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Ruminal Acidosis in Dairy Calves During the Weaning Transition

by

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DEDICATION

I dedicate this thesis to my parents, for all their support and encouragement.

ABSTRACT

The goal of this research was to evaluate the effects of calf starter fermentation on rumen pH, metabolic adaptation of ruminal epithelia and growth of calves during the weaning transition. In study 1, calf starter consumption increased short chain fatty acid concentration in the rumen and changed expression of genes involved in ketogenesis and intracellular pH regulation but did not affect rumen pH. In study 2, decreasing dietary starch content did not increase rumen pH. However, feeding a calf starter that had the highest in situ dry matter disappearance lowered rumen pH without adversely affecting feed intake and growth of calves. These findings suggest that rumen epithelium can metabolically adapt to increased fermentation in the rumen at molecular level, and that sub-acute rumen acidosis may not adversely affect feed intake and productivity of dairy calves during the weaning transition.

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LIST OF ABBREVIATIONS

AcAc	Acetoacetate
ACAT	Acetoacetyl CoA thiolase
ACTB	β-actin
ADG	Average daily gain
ARA	Acute ruminal acidosis
AUC	Area under the curve
BDH	β -hydroxybutyrate dehydrogenase
BHBA	β –hydroxybutyrate
BP	Calf starter where dry ground corn was partially replaced by beet pulp at 10.2% dry matter
BW	Body weight
CA	Carbonic Anhydrase
CORN	Calf starter with dry ground corn at 27.2% dietary dry matter
CP	Crude protein
d	day(s)
DDGS	Calf starter where dry ground corn was partly replaced by triticale dried distillers' grains with soluble at 18.6% dry matter

DM	Dry matter
DMI	Dry matter intake
EE	Ether extract
g	gram(s)
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HMGL	3-hydroxy-3-methyglutaryl-CoA lyase
HMGS	3-hydroxy-3-methyglutaryl-CoA synthase
LCFA	Long chain fatty acid
MCT	Monocarboxylate transporter
min	Minute(s)
MR	Milk replacer
MR+S	Milk replacer and starter
NBC	Sodium/bicarbonate co-transporter
NDF	Neutral detergent fibre
NHE	Sodium/proton exchanger
OM	Organic matter
pH_i	Intracellular pH

RPLP0	Ribosomal Protein Large, P0
SARA	Sub-acute ruminal acidosis
SCFA	Short chain fatty acid(s)

1.0 LITERATURE REVIEW

1.1 General Introduction

Neonatal calves do not have a functional rumen, yet adult cows rely on ruminal fermentation end-products for over 60% of their energy needs (Bergman, 1990). The neonatal calves need to develop the rumen morphologically and physiologically. The course of rumen development is divided into 3 major phases: the liquid-feeding phase, the transition phase and the ruminant phase (NRC, 2001). The liquid-feeding phase generally lasts 2-3 weeks and ends when calves begin to consume some solid feed such as calf starter, at which point the transition phase begins, and it lasts until weaning (Drackley, 2008). Weaning marks the beginning of the ruminant phase, which lasts for the rest of the bovine's life (Drackley, 2008). Pre-weaning mortality of heifer calves has been found to be approximately 4.5 % in Ontario (Waltner-Toews et al., 1986) and in the Netherlands (Perez et al., 1990); bull calf mortality has been reported at 20% in the first four months of life with the majority of deaths occurring in the first four weeks (Perez et al., 1990).

The first phase of rumen development, the liquid feeding phase, is characterized by a complete reliance on milk as the primary source of nutrients of the young calf. The reflex closure of the esophageal groove shunts milk past the rumen and into the omasum and abomasum, where milk starts to be digested efficiently (Drackley, 2008). During the liquid-feeding period, average daily gain (ADG) is higher for calves fed greater amounts of milk (Jasper and Weary, 2002). Currently, however, Dairy NRC (2001) recommends that milk provision be limited to 8 – 10% of birth body weight (BW), around half of ad libitum intake, so that calves will remain hungry, causing starter intake to be maximized earlier in life. When starter intake reaches 680 g/d for 3 days, the rumen is considered sufficiently developed for calves to be weaned (NRC, 2001). Earlier weaning is more economically favourable as

the pre-weaning period is characterized with much higher feed and labour costs as well as a higher incidence of morbidity (Davis and Drackley, 1998), though the morbidity is more likely to be a function of age and immune system immaturity than feeding programs.

The consumption of dry feed, while beneficial to rumen development, may be associated with risk of rumen acidosis. Fermentation of calf starter produces short chain fatty acids (SCFA). In the rumen of a mature cow, over 50% of all protons and 60 – 80% of all SCFA are transported across the rumen wall, while in the calf rumen, 80 – 85% of all SCFA are transported across the rumen wall (Allen, 1997), so the luminal side of the rumen epithelium puts a considerable acidotic stress on the rumen epithelium. If the proton removal rate from the rumen is lower than the rate of proton production, rumen pH drops and the rumen environment can become acidotic. Transepithelial proton transport and the repercussions of rumen acidosis will be covered in more depth later, but it is important to be aware of the risks associated with starter intake during rumen development.

1.2 Calf Management

1.2.1 Milk and Milk replacers

During the first 3 weeks of life, the liquid-feeding phase, milk or milk replacer is the main energy and protein source, and the growth of calves in this time frame is directly proportional to the amount of liquid provided (Khouri and Pickering, 1968). Whether milk is provided once or twice a day does not appear to have a significant impact on calf performance or glucose metabolism (Stanley et al., 2002). However, the feeding frequency and consistency of feeding times are very important. Calves fed milk replacer (MR) consistently at 0.68 kg/d of MR powder had greater ADG pre- and post-weaning, greater starter intake pre- and post-weaning, and greater feed efficiency pre-weaning than calves fed an average of 0.68 kg/d of MR powder at random, variable feeding rates (Hill et al., 2009b).

Calves are normally fed whole milk or milk replacer during the liquid-feeding phase. In the US, slightly more than half of farmers feed milk replacer to calves, though the crude protein (CP) and fat content of the MR vary (Heinrichs et al., 1995). It should be noted that MR contains 13% less metabolizable energy (ME) than the same volume of milk solids (NRC, 2001), so similar amounts of milk replacer and whole milk represent two very different planes of nutrition. Historically, whole milk is considered to be the better liquid feed for pre-weaned calves as it can have health benefits such as reducing incidence of diarrhaea (Blome et al., 2003), although these benefits depend on MR quality. Since whole milk is generally sold for human consumption, MR and non-saleable whole milk can be used as a cheaper alternative to whole milk.

Waste milk is whole milk that cannot be sold for human consumption; its sources include lactating cows being treated with antibiotics and cows immediately postpartum (after colostrum). Although waste milk has been shown to support greater ADG and lower morbidity and mortality than conventional milk replacers there is considerable variation in the quality of waste milk (Moore et al., 2009). Spoilage occurs frequently and total solid concentration in waste milk is often lower than expected, while non-pasteurized waste milk also often contains large numbers of bacterial pathogens (Moore et al., 2009). The variation in quality of whole milk may be disadvantageous; calves fed MR, which had lower bacterial counts than whole milk or half milk and half MR, experienced greater ADG and starter intake pre-weaning (Hill et al., 2009b). Due to the inherently variable nature of waste milk, milk replacer can be an appealing alternative.

In the US, conventional milk replacers contain 20% CP and 20% fat. Increasing CP in MR to 22 and 26% increased average daily gain and plasma glucose, insulin, and IGF-1 concentrations (Bartlett et al., 2006) and increased the gain to feed ratio (Blome et al., 2003). Further, decreasing CP content of MR from 27% to 25% in dairy calves decreased average daily gain (ADG) even when lysine, methionine and threonine concentrations were maintained (Hill et al., 2009a). Intake of CP, however, is

not only a function of CP content of the MR, but also affected by type of liquid-feeding program. Conventional liquid feeding programs feed considerably less MR than intensive liquid feeding programs, so CP intake in conventional liquid feeding programs are lower because the volume fed is lower.

1.2.2 Liquid-feeding Programs

Conventional liquid-feeding programs are developed mostly based on findings that rumen development and metabolic activity are stimulated by end-products of solid feed fermentation such as propionate and butyrate (Sander et al., 1959). The sooner a calf consumes solid feed, the sooner the rumen papillae will grow, and limit-feeding milk at 10% of BW is recommended to encourage starter consumption (NRC, 2001). During the first two weeks of life, however, calves fed 3.1 and 4.8 kg/d of milk replacer – approximately 10% of birth BW – had lower ADG than calves fed 6.6 or 8.3 kg/d (Kristensen et al., 2007). When reconstituted whole milk solid was fed ad libitum, calves consumed 19% of BW (Khouri and Pickering, 1968), nearly twice the rate suggested by the NRC (2001). In a conventional liquid-feeding program, calves are likely in a starvation state, which may undermine resistance to disease. Calves fed at 50% of maintenance requirements in the first 4 weeks of life depressed immune response amplitude (Griebel et al., 1987), making them more prone to disease. During the first 6 months, calves suffering from septicimia and pneumonia can take 2 weeks longer to reach the same body weight as healthy calves (Donovan et al., 1998). Increased susceptibility to disease when calves are underfed risks delaying growth, undermining the economic goals of conventional liquid-feeding programs.

Recently, intensified liquid feeding, in which high amounts of milk are provided early in life, has provided an alternative to conventional liquid feeding programs. Average daily gain post-weaning

is significantly higher under an intensified nursing program than under a conventional nursing program (Khan et al., 2007b). Moreover, papilla length, width, density and rumen wall thickness were all significantly greater under the intensified program than under the conventional program (Khan et al., 2007a). These results suggest that rumen development can be significantly enhanced by the intensified program. After several weeks of consistent feeding, a reduction in MR causes starter intake to increase (Khan et al., 2007b), regardless of whether the reduction in MR represents partial or complete elimination of MR provision.

Previously, ad libitum milk feeding has been found to decrease starter intake pre-weaning (Huber et al., 1984, Jasper and Weary, 2002, Khan et al., 2007b). Starter intake depression may be attributed to the high fat content of milk. When calves were fed MR that contained 17 – 20% fat, pre-weaning starter intake was higher than in calves fed MR containing 23% fat (Hill et al., 2009c). In whole milk, fat content can be over 30% of DM (Huber et al., 1984), which may explain why some studies found lower starter intakes when whole milk was fed pre-weaning. While some studies have found a lower rate of starter intake increase pre-weaning as a possible result of high MR fat content, the depressed rate of increase of calf starter intake disappears after weaning (Jasper and Weary, 2002, Khan et al., 2007b).

Calves can be weaned as early as 28d of age (NRC, 2001), and although early weaning is associated with increased starter intake, it does not necessarily increase body weight gain (Hopkins, 1997). In Canada, half of dairy producers wean calves at 6 – 8 week old (Vasseur et al., 2010). However, since rumen development can be enhanced by intensified nursing programs, conventional nursing programs may not foster optimal growth rates in the long term. Nonetheless, as with selection of nursing program, optimum age for weaning is partly influenced by economics, as both intensified nursing programs and delayed weaning increase the cost of liquid feed per calf.

Some of the additional pre-weaning feeding costs can be compensated as the provision of extra energy pre-weaning has long-term benefits. Specifically, when fed whole milk or milk replacer in equal amounts, heifer calves receiving whole milk pre-weaning, when mammary gland growth is isometric, produced 3.1 kg/d more milk in their first lactation than calves fed MR, and heifer calves receiving whole milk had a greater protein and lactose content in their milk in the first lactation (Moallem et al., 2010). It should be noted that the fat content in whole milk was 13% higher than in MR, so the observed differences in milk production are likely attributed to the greater pre-weaning energy intake. Historically, elevated prepubertal energy supplies had been discouraged because higher planes of nutrition during the prepubertal phase, when mammary growth is allometric, were associated with decreased mammary development (Sejrsen et al., 1982).

1.2.3 Calf Starter

Intake of calf starter, a readily fermentable solid feed source, stimulates the fermentation necessary to facilitate metabolic and morphological rumen development. A minimum particle size of 2000 µm is recommended to facilitate rumination (Porter et al., 2007). Calves fed coarse mash calf starter initiated rumination earlier and have faster ADG than calves fed pelleted starter (Porter et al., 2007), while calves fed texturized calf starter consumed more grain and have a greater BW at 6 weeks of age than calves fed pelleted starter (Franklin et al., 2003). Physical characteristics such as texture and particle size are important factors affecting calf starter intake. Intake can also be affected by ingredients of calf starter; calves fed a corn-based calf starter had greater dry matter, starch and CP intakes and higher ADG than calves fed barley-, oat- or wheat-based calf starters (Khan et al., 2007c). Previously, calf starter containing 12% molasses has been shown to decrease BW gain, heart girth and starter intake as compared to calf starter containing 5% molasses (Lesmeister and Heinrichs, 2005).

