that ruminal maturation may depend upon changes in relative cell circumference regulation. With no change in MCT1 or NBC1 abundance during weaning, an increase in relative cell circumference may impose a dilution effect on the density of these transporters. The reduction of NHE3 abundance during weaning was discussed previously and may be regulated by other mechanisms.

Limitations of the relative cell circumference measurements exist and should be considered. The images taken to assess transporter abundance are captured in a single plane, and therefore the relative cell circumference that was measured is a cross-sectional slice of each cell. Similar methods are used to measure the cross-sectional area of muscle fibers (Round et al., 1982), and may be applicable to the rumen epithelium. Further refinement of measurement techniques are required to understand fully what is happening in the developing rumen in terms of cell size and relative cell circumference, perhaps expanding to cell volume and membrane surface area. At this time, there appears to be no effect of increasing relative cell circumference on transporter abundance and density.

3.6 Conclusion

The weaning transition did not have an effect on rumen pH despite vast increases in calf starter intake, possibly because of changes in pH_i regulation, changes in cell size, and the buffering capacity of ad libitum hay. VFA concentration and fermentation rate in the rumen do not appear to alter VFA transport capacity in the developing rumen, nor does incidence of acidosis. Increased incidence of acidosis did not affect NHE3, NBC1, or MCT1 abundance, though calves may need to be followed for longer than one week post supplementation to see an effect. Without changes in transport proteins and sufficient regulation of pH_i, the rumen epithelium may be acidified during the weaning transition; the long term effects of this acidification are unknown. Additionally, the buffering capacity of hay seems to have a limit as additional starter intake caused a drop in rumen pH and a greater incidence of subacute ruminal acidosis. Whether increased hay consumption or an increase in NBC1 and NHE3 could mitigate the drop in pH when further increasing starter intake is unclear. Either way, the weaning transition is improved by butyrate supplementation but its effects on total VFA uptake capacity and cell size require additional research.

3.7 References

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Chapter 4: General Discussion

4.1 Study Summary

This research examined the impact of butyrate on absorption of IgG and VFA in young calves. Two studies were carried out: Study 1 examined the impact of supplemental butyrate on absorption of IgG in newborn calves, while study 2 examined the impact of supplemental butyrate on VFA transport capacity during the weaning transition. These studies provide information on the role of nutritional management in improving calf health and development.

Study 1 examined the effects of supplemental butyrate on colostrum production in dams and on passive transfer of immunity in dairy calves. We hypothesized that supplemental butyrate would increase colostral IgG concentrations and also would increase IgG absorption in calves, as observed in sows and piglets. No differences were observed in dam DMI or in serum and colostrum IgG concentrations. In calves, colostrum supplementation with butyrate decreased IgG concentrations starting at 24 hours of age and sustained through week 2 of life. Despite lower blood IgG concentrations, butyrate-supplemented calves did not differ from control calves in ADG, serum glucose, and serum BHB concentrations. Study 1 showed a negative effect of supplemental butyrate in colostrum fed to neonates, while showed no effect on colostrum production in dams.

Study 2 examined the effects of weaning and supplemental rumen-protected butyrate on rumen fermentation characteristics, growth, and rumen epithelial transporter abundance. We hypothesized that supplemental butyrate would increase transporter abundance and calf starter intake during the weaning transition. The addition of solid feed to the pre-weaning diet tended to increase total VFA and NBC1 abundance. During the weaning transition, calf starter intake and total ruminal VFA concentrations increased, while mean pH remained unaffected, and NHE3 abundance, a sodium/proton exchanger, decreased. Supplemental butyrate during weaning increased calf starter intake and ADG, but decreased rumen pH and extended the length of subacute ruminal acidosis. Despite the increased acidosis, transporter abundance did not differ post-weaning. Study 2

demonstrated adaptive capabilities of the rumen during the weaning transition and increased weight gain with supplemental butyrate, and increased incidence of subacute ruminal acidosis did not appear to affect transporter abundance.

4.2 Butyrate Supplementation Shortens Window of IgG Absorption

In study 1, supplementation of butyrate in the colostrum reduced IgG AEA and serum IgG levels in neonatal calves. IgG absorption in neonatal calves is nonselective (Staley et al., 1972) and does not appear to be paracellular due to a lack of change in tight junctions during the first 24 hours of life (Jochims et al., 1994). Likely the method of absorption involves pinocytosis, as pinocytotic activity is lost when the epithelial cells mature (Jochims et al., 1994). Butyrate has been shown to increase mitosis:apoptosis ratios in small intestinal epithelium, indicating a faster maturation rate (Górka et al., 2014). It is likely that the butyrate supplemented in this study increased the maturation rate of the intestinal epithelium, thereby decreasing the window of nonselective pinocytotic activity and thus limiting IgG absorption. Further research should focus on the stimulus of cell maturation that causes an end to pinocytotic activity and closes the window for IgG absorption.

4.3 Solid Feed Intake Does Not Effect VFA Absorption Capacity

Study 2 suggests that the rumen has an immense capacity to buffer pH when highly fermentable starches are consumed during the weaning transition, but may not have the capacity to absorb the increased VFAs. MCT1 abundance did not change during pre-weaning, weaning, or post-weaning in this study, suggesting a limited capacity for fluctuation in VFA absorption, though it is important to note that other VFA transporters and transport mechanisms exist (Dengler et al., 2015). MCT1 abundance is an important determinant of VFA transport (Dengler et al., 2015), often being described as the VFA transport “bottleneck”, and is responsive to butyrate supplementation in adult cattle (Laarman et al., 2012; Laarman et al., 2013a). No change in MCT1 abundance in this study and in similar studies pre-weaning (Yohe et al., 2018) suggest that VFA absorption capacity is driven

by other factors in the developing rumen. Why butyrate affects VFA absorption capacity at the genome level (Laarman et al., 2012), but not the protein level or ruminal absorption kinetics (Yohe et al., 2018) is unclear.