The beginning of solid feed intake coincides with a rapid growth of the rumen relative to the rest of the digestive tract (Walker and Walker, 1961). The growth of the rumen is dependent on end-products of solid feed fermentation (Warner et al., 1956), specifically propionate and butyrate (Sander et al., 1959). When calf starter is fed to calves, as opposed to hay, concentration of propionate and butyrate in rumen fluid increased (Stobo et al., 1966)

The emphasis on encouraging starter intake is based on the idea that calves should be weaned when the rumen is sufficiently developed and that calves with the fully-functioning rumen can consume more calf starter. As such, starter intake can be an indicator of rumen development. It is recommended, under conventional nursing programs, that Holstein calves be weaned once they consume starter at a rate of 700 g/d (NRC, 2001) – 1000 g/d (Drackley, 2008). Starter consumption post-weaning is higher when calves are weaned over a 10-d period of gradual reduction in milk feeding rather than abrupt weaning (Sweeney et al., 2010).

1.2.4 Roughage and Water

Water is an important nutrient, as it maintains a liquid environment for the rumen microbiota. Water intake stimulates starter intake as calves with restricted water intake decreased dry matter intake by 31% (Kertz et al., 1984). Thus, it is recommended that clean, "free" water be provided at least once daily to optimize health and stimulate dry feed intake (Davis and Drackley, 1998). Free water is water that is not part of milk or milk replacer. Free water is vital in maintaining the liquid environment for microbes in the rumen to survive (Quigley, 2001).

Although the importance of free water for pre-weaned calves is widely accepted, the importance of roughage intake is still a matter of debate. Inclusion of roughage in a calf diet prior to weaning did not affect ADG (Suárez et al., 2007) while others found that calves fed hay before weaning decreased

starter intake and ADG compared to those fed starter alone (Hill et al., 2008, Stobo et al., 1966, Warner et al., 1956). When alfalfa hay was fed with the concentrate, as opposed to being fed separately, starter intake increased by more than 5% and BW increased by 10% at 16 weeks of age (Bartley, 1973). However, long hay was less effective than concentrates at stimulating ruminal epithelium development (Stobo et al., 1966), so long hay is not recommended for pre-weaned calves (Davis and Drackley, 1998).

A mature ruminant derives over 60% of total energy needs from fermentation end-products (Bergman, 1990), thus the ability to absorb SCFA is vital. Although rumen capacity can increase to as much as 35 L by 14 weeks of age when a 75%-forage diet is fed to calves, rumen papillae do not grow to the same length (Stobo et al., 1966). Due to variable effects of hay consumption on ADG and rumen papillae growth (Bartley, 1973, Hill et al., 2008, Stobo et al., 1966, Warner et al., 1956), it is unclear what type and amount of hay ought to be provided.

1.3 Rumen pH

Rumen pH, normally ranging from 5.5 to 7.0, is an important factor affecting rumen function. The primary source of protons in the rumen is the fermentation of organic matter of feedstuffs. Protons are removed from the rumen by neutralization, passage through the digestive tract, or absorption across the rumen wall. The major source of buffer is saliva, which has been estimated to neutralize up to 30% of protons in the rumen of mature dairy cows (Allen, 1997). Saliva is rich in HCO_3^-, which acts as a buffer and removes protons from the rumen. Proton passage from the rumen, as $H_2PO_4^-$, NH_4^+, or associated SCFA is estimated to account for 15% of proton removal from the rumen (Allen, 1997). The remaining protons are removed with absorption of SCFA, and trans-epithelial SCFA transport will be discussed more in depth later.

1.3.1 Causes of Ruminal Acidosis

When protons accumulate after fermentation of feedstuffs, rumen pH drops drastically. Ruminal acidosis can be either sub-acute or acute. Sub-acute ruminal acidosis (SARA) is not associated with overt clinical signs, but accompanied by high SCFA concentrations in the rumen without an increase in lactate concentration (Schwartzkopf-Genswein et al., 2003). Acute ruminal acidosis (ARA) is accompanied by elevated rumen lactate concentrations that is caused by proliferation of *Streptococcus bovis* (Russell and Hino, 1985), decreased salivation and redistribution of systemic water (Slyter, 1976) as well as overt clinical signs such as decreased appetite and ruminal motility (Allen, 1997). Various pH thresholds have been proposed, rumen pH ranging from 5.5 to 5.8 is defined as SARA, and rumen pH 5.0 to 5.2 is defined as ARA (Schwartzkopf-Genswein et al., 2003). Because rumen pH is very dynamic, SARA and ARA thresholds of 5.5 and 5.0 are more appropriate when doing single measurements while thresholds of pH 5.8 and pH 5.2 for SARA and ARA, respectively, appear more appropriate when measuring rumen pH continuously (Aschenbach et al., 2010). For instance, a single hypothetical pH measurement of 5.75 is not very informative as the value of 5.75 could be part of the depression, the nadir, or part of the recovery from an episode of SARA, so having a threshold of 5.6 instead of 5.8 allows for a more conservative estimate of whether SARA is occurring or not. When continuous measurements are used, it is much clearer if a measurement of 5.75 is part of a bout of SARA and ARA or not, so the threshold does not need to be as conservative.

It is important to accurately measure the duration and severity of ruminal acidosis that are indicated by the time spent below the threshold pH (e.g., 5.8) and the area (pH × time) under the threshold, respectively (Figure 1.1). Mean pH based on multiple time-point measurements can be a misleading indicator as it does not fully take into account the frequency, duration and severity of acidotic bouts (Schwartzkopf-Genswein et al., 2003). As there is diurnal variation in rumen pH *in vivo* (Wales et al., 2004), continuous pH measurements rather than averaging multiple time-point

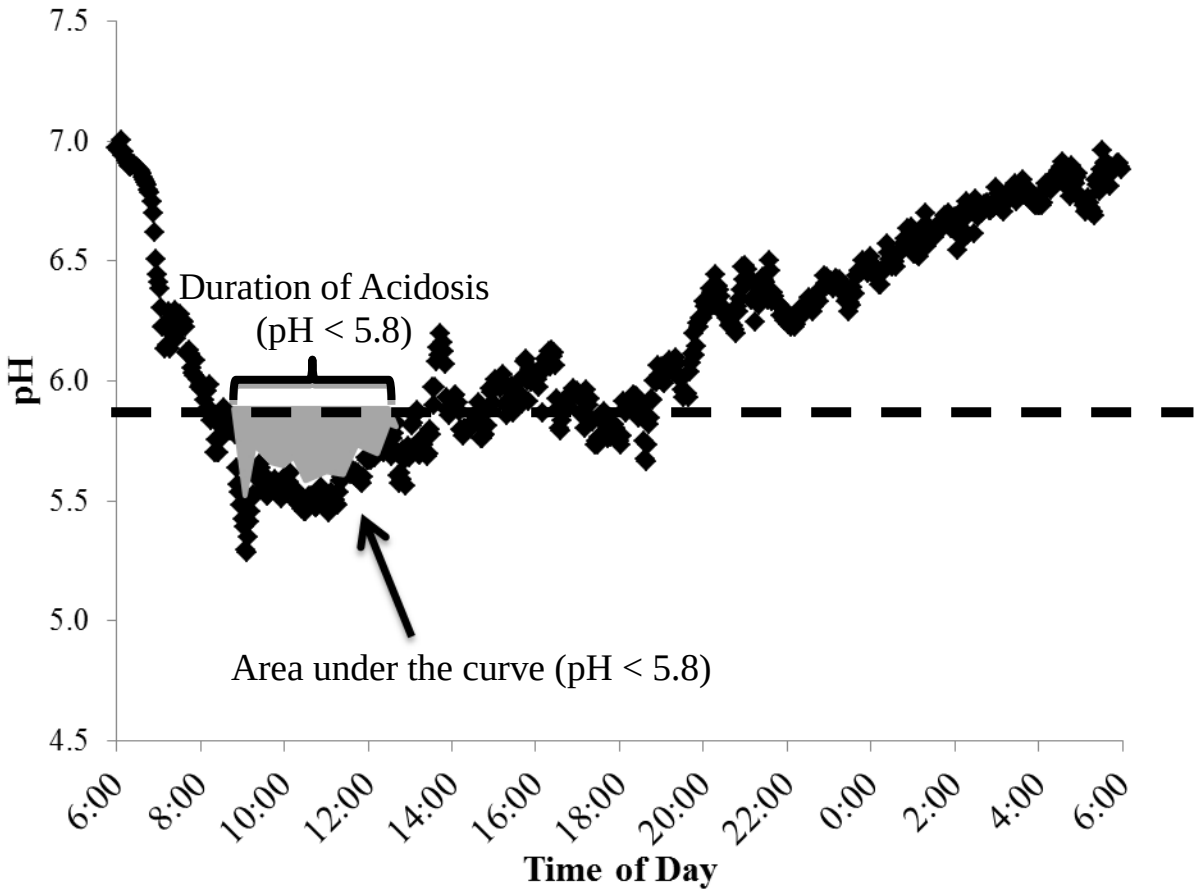

Figure 1.1 Example data from continuous pH measurements using a small ruminant rumen pH logger system (SRS), as described by Penner et al. (2009). Continuous pH measurements allow for the measurement of duration of sub-acute ruminal acidosis (SARA), where rumen pH < 5.8, as well as the area under pH 5.8, which gives an objective measure of SARA severity.

measurements are necessary for diagnosis of rumen acidosis. Continuous measurement of pH allows for the elucidation of baseline pH. In cows where the baseline rumen pH is low, reductions in pH increase the severity of ruminal acidosis compared with cows where baseline rumen pH is high (Dohme et al., 2008).

1.3.2 Effects of Ruminal Acidosis on Digestion

Greater severity of ruminal acidosis is detrimental to rumen health because prolonged ruminal acidosis can cause rumenitis and papillae abnormalities, decreasing the absorptive capacity of the rumen epithelium (McGavin and Morrill, 1976, McManus et al., 1977). In a continuous culture fermenter, in vitro SCFA concentration is lower at pH 5.6 than at pH 6.1 (Wales et al., 2004). In addition, SCFA concentration is also lower when there is diurnal variation in rumen pH than when pH is constant (Wales et al., 2004). Together, the rumenitis, papillae abnormalities and the depressed SCFA production at lower and variable pH show that ruminal acidosis can impede normal rumen function.

Rumen pH also affects extent of fibre digestion. When rumen pH decreases below 6.2, moderate reductions in fibre digestibility were observed (Hoover, 1986). At pH below 6.0 growth rate of cellulolytic bacteria is compromised (Russell and Wilson, 1996). Furthermore, neutral detergent fibre (NDF) digestibility decreases linearly with increased duration of rumen acidosis (Cerrato-Sánchez et al., 2007) and in vitro cellulose digestion ceases at rumen pH below 5.2 (Stewart, 1977). Fibre digestion in the rumen is thus compromised during SARA and cellulose is not digested during ARA.

1.3.3 Dietary Risks and Management of Acidosis

Increased starch concentration in feed and increased starch intake increase the risk of acidosis (Zebeli et al., 2008). This is likely due to the rapid fermentation of starch; rapid fermentation drops rumen pH if protons are not readily neutralized or absorbed. Supplemental dietary glucose has been shown to increase SCFA production in vitro (Piwonka and Firkins, 1996). Greater fermentation in the rumen increases availability of energy to animals, but it also increases acid load in the rumen.

Some nutritional management strategies have been developed to minimize occurrence of SARA. Greater dietary inclusion rate of physically effective NDF (peNDF) increases salivary buffer flow and dietary forage NDF content is positively related to ruminal pH (Allen, 1997). When rumen pH is low, near 5.0, cows actively sort their feed to favour intake of long particle forage (Yang and Beauchemin, 2006), suggesting that adult cows can modify their feeding behaviour to mitigate ruminal acidosis.

1.3.4 Effects of Ruminal Acidosis on Rumen Microbiota and Systemic Immunity

The rumen is an anaerobic environment that contains several major groups of microbes that break down fibre and starch into simple sugars, ferment simple sugars into SCFA and CO_2, and reduce CO_2 into CH_4 (Baldwin and Allison, 1983). Diet is the primary factor that affects bacterial populations (Krause et al., 2003), so changes in diet and microbial profile are closely linked. Dietary changes, especially adaptation to high-grain diets, can be associated with drastic changes in fibrolytic and amylolytic bacteria population and can result in a distinct microbial population structure in the rumen (Fernando et al., 2010).

Systemic immunological stress can also be increased by SARA. Gram-negative bacteria in the rumen are more susceptible to low rumen pH (Nagaraja et al., 1978). Extended exposure to low pH during SARA causes rupturing of the outer membranes of gram-negative bacteria, increasing lipopolysaccharide (LPS) concentration in the rumen by 300% (Gozho et al., 2006). Simultaneously, low pH also increased rumen epithelium permeability (Emmanuel et al., 2007), increasing the risk of LPS absorption into the blood stream. The LPS is recognized by the innate immune system, causing an immune response through production of acute phase proteins such as serum amyloid A and haptoglobin (Plaizier et al., 2008). Some LPS damage lamellae capillaries in the foot, leading to laminitis and liver abscesses (Nocek, 1997, Plaizier et al., 2008). Inflammation, as a result of the immune response, also poses an indirect systemic risk through opportunistic pathogens such as *Fusobacterium necrophorum* and *Arcanobacterium pyogenes*, which are associated with liver abscesses, foot claw lesions, calf diphtheria and mastitis (Nagaraja et al., 2005).

Incidence of SARA can also have other detrimental health effects. Rapid adaptation to a high concentrate diet, which decreases ruminal pH, can cause rumen thiamanase, which breaks down thiamin to levels where polioencaphalomalacia, a disorder of the forebrain, can occur (Brent, 1976). Also, SARA may also affect gelatinoproteinases, causing an elongation of collagen fibre, ultimately making the third phalanx in the hoof more mobile (Cook et al., 2004), which makes cows more susceptible to claw horn lesions and laminitis.

Rumen function is also affected by SARA, as SARA can cause epithelial parakeratosis (Kleen et al., 2003), rumenitis (Enemark, 2008) and greater histamine absorption from the rumen due to high rumen histamine concentrations and increased permeability of the epithelial membrane. The consequent increase in plasma histamine concentrations can lead to bronchial constriction and cardiovascular disorders (Plaizier et al., 2008).

The prevalence and economic costs of SARA are immense. In a survey of Wisconsin dairy herds, SARA prevalence was estimated to range from 12 to 30 % (Krause and Oetzel, 2006). On an annual basis, the US dairy industry is estimated to lose $500 million US dollars due to SARA. In Canada, the daily cost of SARA is estimated to be $1.12/head/d (Mutsvangwa and Write, 2003). Due to the huge economic losses, it is important to establish mitigation strategies for SARA and ARA.

1.4 Transport and Metabolism of SCFA

Our understanding of the molecular aspects of SCFA transport and metabolism in the rumen is limited. Recently, greater capacity for apical uptake of SCFA was found in sheep that are less susceptible to SARA (Penner et al., 2009b), suggesting that transepithelial transport may affect rumen pH. A number of membrane transport proteins have been identified and several rumen SCFA transport models have been proposed (Connor et al., 2010). However, our understanding of the SCFA transport and metabolism in the rumen is still limited. The epithelium function of large intestine is similar to that in the rumen in some aspects. Both digestive organs are fermentation chambers where protein, fibre and fermentable sugars are degraded by microbes, resulting in microbial protein and SCFA production. Ileal fluid and saliva, which flow into the large intestine and rumen, respectively, contain similar concentrations of Na^+, HCO_3^- and PO_4^{3-} (Argenzio and Stevens, 1984), suggesting similar buffering systems. Per unit of surface area, SCFA absorption rates in the bovine rumen and large intestinal mucosa of pig and dog are similar (Argenzio and Stevens, 1984). While some species-to-species and organ-to-organ differences exist, knowledge of SCFA transport and metabolism in the colon is far more advanced and will be discussed to augment our understanding of SCFA transport and metabolic physiology in ruminal epithelium.

1.4.1 Methods of SCFA Uptake

Apical uptake of SCFA from the rumen occurs via passive diffusion of associated SCFA and facilitated transport of dissociated SCFA. The acid dissociation constant of acetate, propionate and butyrate is 4.75, 4.87 and 4.81, respectively (Titus and Ahearn, 1992), meaning that, at these pH values, the ratio of associated to dissociated SCFA is 1:1. At pH 5.8, where fibre digestion begins to be compromised, 90% of each SCFA exists in the dissociated form while 99% of each SCFA exists in dissociated form at pH 6.8. Under optimal physiological conditions where fibre digestion is not compromised, 90 - 99% of SCFA in the rumen exist as dissociated ions.

Historically, it is believed that SCFA diffuse solely in the associated form (Bergman, 1990), which is unlikely to be the case. Exclusive passive diffusion theory would require SCFA transport rates to increase when pH drops and when carbon chain length increases. Actual changes in SCFA transport rates in response to decreased pH and increased carbon chain length are far short of the rates predicted by passive diffusion (Sehested et al., 1999). Further, in vitro SCFA flux rates across rumen epithelium were dependent on direction, which does not agree with exclusive passive diffusion (Sehested et al., 1999).

There is strong evidence supporting facilitated apical SCFA uptake. In a colonic cell line, a mucosal-to-serosal SCFA gradient depresses intracellular pH (pH_i) more than a serosal-to-mucosal SCFA gradient (Gonda et al., 1999), suggesting different transport mechanisms between these membranes. Furthermore, extracellular SCFA load at either the mucosal or serosal side causes intracellular acidification at the apical side and a buffering at the basolateral side of the cytosol (Gonda et al., 1999), suggesting that epithelial cells have membrane ion transporters whose activity will change in response to acid load. Apical acidification following a serosal SCFA load would not be expected from exclusive passive diffusion, as passive diffusion is primarily a function of solutes moving down a

concentration gradient. Lastly, decreasing luminal pH in vitro in bovine rumen epithelia from 7.3 to 6.0 represents a 19-fold increase in the abundance of associated SCFA, yet resulted in only a 1- or 2-fold increase in SCFA flux rate (Sehested et al., 1999), suggesting that rate of SCFA flux is not solely dependent on the concentration of associated SCFA, but that there is some mechanism that alter transepithelial SCFA flux.

1.4.2 Main Transporters Involved in SCFA Transport and pH_i Regulation Flux

Organic matter fermentation continuously produces SCFA and protons, and the rumen continuously removes SCFA to provide energy for host animals and continuously remove protons to provide optimal environment for microbial organisms. In cows, it is estimated that 60 – 80% of SCFA and over 50% of all protons are removed from the rumen via translocation across the rumen epithelium (Allen, 1997). When intracellular acidification of sheep rumen epithelium is induced by butyrate, intracellular pH (pH_i) dropped to 7.0, but recovers to approximately 7.3 within 10 minutes (Müller et al., 2000). The epithelial cells must have pH_i between 7.0 and 7.4 to maintain enzyme activity and protein synthesis capacity (Madshus, 1988). In the rumen epithelium, SCFA absorption would drastically affect pH_i unless protons are exported from the cytosol in response to pH_i drops.

Multiple transport mechanisms exist for apical SCFA uptake in the rumen epithelium. Facilitated SCFA apical uptake occurs through an apical $SCFA^-/HCO_3^-$ exchanger (Gäbel and Sehested, 1997). Apical $SCFA^-/Cl^-$ exchangers may also contribute to SCFA apical uptake, and they have been identified in rat colon (Rajendran and Binder, 1994). Together, $SCFA^-/Cl^-$ exchangers, $SCFA^-/HCO_3^-$ exchangers and passive diffusion increase the concentration of $SCFA^-$ in the rumen epithelium cytosol. Because the $SCFA^-/HCO_3^-$ exchanger removes HCO_3^- from the cytosol, $SCFA^-/HCO_3^-$ exchanger activity depresses pH_i.

Acidification of epithelial pH_i and $SCFA^-$ build-up in the epithelial cytosol is partly mitigated by monocarboxylate transporters (MCT; Kirat et al., 2006). Part of the solute carrier family 16 (SLC16), MCT transport H^+ and $SCFA^-$ to the outside of epithelial cells across the basolateral membrane, thereby increasing pH_i and decreasing epithelial SCFA concentration. There are 14 MCT isoforms identified, of which only MCT 1 – 4 actually transport monocarboxylates (Meredith and Christian, 2008). In particular, MCT-1 is abundant in both rumen and colon (Kirat et al., 2006). The MCT-1 is predominantly located on the basolateral membrane in the stratum basale (Graham et al., 2007) whereas MCT-4 may be located on the apical membrane, facilitating the uptake of $SCFA^-$ and H^+ (Connor et al., 2010).

Other pH_i regulatory mechanisms are Na^+/H^+ exchangers (NHE), Na^+/HCO_3^- co-transporter (NBC) and carbonic anhydrases (CA; Figure 1.2). The NHE are antiporters that transport Na^+ and H^+ in opposite directions and expel protons from the cytosol, thereby raising pH_i. Thus far, NHE isoforms 1, 2, 3 and 8 have been found in the rumen epithelium (Graham et al., 2006). The NHE1 and NHE3 that are located on the luminal side of the stratum granulosum contribute to the proton recycle to the rumen, decreasing rumen pH. Elevation of pH_i following acidification is not only possible by exporting protons from the cytosol, but also by increasing cytosolic concentrations of HCO_3^-, which is the primary effect of NBC. Specifically, NBC is located on the basolateral side of the rumen epithelium (Connor et al., 2010) and transports Na^+ and HCO_3^- into the cytosol, contributing to elevation of pH_i (Huhn et al., 2003).

Regulation of pH_i is thus complex and is highly dependent on the removal of protons from the epithelium. The activity of both NHE and NBC is powered by sodium gradients, created by Na^+/K^+ ATPase. By pumping Na^+ out of epithelia and K^+ into the epithelia, Na^+/K^+ ATPase serves as an "engine" to create concentration gradients for Na^+ and K^+ that other transporters use to drive trans-membrane solute transport. The Na^+/K^+ ATPase is localized near the basolateral membrane

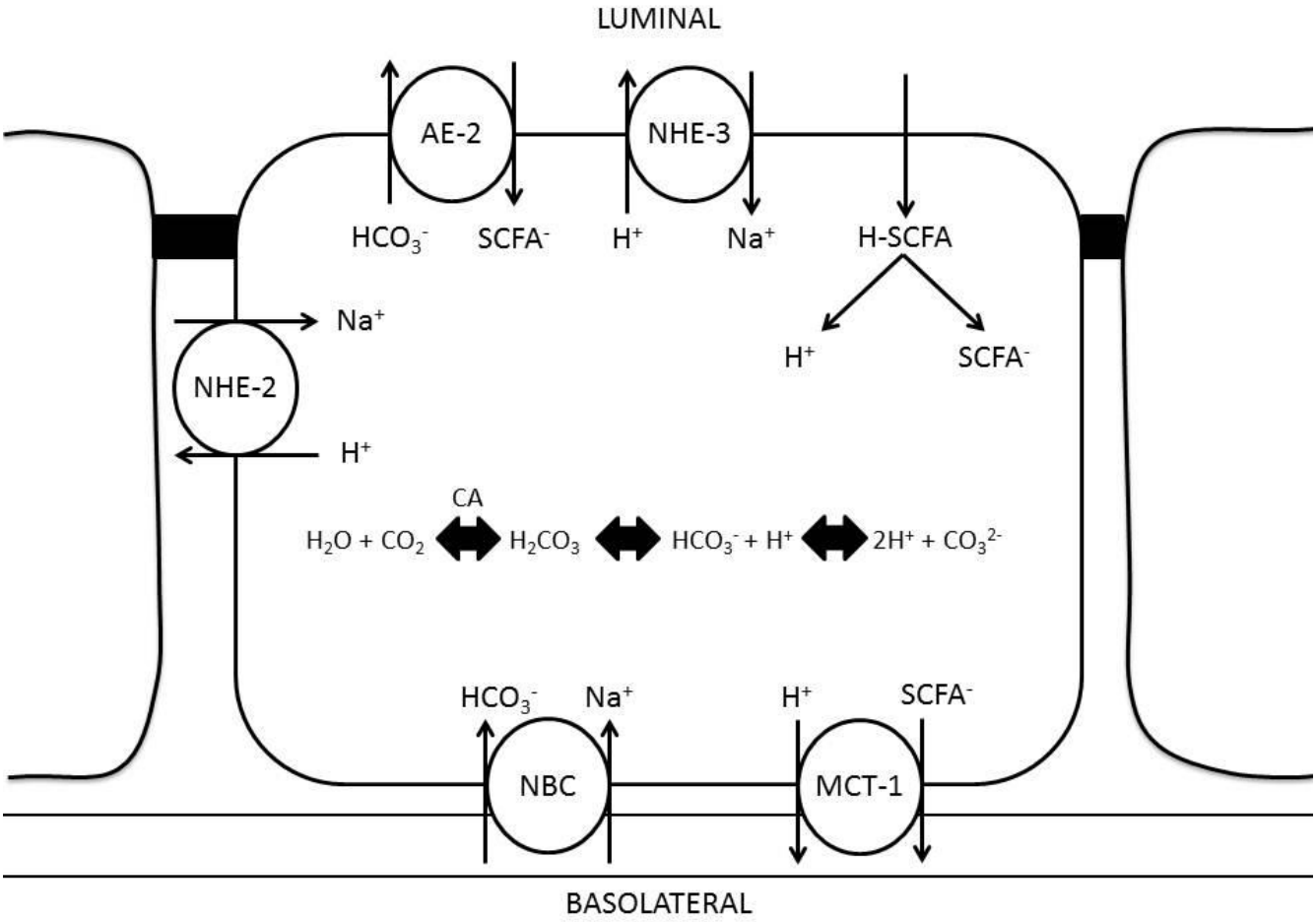

Figure 1.2 Transport proteins involved in short chain fatty acid absorption and intracellular pH regulation in ruminal epithelium (adapted from Gaebel et al., 2002). SCFA – short chain fatty acid; AE-2 – Anion exchanger isoform 2 (SLC4A2); NHE-2 – Na^+/H^+ exchanger isoform 2 (SLC9A2); NHE-3 – Na^+/H^+ exchanger isoform 3 (SLC9A3); NBC – Na^+/HCO_3^- co-transporter (SLC4A4); MCT-1 – Monocarboxylate transporter, isoform 1 (SLC16A1); CA – Carbonic anhydrase.

of the stratum basale (Graham and Simmons, 2005). Inhibition of Na^+/K^+ ATPase by ouabain causes remarkable reductions in SCFA transport, suggesting a strong link between SCFA uptake and Na^+/K^+ ATPase (Titus and Ahearn, 1992).