4.4 Solid Feed Intake Affects Transporters Involved in pH_i Regulation

Increased intracellular acidotic pressure would necessitate an additional influx of alkalotic molecules, or additional output of protons (Aschenbach et al., 2011). Pre-weaning, increased calf starter intake is associated with a "protective" effect of pH_i homeostasis; bicarbonate import likely increased with increased NBC1 abundance. Through the weaning transition, changes in osmolarity caused by increased fermentation appear to inhibit NHE3 abundance, decreasing proton export and possibly acidifying the rumen epithelium. This effect has been previously reported in sheep (Schweigel et al., 2005) but is novel to calves and the weaning transition. Post-weaning, increased calf starter intake had no effect on pH_i homeostasis. Why the developing rumen is capable of regulating pH_i pre-weaning during increased fermentation, but not during weaning, is unknown. Pre-weaning, weaning, and post-weaning occurred within a three-week span; how the developing rumen is able to regulate pH_i, then lose regulatory capacity, and then not change in response to increases in calf starter intake is also unknown and requires further study. The weaning transition and rumen development are intimately linked and understanding fluctuations in rumen parameters during development will help improve the weaning transition.

4.5 Future Research

Study 1 demonstrated that the window of IgG absorption in neonatal calves can be manipulated. Though the results of this study were negative, further research on how butyrate increases the maturation rate of intestinal epithelial cells is needed to understand the cessation of pinocytotic activity.

Butyrate supplementation had positive effects on calf starter intake in study 2, and understanding the effect of butyrate on intestinal epithelial maturation may help explain the positive effects it has during weaning. It also would give an answer as to when butyrate supplementation could be started in young calves, or whether butyrate supplementation should be fed at all. For early weaning programs, ensuring calves eat starter is essential for early rumen development (Anderson et al., 1987). Butyrate may be a tool to encourage starter intake and reduce the time to weaning in commercial operations, but should be avoided in the first few days of life due to its detrimental effects on IgG absorption.

Ruminal fermentation and pH results from study 2 give a snapshot of the rumen environment before and after the weaning transition, but do not accurately portray the VFA profile and pH of the rumen during the weaning transition. Further research that investigates pH changes during the weaning transition itself will be important to understanding both the transporter abundance changes observed in study 2 and the lack of rumen pH response before and after weaning.

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Appendix 1

IACUC Approval for Experiment 1

Laarman, Anne (annelaarman@uidaho.edu)

Van: Institutional Animal Care and Use Committee (iacuc@uidaho.edu)
Verzonden: dinsdag 7 juni 2016 4:38
Aan: Laarman, Anne (annelaarman@uidaho.edu)
Onderwerp: IACUC protocol 2016-32 Approved

University of Idaho
Institutional Animal Care and Use Committee

Date: Friday, June 3, 2016
To: Anne Laarman
From: University of Idaho
Institutional Animal Care and Use Committee
Re: Protocol 2016-32
Seed Grant: Mechanism of butyrate action on gene expression in rumen tissue

Your animal care and use protocol for the project shown above was reviewed and approved by the Institutional Animal Care and Use Committee on Friday, June 3, 2016.

This protocol was originally submitted for review on: Wednesday, May 18, 2016
The original approval date for this protocol is: Friday, June 3, 2016
This approval will remain in effect until: Saturday, June 3, 2017
The protocol may be continued by annual updates until: Monday, June 3, 2019

Federal laws and guidelines require that institutional animal care and use committees review ongoing projects annually. For the first two years after initial approval of the protocol you will be asked to submit an annual update form describing any changes in procedures or personnel. The committee may, at its discretion, extend approval for the project in yearly increments until the third anniversary of the original approval of the project. At that time, the protocol must be replaced by an entirely new submission.

Barrie Robison, IACUC Chair

Appendix 2

IACUC Approval for Experiment 2

University of Idaho
Institutional Animal Care and Use Committee

Date: August 25, 2017
To: Dr. Anne Hermen Laarman
From: University of Idaho
Institutional Animal Care and Use Committee
Re: Approval of personnel amendment request for Protocol IACUC-2017-33 *Impact of butyrate supplementation on colostrum quality and passive transfer of immunity*

Your personnel amendment requested submitted on 08/23/2017 04:48:15 PM PDT to the animal care and use protocol listed above was administratively reviewed and approved by the Institutional Animal Care and Use Committee on 08/25/2017.

The original approval date for this protocol was: 07/24/2017
This protocol approval will remain in effect until: 07/23/2018
The protocol may be continued by annual updates until: 07/23/2020

Currently approved personnel on this protocol are: Hiltz, Rebecca; Laarman, Anne Hermen; Lowe, Summer; McCurdy, Dana; Stevens, Allison; Vinyard, James; Watts, Corinna; Wilkins, Katie

Federal laws and guidelines require that institutional animal care and use committees review ongoing projects annually. For the first two years after initial approval of the protocol you will be asked to submit an annual update form describing any changes in procedures or personnel. The committee may, at its discretion, extend approval for the project in yearly increments until the third anniversary of the original approval of the project. At that time, the protocol must be replaced by an entirely new submission.

Craig McGowan, IACUC Chair

Appendix 3

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