1.5 Growth and Development of the Rumen

1.5.1 SCFA Production in the Rumen

A neonatal calf does not have the functioning rumen. Milk consumed in the early weeks of life bypasses the rumen via the esophageal groove, which is active for the first 12 weeks of a calf's life (Quigley, 2001). Lactase in the intestine can effectively digest lactose provided in milk (Walker, 1959). During the pre-ruminant phase in lambs, intestinal lactase activity is constant (Walker, 1959). One of the main products of lactase activity is glucose, which is absorbed through the intestinal wall by Na^+-glucose linked transporter (SGLT-1). Both SGLT-1 protein abundance and sodium-dependent glucose transport activity in the ovine intestine decrease dramatically over the weaning transition to being barely measurable post-weaning (Lescale-Matys et al., 1993). After weaning, ruminants are highly dependent on the rumen for the digestion of structural carbohydrates by bacteria (Walker, 1959). Inoculation of the rumen with bacteria begins during the birth; after 1 day of age, the concentration of bacteria does not change drastically, but the bacterial number and profile do, especially as the calf begins to consume solid feed (Quigley, 2001). By the time lambs are weaned, the microbiota become as efficient in hydrolyzing carbohydrates and proteins as adult sheep (Walker and Walker, 1961).

Development of the rumen coincides with increased absorption of SCFA, the main end-products from solid feed fermentation and the primary energy source of weaned ruminants. It is estimated that net glucose uptake represents less than 3% of all energy absorbed daily through the rumen wall (Rémond et al., 1995), highlighting the importance of SCFA in providing energy to the

ruminants. Feeding calf starter significantly increases total SCFA concentration in the rumen (Lane et al., 2000), so the consumption of starter feed contributes to rumen development.

1.5.2 Morphology of the Rumen Wall

The muscular layer of the rumen wall plays an important role in contractions that aid mixing, moving, and regurgitating rumen contents. Musculature is primarily developed by inclusion of forages and other long-particle roughage in calf diets (Heinrichs, 2005). Calves fed chopped hay increased the relative proportion of muscle by 10% and decreased the relative proportion of mucosa by 10% compared to calves fed concentrate only (Nocek et al., 1984).

The epithelia have 4 strata; from the serosal to the luminal side, stratum basale, stratum spinosum, stratum granulosum and stratum corneum. The strata closest to the serosa contain the most mitochondria and organelles, and most of the metabolic activity takes place there (Baldwin VI, 1998). Tight-gap junctions, the extracellular proteins that provide epithelial barrier function, are found in the stratum granulosum (Graham and Simmons, 2005).

The luminal surface of the rumen wall contains numerous papillae, which increase the available surface area for absorption. In bovine, the papillae can be up to 15 mm in length and slough off extensively (Graham and Simmons, 2005). Papillae proliferation increases in response to consumption of a highly fermentable diet, which increases papillae size and number, and hence the surface area available for SCFA absorption (Gäbel et al., 2002). Papillae length and width increase in calves that are completely weaned at 6 weeks compared with those not weaned at 6 weeks of age (Zitnan, 2005), indicating that fermentation of starter feed likely promotes papillae growth. Infusing SCFA into the rumen results in shorter papillae than when solid feed is consumed, suggesting that SCFA may not be

solely responsible for the metabolic and morphological adaptations during rumen development (Lane and Jesse, 1997).

1.5.3 Ketogenesis in the Rumen Epithelium

Consumption of solid feed is associated with a sharp increase in butyrate utilization and can be nearly 60% higher in the first several weeks after weaning than utilization rates in mature ruminants (Walker and Simmonds, 1962). It is suggested that SCFA play an important role in butyrate metabolism and ketone body production in the rumen epithelium (Walker and Simmonds, 1962), possibly through alterations in gene regulation (Kiela et al., 2007) or through butyrate metabolism via ketogenesis. Ketogenesis in the rumen epithelium is essentially non-existent at birth (Walker and Simmonds, 1962), yet it can increase six-fold from 42 to 56 days of age in lambs (Baldwin and Jesse, 1992). The development of ketogenesis in rumen epithelium is an indicator of ruminal epithelium development (Lane et al., 2002) and results in the production of ketone bodies, primarily acetoacetate (AcAc) and β–hydroxybutyrate (BHBA; Figure 1.3). The consumption of starter feed enhanced AcAc and BHBA production (Giesecke et al., 1979), and intraruminal SCFA infusion increased AcAc, but not BHBA, production (Lane and Jesse, 1997), suggesting decreased reduction of AcAc to BHBA by β–hydroxybutyrate dehydrogenase (BDH), which may imply regulation of BHBA production. It is possible that plasma BHBA concentrations are regulated because subcutaneous BHBA injections have been shown to be anorexigenic (Laeger et al., 2010). Not only is BHBA an energy source for the brain of suckling mammals, it is also a more important precursor for amino acid synthesis than glucose in the immature brain of the mammal (Laeger et al., 2010). Since BHBA may play an important function in brain development, ketogenesis is considered as an important metabolic process.

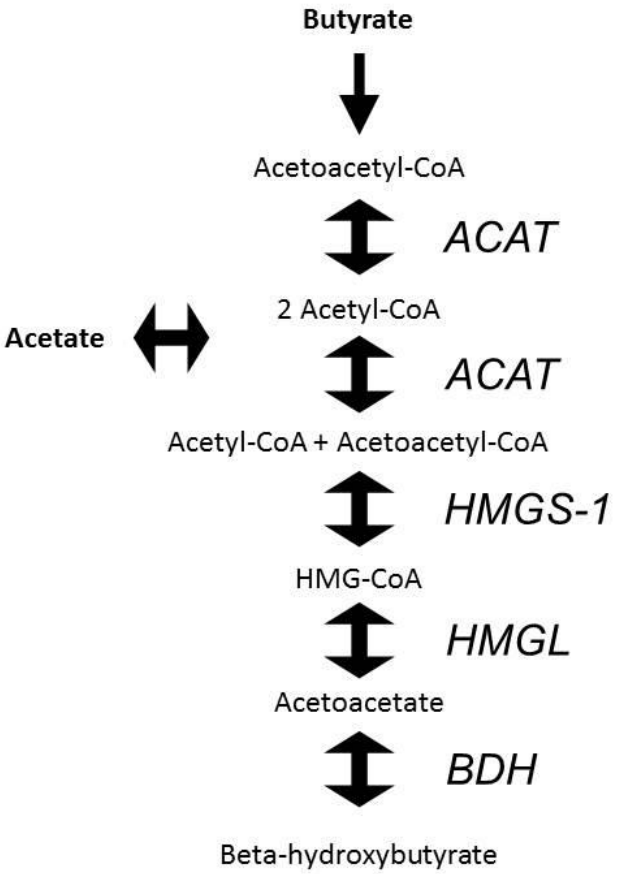

Figure 1.3 Ketogenesis pathway (McGarry and Foster, 1980). The conversion of butyrate to acetoacetyl-CoA involves several reactions and intermediates not shown in this diagram. ACAT – Acetoacetyl-CoA thiolase; HMGS - 3-hydroxy-3-methylglutaryl-CoA Synthase; HMGL – 3-hydroxy-3-methylglutaryl-CoA Lyase; BDH - β-hydroxybutyrate dehydrogenase

In ruminal epithelia, mRNA abundance of ketogenesis enzymes changes during the weaning transition. In growing lambs, 3-hydroxy-3methylglutaryl-CoA Synthase (HMGS) mRNA abundance increases when lambs start to consume solid feed (Lane et al., 2002). Previously, it has been found both β-hydroxybutyrate dehydrogenase (BDH) and HMGS activity increase with age in isolated sheep rumen (Lane et al., 2000). Acetoacetyl-CoA thiolase (ACAT) is the first step in ketogenesis. For both ACAT and HMGS, transcription is a long-term regulator of activity (Lane et al., 2002, Quant et al., 1991), so their relative abundance can be a useful indicator of metabolic development of the rumen. However, relative mRNA abundance of ACAT was not affected by consumption of starter in lambs post-weaning (Lane et al., 2002).

1.6 Summary

The rumen continuously produces protons and SCFA through fermentation. The production of protons increases acidic load in the rumen and can decrease rumen pH. Mature ruminants have several mechanisms to remove protons, primarily through buffer from saliva and ruminal epithelia, and absorption of dissociated SCFA across the rumen wall. It is not clearly understood how and when these metabolic mechanisms develop. The ruminal epithelia, especially, undergoes dramatic morphological and metabolic adaptations. While extensive research has been done on morphological development of the ruminal epithelia, our current understanding of the metabolic adaptation is limited. As calves begin to consume solid feed, fermentation in the rumen produces SCFA, which stimulate papillae growth. The majority of SCFA and protons produced are removed from the rumen by absorption across the rumen wall. Currently, our understanding of developmental changes in pH_i regulation and the major genes involved in rumen epithelial development is limited.

Another knowledge gap is the regulatory mechanism of rumen pH in calves. Many studies in literature evaluated rumen pH by multiple time-point measurements and did not account for diurnal variation in rumen pH, thus cannot accurately measure the duration or severity of SARA. In lactating dairy cows and beef cattle, SARA is detrimental to digestion, ruminal health and systemic health and can lead to economic losses. The effect of SARA on growth in calves during weaning transition is not extensively investigated, thus warrants investigation due to the detrimental consequences of SARA in mature cows. Because of the stimulatory effects of starter feed fermentation on rumen development, it is possible that a low rumen pH may actually stimulate rumen development (Stobo et al., 1966).

The objective of this thesis work is to investigate the effects of calf starter fermentation on rumen pH and metabolic responses in the rumen epithelium and to investigate the relationship between rumen pH and growth performance during the weaning transition in dairy calves. We hypothesized that calf starter fermentation would decrease rumen pH and up-regulate genes involved with pH_i regulation and ketogenesis. Furthermore, we hypothesized that SARA in calves would adversely affect growth performance.

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2.0 EFFECTS OF CALF STARTER ON RUMEN pH AND METABOLIC ADAPTATIONS IN THE RUMINAL EPITHELIUM OF HOLSTEIN DAIRY CALVES AT WEANING

2.1 Abstract

The objective of this study was to elucidate the effect of feeding calf starter on expression of genes involved in intracellular pH regulation and ketogenesis in the rumen epithelium, short chain fatty acid (SCFA) profile and pH in the rumen during weaning transition. Twenty Holstein bull calves were fed either milk replacer and hay (MR) or milk replacer, hay and a commercial texturized calf starter (MR+S) in a randomized complete block design. All calves were fed 750 g/d of MR. Calves on MR+S treatment fed starter ad libitum, and energy intake of calves within blocks was maintained by supplementing MR group with extra milk replacer that is equivalent to energy intake from calf starter. When MR+S consumed 680 g/d of calf starter for 3 consecutive d, rumen pH was measured continuously for 3 d using a small ruminant rumen pH logger system prior to slaughter. Treatment did not affect minimum pH, mean pH and duration or area under pH 5.8. Calves fed MR+S had higher SCFA concentrations in the rumen and a higher proportion of butyrate. Abundance of mRNA for monocarboxylate transporter isoform 1 was higher, and that for Na^+/H^+ exchanger isoform 3 and 3-hydroxy-3-methylglutaryl synthase was lower for the MR+S treatment compared with MR treatment. Calf starter consumption appears to increase fermentation in the rumen and affect expression of genes involved in ketogenesis and intracellular pH regulation without affecting rumen pH.

2.2 Introduction

Neonatal calves do not have a functioning rumen. When calves begin to consume solid feed, the weight of the rumen as a fraction of live weight increases (Walker and Walker, 1961). Fermentation of calf starter increases ruminal concentrations of short chain fatty acids (SCFA), especially propionate and butyrate (Stobo et al., 1966, Warner et al., 1956), which most potently stimulate papillae development in the rumen (Sander et al., 1959). To foster rumen development, it is recommended that calves maximize intake of calf starter (NRC, 2001), which is easily fermented in the rumen. However, rapid ruminal fermentation can lower rumen pH when proton production outweighs proton removal in the rumen. While low rumen pH has been associated with detrimental health effects in mature ruminants (Nocek, 1997), greater fermentation in the rumen of calves might promote papillae development (Stobo et al., 1966) because of greater proportions of butyrate and propionate. Impacts of calf starter feeding on rumen pH and animal health were not well understood in calves during weaning transition.

Whereas the neonatal rumen epithelium relies heavily on glucose as a metabolic fuel, the mature rumen epithelium derives most of its energy from SCFA oxidation (Rémond et al., 1995), representing a remarkable shift in metabolic substrates in the rumen epithelium. While the effects of calf starter on morphological development of the rumen are well established, effects of calf starter consumption on metabolic adaptation of ruminal epithelia are not well understood. Mature cows absorb 60 – 80% of all SCFA and 53% of protons across the rumen wall (Allen, 1997). Rumen epithelium does not only transport SCFA, but also metabolizes some of it, especially butyrate. Between 75 and 90% of absorbed butyrate is metabolized in ruminal epithelia, and 83% of metabolized butyrate is converted into ketone bodies (Rémond et al., 1995). The neonatal rumen, however, is non-ketogenic (Lane et al., 2002), thus the ketogenic capacity of the rumen must develop over time. While

development of ruminal ketogenesis capacity is partially dependent on age (Lane et al., 2002), the effects of calf starter on the development of ketogenic capacity is unclear.

The metabolic adaptation of rumen epithelium during the weaning transition is not well understood. We hypothesized that the high fermentability of calf starter would decrease rumen pH and increase the expression of genes involved in ketogenesis and regulation of intracellular pH (pH_i).The objective of this study was to investigate the effects of feeding calf starter on rumen pH and the metabolic adaptation of rumen epithelium.

2.3 Materials and Methods

2.3.1 Animals and Diets

All procedures for the animal study were approved by the Livestock Animal Care and Use Committee of the University of Alberta, using the guidelines of the Canadian Council for Animal Care (Ottawa, ON). Twenty Holstein bull calves were obtained in October, 2008 (n = 10) and May, 2009 (n = 10) from a commercial dairy farm at 46.7 ± 1.0 kg BW and 13.1 ± 0.4 d of age. Prior to arrival, calves were individually housed and were given up to 4L of whole milk daily but had no access to calf starter. After calves were moved to the Metabolic Unit at the University of Alberta Edmonton Research Station, all animals were housed in 3.05 x 3.66 m individual pens with wood shavings bedding. All animals had ad libitum access to water and hay throughout the study. Due to a logistical error, calves obtained in October, 2008 were fed Timothy hay (7.7% CP, 65.6% NDF) while calves obtained in May, 2009 were fed mixed legume hay (15.0% CP, 56.8% NDF). Calves were blocked by starting date of the study and BW, and one calf was assigned to milk replacer (MR) treatment, and the other calf in the same block was assigned to milk replacer and starter (MR+S) treatment.

All calves received milk replacer (22% CP, 17% fat, and 45% lactose; High Performance Milk

Replacer, Grober Nutrition, Cambridge, ON, Canada) at 750 g/d (12.5% w/v). The MR+S calves were offered a commercial texturized calf starter containing canola meal, soybean meal, wheat, barley, corn, peas and vitamin and minerals (29.5% CP, 27.1% starch; Wetaskiwin Co-Op Country Junction, Wetaskiwin, AB, Canada) ad libitum. The metabolizable energy (ME) of the milk replacer and starter, respectively, were calculated using the formulae below:

ME= 0.93× (0.057×%CP + 0.092×%fat + 0.0395×%lactose)

ME= ((1.01 × DE)- 0.45) + (0.0046 × (%EE – 3))

Where digestible energy (DE) = 0.7 x ((0.057 x %CP) + (0.092 x %EE) + (0.0415 x %carbohydrates))

Carbohydrate content = 100 – EE – CP – Ash.

Values for CP, fat, lactose, and ether extract (EE) were obtained from the manufacturer. For the MR+S calves, starter intake was recorded daily, and ME intake from starter was calculated. The MR calves were fed additional milk replacer that supplies ME that is equivalent to ME intake from starter in calves within the same block. Therefore, calves within each block had similar ME intake. Milk replacer was offered twice daily in two equal portions at 0515 and 1515 h, and starter was offered once daily at 0515 h. All animals were weighed twice weekly and blood was sampled once weekly at 0930 h. Blood was collected from the jugular vein into a Vacutainer containing heparin (Becton Dickinson, Franklin Lakes, NJ, USA) and immediately centrifuged at 3,000 x *g* for 20 min and plasma was harvested and stored at -20^{o}C until analysis.

When a calf on MR+S treatment consumed starter at 680 g/d for 3 consecutive d, a small ruminant ruminal pH measurement system (SRS) with a diameter of 20.6 mm, 138 mm length and 245 g weight (Penner et al., 2009a), was orally dosed to both calves within a block; calves were placed in a

reclining position during dosing, to ensure that the SRS entered the reticulorumen dorsally and was not caught in the reticulum. Rumen pH was measured every 2 min continuously for 3 d. After the rumen pH measurement period, both calves within the same block were slaughtered at 1100 and 1200 h by captive bolt gun stunning followed by exanguination. The order of slaughter was randomized throughout the trial. Ruminal epithelial tissue was harvested; the epithelium was manually peeled off from the muscle layer, rinsed in PBS at pH 7.4 and snap-frozen in liquid nitrogen and kept on dry ice at -80^{o}C. Rumen contents were strained through a layer of porous material *(*Peetex, pore size = 355 μm; Sefar Canada Inc., Scarborough, Ontario, Canada*)* and rumen fluid was snap frozen in liquid nitrogen and kept on dry ice at -80^{o}C. Both rumen tissue and fluid were kept at -80^{o}C until analysis.

2.3.2 Sample Analysis

Calf starter was sampled at the arrival date of each group of calves, dried at 55^{o}C for 48 h then ground through a 1-mm screen using a Wiley mill (Thomas-Wiley, Philadelphia, PA, USA) and analyzed for chemical composition. Dry matter content was determined by drying samples at 135^{o}C for 2 h (AOAC, 2002; method 930.15) and OM was analyzed by 2h combustion at 600^{o}C (AOAC, 2002). CP was analyzed using flash combustion (TruSpec analyzer, Leco Corp., St. Joseph, MI, USA; Wrolstad et al., 2004). Starch was analyzed using an enzymatic method (Karkalas, 1985). Concentration of NDF was analyzed using heat-stable amylase and sodium-sulfite as described previously (Van Soest et al., 1991). Ether extract was analyzed using a Goldfisch extractor (Labconco, Kansas City, MO; Wrolstad et al., 2004).

Plasma samples were analyzed for concentrations of β-hydroxybutyrate (BHBA), insulin and glucose. Plasma BHBA was analyzed using a β-hydroxybutyrate dehydrogenase enzyme (Roche, Mannheim, Germany) and glucose was analyzed using a glucoxidase/peroxidase enzyme reaction

(Sigma-Aldrich Canada, Oakville, ON, Canada) as described previously (Penner et al., 2009c). BHBA and glucose assays were conducted using a plate reader (SpectraMax; Molecular Devices, Inc., Sunnyvale, CA, USA). Insulin concentration was analyzed using a commercial radioimmunoassay kit (Siemens Medical Diagnostics, Los Angeles, CA, USA).

Rumen fluid samples were thawed, and centrifuged at 15,000 x *g* at 4^{o}C to obtain clear supernatant. Concentration of SCFA was determined using isocaproic acid as an internal standard with gas chromatography (Varian, Walnut Creek, CA, USA; Penner et al., 2009c).

2.3.3 Real-time PCR

The RNA was extracted using a Trizol/chloroform (Invitrogen, Burlington, ON, Canada) extraction method (Chomczynski and Sacchi, 1987). After the purity of the RNA was measured with a spectrophotometer (ND-1000, Nanodrop Technologies, Wilmington, DE, USA), 1.6 µg of RNA was reverse-transcribed using Superscript II and Oligo-d(T) (Invitrogen). Real-time PCR was performed using recombinant Taq DNA polymerases. Primers (Invitrogen) and probes (Applied Biosystems Inc., Foster City, CA, USA) were designed using Primer Express v.3.0 (Applied Biosystems, Inc.; Table 2.1) and analyzed by BLAST (NCBI) to verify primer specificity. The target genes were acetoacetyl-CoA thiolase (ACAT), 3-hydroxy-3-methylglutaryl-CoA synthase (HMGS), 3-hydroxy-3-methylglutaryl-CoA lyase (HMGL), β-hydroxybutyrate dehydrogenase isoforms 1 (BDH-1) and 2 (BDH-2), all of which are enzymes involved in ketogenesis. The target genes involved in pH_i were monocarboxylate transporter isoform 1 (MCT-1) and Na^+/H^+ exchanger isoforms 2 (NHE-2) and 3 (NHE-3). The thermocycler (StepOnePlus, Applied Biosystems Inc.) was set to 40 cycles of 95^{o}C for 1 s, and 60^{o}C for 20 s.

Abundance of mRNA for target genes was normalized to 3 reference genes rather than 1, as

Table 2.1 Gene name, primer and probe sequence and NCBI Accession number for genes used in real-time PCR analysis

Gene name	Primer and Probe Sequence		Accession number
Monocarboxylate Transporter, isoform 1 (MCT-1)	Forward:	ATC TAC GCG GGA TTC TTT GGA T	NM_001109980
	Reverse:	AAG GTC CAT CAG CGT TTC AAA C	
	Probe:	TGC TTT TGG GTG GCT C	
Sodium/proton exchanger, isoform 2 (NHE-2)	Forward:	TTG TGC GAT GAC CAT GAA TAA GT	XM_604493
	Reverse:	TGA TGG TCG TGT AGG ATT TCT GA	
	Probe:	CGT GGA AGA GAA CGT G	
Sodium/proton exchanger, isoform 3 (NHE-3)	Forward:	AGC CTT CGT GCT CCT GAC A	AJ131764.1
	Reverse:	TGA CCC CTA TGG CCC TGT AC	
	Probe:	TGC TCT TCA TCT CCG	
Acetoacetyl-CoA Acyltransferase (ACAT)	Forward:	CAC TGG CTT CCC AGC AAA A	BC102927
	Reverse:	ACG ATC TCG GCC TGG AAA C	
	Probe:	AGC CAG AGC CCA GAG A	
3-hydroxy-3-methylglutaryl-CoA Synthase (HMGS)	Forward:	AGG ATA CTC ATC ACT TGG CCA ACT	AY581197
	Reverse:	CAT GTT CCT TCG AAG AGG GAA T	
	Probe:	CAT TCC CCA GAG TTC CA	
3-hydroxy-3-methylglutaryl-CoA Lyase (HMGL)	Forward:	TGC AGA TGG GAG TGA GTG TCA	NM_001075132
	Reverse:	GAC GCC CCC TGT GCA TAG	
	Probe:	TGG CAG GAC TGG GAG	
β -hydroxybutyrate dehydrogenase, isoform 1 (BDH-1)	Forward:	GAC TGC CAC CAC TCC CTA CAC	NM_001034600
	Reverse:	TCC GCA GCC ACC AGT AGT AGT	
	Probe:	CGC TAC CAT CCC ATG	
β -hydroxybutyrate dehydrogenase, isoform 2 (BDH-2)	Forward:	CTG TGG CTT CCA GCA TCA AA	NM_001034488
	Reverse:	CGC CTT GGT TGT GCT GTA CA	
	Probe:	CGT TGT GAA CAG GTG C	
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Forward:	TGC CGC CTG GAG AAA CC	NM_001034034
	Reverse:	CGC CTG CTT CAC CAC CTT	
	Probe:	CCA AGT ATG ATG AGA TCA A	
β-actin (ACTB)	Forward:	TCA CGG AGC GTG GCT ACA G	NM_173979
	Reverse:	TTG ATG TCA CGG ACG ATT TCC	
	Probe:	CAC CAC CAC GGC CG	
Ribosomal Protein, Large P0 (RPLP0)	Forward:	AGG GCG TCC GCA ATG TT	NM_001012682
	Reverse:	CGA CGG TTG GGT AAC CAA TC	
	Probe:	CCAGCGTGTGCCTG	

previously described (Vandesompele et al., 2002). Using multiple reference genes to normalize mRNA quantification is more robust than using a single reference gene because single-reference quantification is dependent on a housekeeping gene whose expression is not regulated. A growing body of evidence suggests that multiple reference genes, rather than a single reference gene, be used for mRNA abundance normalization (Robinson et al., 2007, Tricarico et al., 2002, Vandesompele et al., 2002) as oft-used housekeeping genes can vary by diet, physiological state (Janovick-Guretzky et al., 2007), tissue type (Kessler et al., 2009, Warrington et al., 2000), individual (Tricarico et al., 2002), disease state (Huggett et al., 2005) and age (Chen et al., 2006). In this study, to normalize the relative mRNA abundance in a sample using multiple reference genes, the relative mRNA abundance (Q) was adjusted by a normalization factor (NF), the geometric average of mRNA abundance of 3 reference genes: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin, and ribosomal protein large P0 (RPLP0).

Briefly, all samples from both treatments were analyzed on the same plate for one gene. For each sample, the cycle threshold (C_t) was calculated and then subtracted from the minimum C_t in the treatment. Then, the relative abundance of mRNA and the normalized mRNA abundance (Qn), respectively, were calculated by the formulae:

$Q = \text{Amplification efficiency}^{\Delta Ct}$

$Q_n = Q/NF$

where NF is the normalization factor, calculated as the geometric average of the Q values for GAPDH, β-actin and RPLP0 for each sample.

2.3.4 Statistics

Average daily gain (ADG) was calculated via regression of weekly body weights using PROC REG in SAS (v. 9.1; SAS Institute, Cary, NC, USA). Data were tested for normality using PROC GLM

of SAS. Non-normally distributed data (i.e., BW at slaughter) were analyzed by Signed Rank test. The other data were analyzed as a randomized complete block design using PROC MIXED of SAS. Data were expressed as LSM ± SE. The statistical model used for gene expression, pH and SCFA data was $Y= \mu + Block_i + Treatment_j + e_{ij}$. For weekly blood plasma metabolites, the statistical model was $Y = \mu + Block_i + Treatment_j + Week_k + Treatment \times week_{jk} + e_{ijk,}$ where week was a repeated measure. Significance was declared at $P < 0.05$ and tendency of treatment effects were declared at $P < 0.10$. Due to a recurring respiratory illness, one block of animals had to be removed from the trial and is not included in statistical analysis. Type of hay offered was not found to significantly affect any of the response variables and thus was not discussed.

2.4 Results

Due to recurring illness, 2 animals had to be removed from the study and were excluded from all statistical analysis.

2.4.1 Growth and Blood Metabolites

There was no difference in ADG between MR and MR+S (0.74 vs. 0.86 kg/d; $P = 0.02$; Table 2.2) than calves in the MR treatment, but there was no treatment effect in final BW ($P = 0.39$). There was no difference between treatments in plasma glucose ($P = 0.38$; Table 2.2), although plasma glucose increased between 6 and 8 weeks of life ($P < 0.01$; Fig. 2.1), though no treatment by time interaction was found ($P = 0.86$). Plasma insulin concentration was also not affected by treatment ($P = 0.23$; Table 2.2), although plasma insulin increased between 6 and 8 weeks of life, rising from 25.2 to 92.2 µIU/mL ($P < 0.01$; Fig. 2.2). No treatment by time interaction existed for plasma insulin ($P = 0.16$). Plasma BHBA concentration was not affected by treatment ($P = 0.53$; Table 2.2)

Table 2.2 Average daily gain and BW at slaughter and average plasma metabolite concentrations of Holstein bull calves fed calf starter ad libitum with milk replacer and hay (MR+S), and those fed milk replacer and hay only (MR)

	MR	MR+S	*P*
ADG, kg/d	0.86 ± 0.07	0.80 ± 0.08	0.45
BW at slaughter, kg	78.4 ± 3.0	76.8 ± 3.5	0.30
Plasma insulin[1], μIU/mL	57.6 ± 14.7	31.1 ± 14.7	0.23
Plasma glucose[1], mg/dL	106.6 ± 7.0	98.5 ± 7.0	0.38
Plasma BHBA[1], mg/dL	6.2 ± 0.1	6.1 ± 0.2	0.53

[1]Average concentration between week 3 and 8 of age

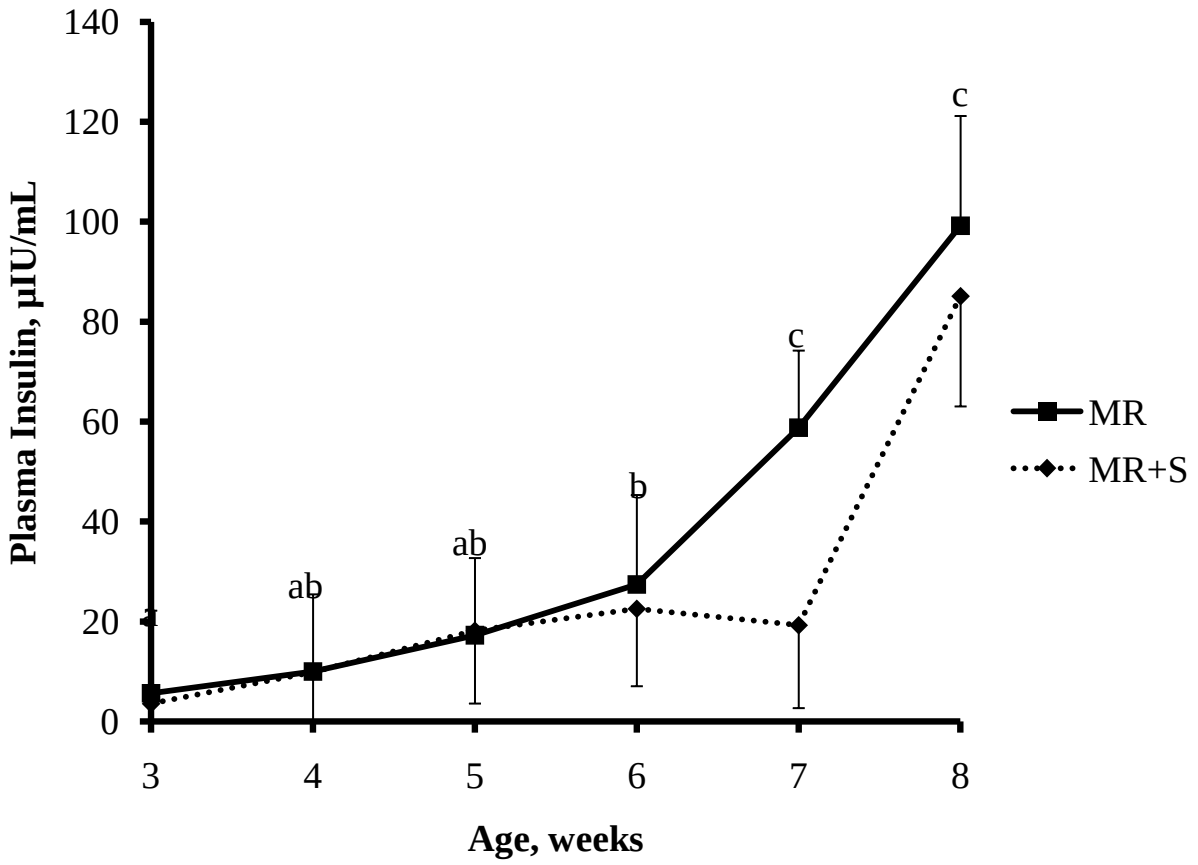

Figure 2.1 Plasma insulin concentrations between 3 and 8 weeks of age in Holstein bull calves fed milk replacer and hay without calf starter (MR) or with calf starter ad libitum (MR+S). Treatment did not affect plasma insulin levels over time ($P = 0.23$) and there was no interaction between treatment and week of life ($P = 0.16$). Superscripts denote significant differences between weeks ($P < 0.01$).

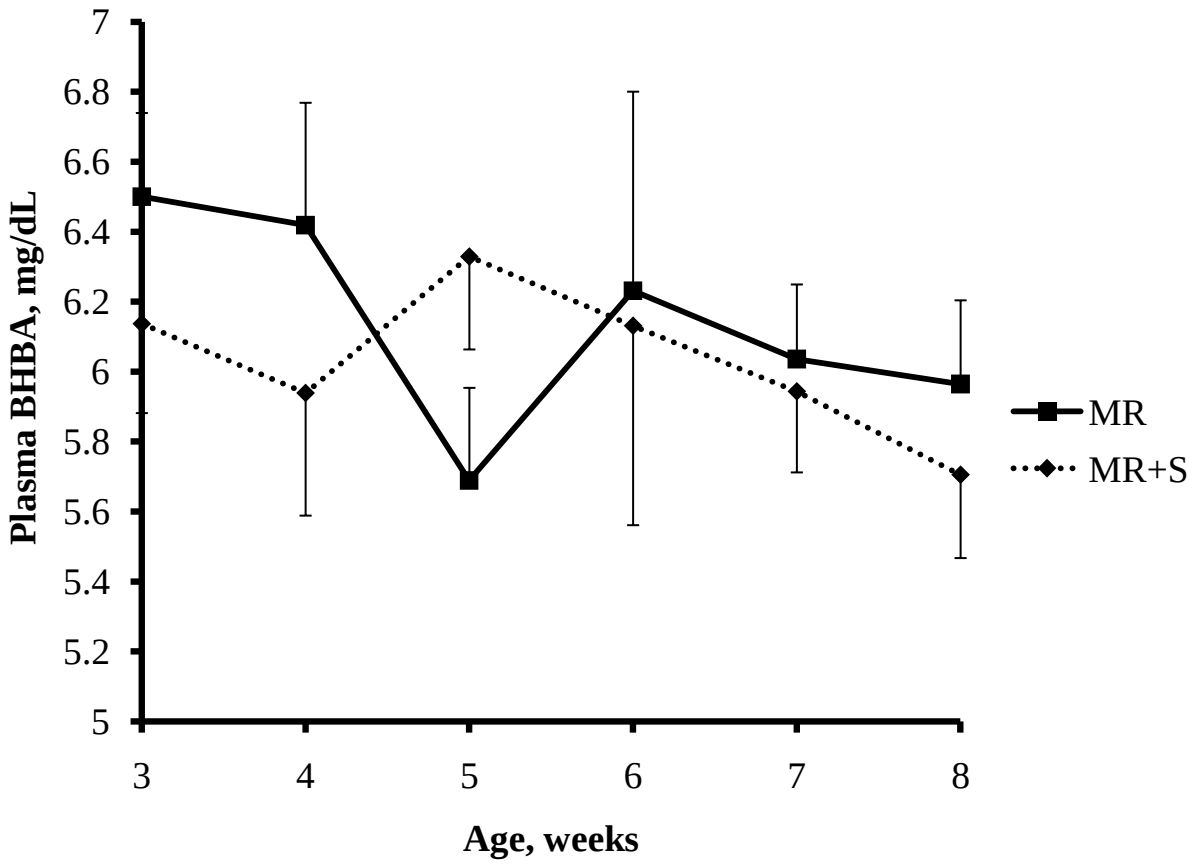

Figure 2.2 Plasma β-hydroxy-butyrate (BHBA) concentrations between 3 and 8 weeks of age in Holstein bull calves fed milk replacer and hay without calf starter (MR) or with calf starter ad libitum (MR+S). Treatment did not affect plasma BHBA concentrations (*P* = 0.53). Between 3 and 8 weeks of life, no statistical difference in average weekly plasma BHBA concentrations was found (*P* = 0.65) and no treatment × week interaction was found (*P* = 0.62).

or by time (P = 0.65; Fig. 2.3) and there was no treatment by time interaction (P = 0.62). At slaughter, there was no significant difference in plasma glucose nor plasma BHBA, but plasma insulin was higher in the MR calves (146.4 vs. 47.9 μIu/mL; P = 0.02) compared with the MR+S calves.

2.4.2 Rumen Fermentation and pH

Minimum, mean, maximum pH, standard deviation of pH, and duration and area under pH 5.8 were not affected by treatment (Table 2.3). Although hay intake in MR+S did not differ from MR during the rumen pH measurement period, hay intake was negatively correlated to the area under the curve at pH 5.8 (r =-0.78; P < 0.01; Fig. 2.4). Because of the correlation between hay intake and area under the curve, pH data were also analyzed using hay intake as a covariate, and treatment effect on area under pH 5.8 became significant, but the other pH response variables did not.

The MR+S treatment also altered SCFA profile in the rumen fluid (Table 2.4). The MR+S calves had lower molar proportion of acetate (57.6 vs. 69.3%; P <0.01), higher molar proportion of butyrate (15.6 vs. 7.9 %; P = 0.03), and higher total SCFA concentration (99.1 vs. 64.6 mM; P < 0.01) than MR calves. The MR+S calves also tended to have a lower acetate to propionate ratio (2.82 vs. 3.96; P = 0.07) than MR calves.

2.4.3 Rumen Epithelium Gene Expression

Abundance of mRNA was calculated as a relative value with arbitrary units. In the MR+S calves, mRNA abundance was higher for MCT-1 (1.45 vs. 0.53; P < 0.01) and lower for NHE-3 (0.37 vs. 0.82; P < 0.01) and HMGS-1 (0.40 vs. 0.94; P = 0.01) than in MR calves (Table 2.5). Relative mRNA abundance of NHE-2, ACAT, HMGL, BDH-1 and BDH-2 were not affected by treatment.

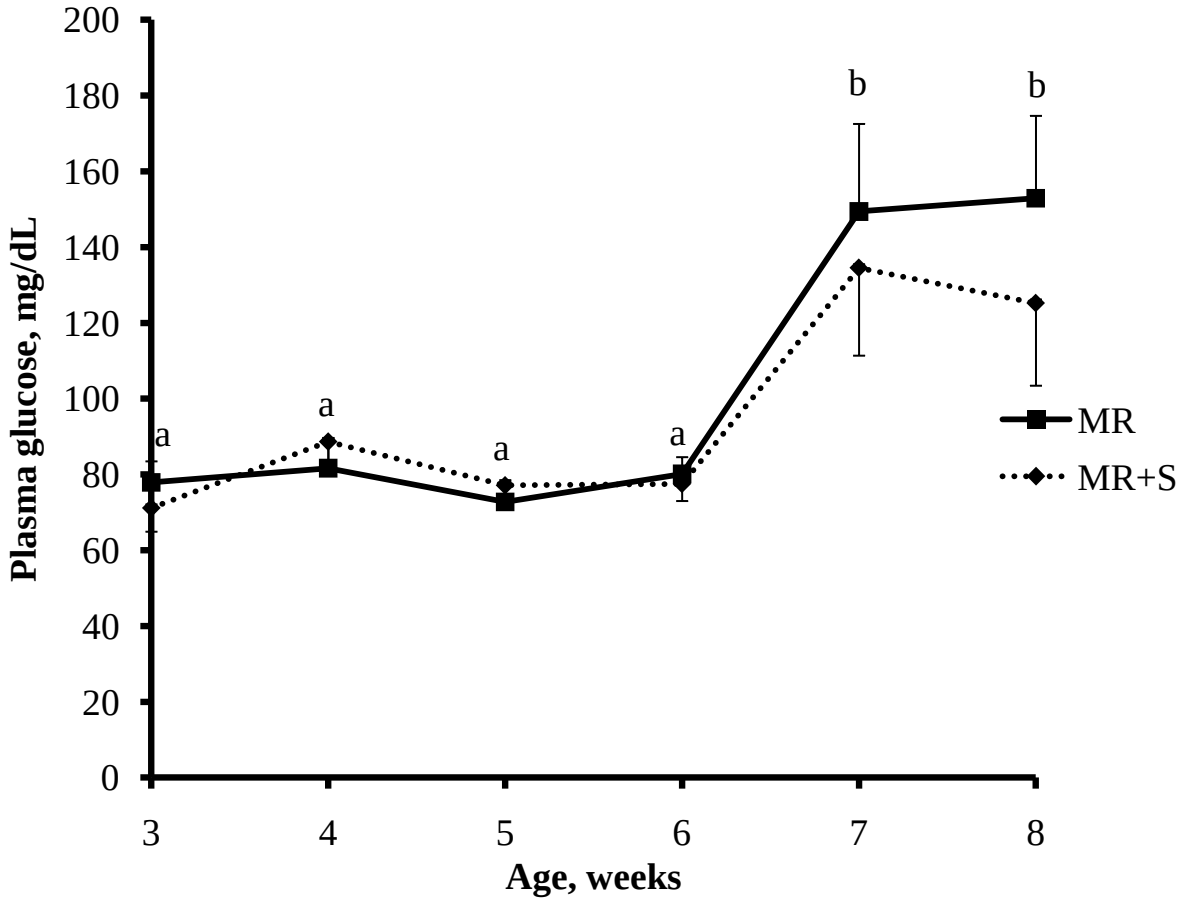

Figure 2.3 Plasma glucose concentrations between 3 and 8 weeks of age in Holstein bull calves fed milk replacer and hay without calf starter (MR) or with calf starter ad libitum (MR+S). Plasma glucose was not affected by treatment ($P = 0.38$). Average plasma glucose did increase significantly between week 6 and 7 of life ($P < 0.01$), but there was no treatment × week interaction ($P = 0.86$). Weekly averages with different superscripts are significantly different.

Table 2.3 Rumen pH profile at weaning of Holstein bull calves fed calf milk replacer and hay only (MR) or milk replacer and hay and calf starter ad libitum (MR+S)

	MR	MR+S	*P*
Min pH	5.35 ± 0.20	5.47 ± 0.25	0.63
Mean pH	6.37 ± 0.10	6.30 ± 0.12	0.64
Max pH	6.87 ± 0.09	6.86 ± 0.11	0.94
SD pH	0.31 ± 0.05	0.26 ± 0.07	0.53
Duration pH < 5.8, min/d	101.2 ± 2.3	14.6 ± 60.3	0.26
AUC[1] pH < 5.8, pH*min/d	4.3 ± 1.8	1.3 ± 2.2	0.32
Hay DMI[2], g/d	229 ± 66	344 ± 76	0.13
Starter DMI[2], g/d	N/A	759 ± 44	N/A
Milk Replacer DMI[2], g/d	1443 ± 46	750	N/A

[1]Area under the curve (AUC) is calculated by the difference between pH and pH threshold multiplied by the time that pH is under the SARA or ARA threshold.
[2]Dry matter intake (DMI) is the average intake during the pH measurement period

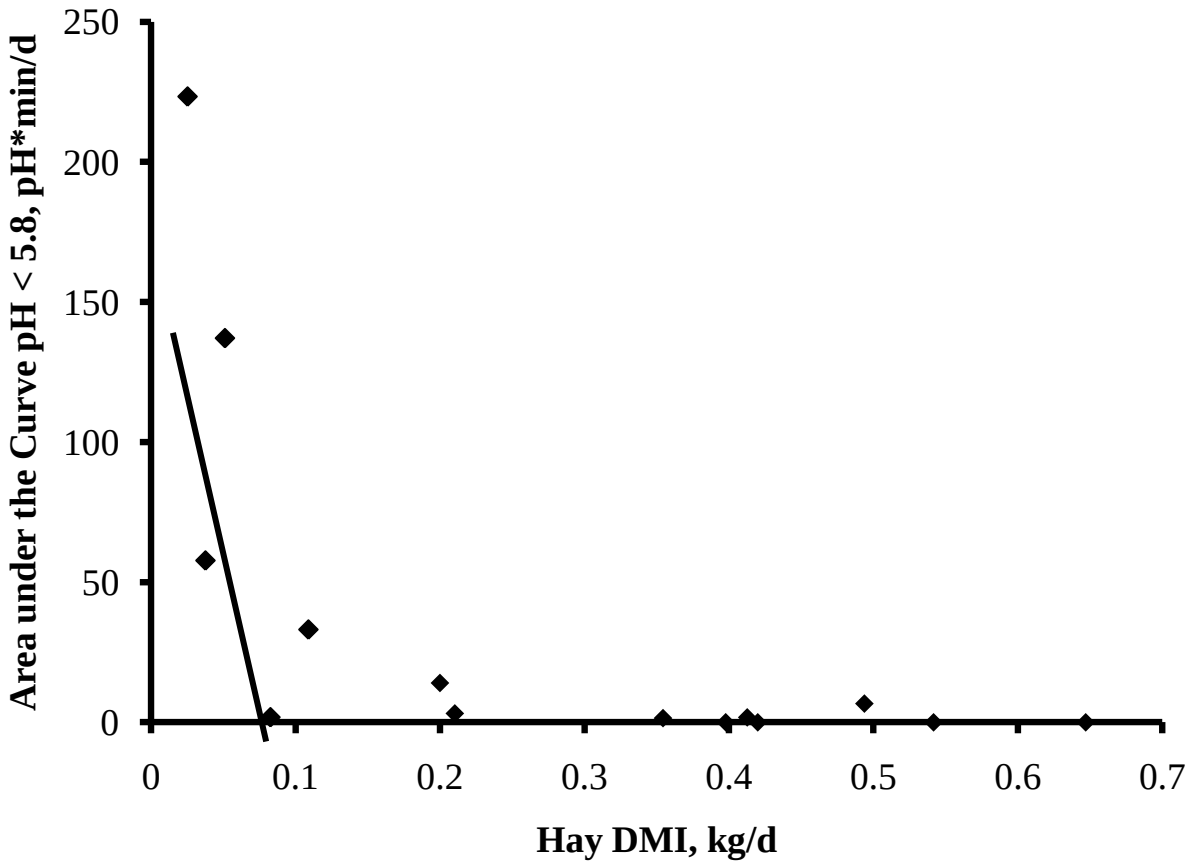

Figure 2.4 Relationship between hay DMI and area under pH 5.8 for dataset containing all treatments(r = -0.78; P < 0.01). The breakpoint analysis showed that area under pH 5.8 becomes zero at hay DMI of 0.080 kg/d.

Table 2.4 Rumen short chain fatty acid (SCFA) profile[1] and blood plasma metabolites[2] on the day of slaughter in Holstein bull calves fed milk and hay only (MR) or calf starter ad libitum, milk and hay (MR+S) at the start of weaning

	MR	MR+S	*P*
Total SCFA, m*M*	64.6 ± 8.6	99.1 ± 8.1	< 0.01
Acetate, mol/100mol SCFA	69.3 ± 1.9	57.6 ± 1.9	< 0.01
Propionate, mol/100mol SCFA	18.7 ± 1.3	21.3 ± 1.2	0.32
Butyrate, mol/100mol SCFA	7.9 ± 1.9	15.6 ± 1.7	0.03
Plasma glucose, mg/dL	84.5 ± 5.5	84.2 ± 5.5	0.97
Plasma BHBA, mg/dL	7.6 ± 0.4	7.1 ± 0.4	0.33
Plasma insulin, µIU/mL	146.4 ± 26.7	47.9 ± 28.6	0.02

[1]Sampled 6 hours after morning feeding
[2]Sampled 4 hours after morning feeding

Table 2.5 Abundance of mRNA[1] involved in intracellular pH regulation and ketogenesis in the rumen epithelium of Holstein bull calves fed milk replacer and hay only (MR) or milk replacer, hay and ad libitum calf starter (MR+S)

	MR	MR+S	*P*
Monocarboxylate transporter, isoform 1	0.53 ± 0.23	1.45 ± 0.22	< 0.01
Na^+/H^+ exchanger, isoform 2	0.68 ± 0.16	0.81 ± 0.16	0.36
Na^+/H^+ exchanger, isoform 3	0.82 ± 0.12	0.37 ± 0.12	< 0.01
Acetoacetyl-CoA thiolase	1.44 ± 0.19	0.99 ± 0.21	0.2
3-hydroxy-3-methylglutaryl-CoA lyase	1.15 ± 0.16	1.26 ± 0.16	0.36
3-hydroxy-3-methylglutaryl-CoA synthase	0.94 ± 0.16	0.40 ± 0.17	0.01
β-hydroxybutyrate dehydrogenase, isoform 1	0.85 ± 0.20	0.93 ± 0.20	0.78
β-hydroxybutyrate dehydrogenase, isoform 2	0.94 ± 0.14	0.92 ± 0.13	0.91

[1]Relative to the sample with the highest abundance (lowest C_t); values were normalized by geometric means of non-normalized mRNA abundance of three reference genes (glyceraldehyde-3-phosphate dehydrogenase, β-actin, and ribosomal protein large P0).
[2]n=18

2.5 Discussion:

2.5.1 Calf Starter and Rumen pH Regulation

In this study, rumen pH did not differ between the MR and MR+S treatments at weaning despite higher SCFA concentration in the MR+S treatment. In pre-weaned calves, rumen pH was not different between calves fed an all-concentrate diet and calves fed a 70% concentrate and 30% roughage diet, although no difference in total SCFA was found. (Suárez et al., 2007). Rumen pH did not change in response to increased barley grain inclusion from 33% to 61% in calf starter whereas rumen pH was increased between 5 and 12 weeks of age (Murdock and Wallenius, 1980). Judging from these studies, rumen pH may not be heavily dependent on diet but may be age-related.

Studies evaluating rumen pH in calves have sampled rumen fluid at one time point between 1h and 5 h post-feeding (Khan et al., 2007a, Suárez et al., 2007); such an approach unfortunately does not measure diurnal variation nor the length and extent of ruminal acidotic conditions. Measuring length and severity of ruminal acidosis is advantageous because in vitro OM digestion decreased as the time that pH remains suboptimal increased (Cerrato-Sánchez et al., 2007). By comparing the length and severity of ruminal acidosis between treatments, we get a better understanding of how digestion of OM compares between treatments.

We hypothesized that MR+S would have greater duration and severity of sub-acute rumen acidosis (SARA) due to rapid fermentation of starch in the rumen, but the duration and severity of SARA were not significantly different between MR and MR+S treatments. When hay DMI was included as a covariate in statistical analysis of pH response variables, the area under pH 5.8 became significantly lower for MR+S treatment, providing no evidence to support that starter feeding increases the severity of SARA.

of SARA. It is possible that, for the MR calves, substantial leakage of milk from the esophageal groove into the rumen caused fermentation decreasing rumen pH to a similar extent for calves fed MR+S. However, similar rumen pH for MR and MR+S may not be attributed to the possible leakage since total SCFA concentration for MR+S treatment were 53% higher than MR treatment, suggesting that fermentation in the rumen was likely greater for the MR+S calves.

If fermentation is greater in the MR+S treatment than the MR treatment, but duration and severity of SARA is similar, then protons must be removed from the rumen at different rates. In mature cows, neutralization by HCO_3^- accounts for 30% of proton removal from the rumen (Allen, 1997). The primary source of HCO_3^- in the rumen is saliva, which is stimulated by chewing activity. The negative correlation between hay intake and area under pH 5.8 (Figure 4) indicates that hay consumption may stimulate salivary buffer flow in calves during weaning transition. However, since hay intake did not differ between treatments, proton neutralization by salivary buffer may not be different between treatments.

Another factor that may have contributed to increased proton removal from the rumen is proton uptake across the rumen epithelium. In lactating dairy cows, more than 50% of proton is removed with absorption of undissociated SCFA across the rumen wall (Allen, 1997). Another mechanism that may affect net proton removal from the rumen is the NHE, which exchange extracellular Na^+ ions and intracellular H^+ ions.

One particular isoform of interest is apical NHE-3, the expression of which is up-regulated by butyrate (Kiela et al., 2007). Long-term regulation of NHE-3 is primarily accomplished through changes in transcription (Zachos et al., 2005). In disagreement with findings of Kiela et al. (2007), abundance of NHE-3 mRNA was lower in the MR+S treatment despite a higher butyrate concentration in rumen fluid. The reason for lower NHE-3 mRNA abundance in rumen epithelium from the MR+S

calves is not known, but lower NHE-3 expression may indicate decreased proton recycling into the lumen and greater net proton uptake by ruminal epithelium for the MR+S calves. Lower proton recycling into the rumen in the MR+S treatment may explain why rumen pH was the same in spite of greater SCFA concentrations. By having greater net proton uptake, the proton concentration in the rumen of MR+S would be more similar to MR, thus showing similar pH values.

Increased expression of MCT-1, situated on the basolateral membrane (Connor et al., 2009), in MR+S supports the idea that net proton uptake may be higher in MR+S. The MCT-1 is a monocarboxylate/H^+ co-transporter, so MCT-1 contributes to proton movement away from the rumen. The increased expression of MCT-1 appears to complement the decreased expression of NHE-3 by changing the genetic response to increasing net proton movement away from the rumen. It is important to note, however, that the gene expression does not imply protein expression or activity, which limits the scope of the current study.

2.5.2 Calf Starter and Ketogenesis

The primary end-product of ketogenesis is BHBA and there is considerable uptake and metabolism of BHBA by the brain in the pre-weaned mammal (Laeger et al., 2010). Lane et al. (2000) showed that BHBA production in lamb rumen epithelium was dependent on age rather than diet or energy intake (Lane et al., 2000), which may explain why BHBA did not differ between MR+S and MR treatments. In spite of similar plasma BHBA concentrations, ruminal butyrate, a primary substrate for rumen ketogenesis (Heitmann et al., 1987), was more abundant in MR+S than in MR. It is possible that increased hepatic ketogenesis in MR could compensate for the lower butyrate concentration; however, MR also had higher plasma insulin concentrations. Insulin has been shown to inhibit hepatic ketogenesis (Foster and McGarry, 1982, Fukao et al., 2004), so hepatic ketogenesis may not be greater

in MR. If plasma BHBA concentrations are similar between treatments, then a possible alternative is that the rumen epithelium in MR produces BHBA more efficiently than in MR+S.

In the rumen epithelium, ketogenesis is limited by HMGS activity, which is restricted by HMGS mRNA abundance (Lane et al., 2000). Abundance of HMGS mRNA was higher in MR than in MR+S treatment despite a lower proportion of butyrate in the rumen of MR treatment. The increased HMGS mRNA abundance could be due to several factors. It is noteworthy that the MR calves had greater insulin concentration in plasma compared with the MR+S calves. Insulin inhibits ketogenesis in the liver (Foster and McGarry, 1982, Fukao et al., 2004), but this may not hold true in ruminal epithelia. It is also possible that ruminal ketogenesis was more efficient in MR because it had greater amounts of alternate substrates. For example, free fatty acids, which have been shown to upregulate HMGS in hepatic tissue (Hegardt, 1999), may have been higher in MR than in MR+S as the milk replacer contained 18% fat compared to 4% in the calf starter; however, long chain fatty acids are only known to be oxidized in the rumen in a fasting state (Jesse et al., 1992). Another possible substrate is glucose, which can be an important substrate for ruminal ketogenesis pre-weaning (Rémond et al., 1995). Which alternate substrates are actually used by the rumen epithelium is unknown and is an interesting focus of future studies.

2.5.3 Age and Rumen Development

Between 3 and 8 weeks of age, there were no differences in plasma insulin, BHBA and glucose concentrations between MR+S and MR. Plasma glucose is highly regulated in non-diabetic mammals. Interestingly, plasma glucose increased between 6 and 7 weeks of age to approximately 120 mg/dL, regardless of treatment, which is nearly double the concentration of that found in lactating dairy cows (Silveira et al., 2007a). There was a significant effect of age on plasma insulin and glucose, but not

BHBA concentrations. The lack of change in plasma BHBA concentrations over time agrees with previous findings that plasma BHBA concentrations are dependent on age, not diet or energy intake (Lane et al., 2000). While average starter intake increased steadily from week 3 to week 8 of life, plasma insulin and glucose concentrations spiked between 6 and 8 weeks of life, suggesting that both plasma glucose and plasma insulin, prior to weaning, appear to be a function of age rather than diet. At slaughter, there was a difference in plasma insulin concentrations, suggesting that insulin may become diet-dependent at higher intakes of calf starter.

2.6 Conclusion

Feeding calf starter does not appear to affect rumen pH in pre-weaned dairy calves. Calf starter fermentation does alter expression of genes involved in pH_i regulation and ketogenesis in the rumen epithelium, signalling metabolic adaptation. While rumen epithelium did show evidence of metabolic adaptation to calf starter, plasma glucose, insulin and BHBA did not appear to be affected by calf starter fermentation and plasma glucose and insulin concentrations may be a function of age rather than diet.

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3.0 EFFECTS OF HIGH-FIBRE BY-PRODUCTS INCLUSION IN CALF STARTER ON GROWTH AND RUMEN pH IN HOLSTEIN DAIRY CALVES DURING THE WEANING TRANSITION

3.1 Abstract

The objective of this study was to evaluate the effects of substituting high fibre by-products for dry ground corn in calf starter on growth and rumen pH during the weaning transition. Holstein bull calves were raised on an intensified nursing program using milk replacer containing 26% CP and 18% fat. Calves were fed either a calf starter containing dry ground corn at 18.8% of dry matter (DM) (CORN), a starter with beet pulp replacing dry ground corn at 10.2% dietary DM (BP), or a calf starter with triticale dried distillers grains with solubles replacing dry ground corn and protein feedstuffs at 18.6% of dietary DM (DDGS). Starch concentrations of CORN, BP and DDGS were 35.3, 33.4 and 31.4%, respectively. When a calf consumed 2500 g of starter for 3 consecutive days, a small ruminant rumen pH logger was inserted orally and rumen pH was measured continuously for 4 days. Calves were then slaughtered and rumen fluid was sampled to determine short chain fatty acid profile. There was no difference in overall average daily gain or rates of hip height, wither height or heart girth growth. During the weaning transition, rate of increase in calf starter intake was greater for calves fed DDGS compared with those fed CORN (87.7 vs. 77.5 g/d), but lower for calves fed BP compared with CORN (68.1 vs. 77.5 g/d). The area under pH 5.8 (470 vs. 295 min×pH/d) or pH 5.2 (72.7 vs. 16.4 min×pH/d) was greater for calves fed DDGS than those fed CORN. Rumen pH profile was not affected by BP treatment compared with CORN, but calves fed BP tended to have greater water intake than those fed CORN (6.6 vs. 5.8 L/d). Short chain fatty acid profile was not affected by treatment with the exception of molar proportion of butyrate, which tended to be lower for calves fed BP compared with

those fed CORN (15.0 vs. 16.6 %). Hay intake was positively correlated to mean rumen pH for calves used in this study (r = 0.48). These results suggest that decreasing dietary starch concentration may not mitigate rumen acidosis in calves during weaning transition, and that low rumen pH may not adversely affect growth during the weaning transition.

3.2 Introduction

Rumen acidosis is one of the major challenges affecting profitability of dairy industry, with $500 million lost annually in the US dairy industry alone (Donovan, 1997). Rumen acidosis is the result of an accumulation of protons following rapid fermentation of feedstuffs. Sub-acute ruminal acidosis (SARA), with a threshold ranging from pH 5.5 to 6.0 in literature (Schwartzkopf-Genswein et al., 2003), is associated with laminitis, rumenitis, liver abscesses, depressed DMI and milk fat depression (Kleen et al., 2003). Management practices aiming to mitigate SARA is to decrease starch intake (Owens et al., 1998) and ruminal starch degradation (Voelker and Allen, 2003b), or increase rumen pH by stimulating salivary buffer flow (Allen, 1997).

Starch source may also affect rumen pH; inclusion of barley or wheat in calf starter caused a greater depression in rumen pH than that of corn or oats as the primary starch source, which is likely due to the rapid fermentability of barley and wheat starch (Khan et al., 2008). While starch source in calf starter may affect rumen pH, the effect of starch concentration of calf starter on rumen pH was not extensively investigated. There is a paucity of data about factors affecting rumen pH in calves and the effects of lower rumen pH on feed intake and growth in calves during the weaning transition. Furthermore, it is important to note that the studies mentioned above reported one-time rumen pH measurements and did not take diurnal variation into account, so gave no information regarding the duration or severity of ruminal acidosis.

The objective of this study was to investigate the effects of decreased dietary starch content in calf starters on rumen pH and calf performance during the weaning transition. We hypothesized that partially replacing corn grain with high-fibre byproducts would decrease incidence and severity of SARA. As SARA often decreases DMI in mature ruminants, we further hypothesized that lower incidence of SARA in calves would increase DMI and ADG.

3.3 Materials & Methods

3.3.1 Animals & Diets

All animals were cared for according to protocols approved by the University of Alberta Animal Care and Use Committee for Livestock (Edmonton, AB, Canada) using the guidelines of the Canadian Council for Animal Care (Ottawa, ON). Forty-two Holstein bull calves (14.9 ± 0.2 d) were obtained from a commercial dairy farm (AB, Canada) and brought to the Metabolic Unit at the University of Alberta Edmonton Research Station at four different time points from July until September, 2009. Prior to arrival, calves were housed in individual pens and fed up to 4L of milk replacer containing 26% CP and 18% fat (Excel Calf Milk Replacer, Grober Nutrition, Cambridge, ON, Canada) daily. Upon arrival, calves were blocked by arrival date and BW, and randomly assigned to treatment (Table 3.1); a control calf starter (CORN), a calf starter where dry ground corn was replaced with beet pulp at 10.1% DM (BP) or a calf starter where dry ground corn and protein feedstuffs were replaced with triticale dried distillers' grains with solubles at 18.6% DM (DDGS). Calves were individually housed in 3.05 x 3.66 m pens with 2.42 x 2.42 m rubber mats (North West Rubber Mats Ltd., Abbotsford, BC, Canada) and had free access to water throughout the study. Body weight, hip height, wither height and heart girth were measured weekly.

Table 3.1 Ingredient and nutrient composition of experimental texturized calf starters

	CORN	BP[1]	DDGS[2]
%DM			
Barley grain	10.3	10.3	10.3
Corn grain	19.3	19.3	19.3
Molasses	1.5	1.5	1.5
Pellet	69.0	69.0	69.0
Pellet			
Ground corn	18.8	8.6	8.6
Beet pulp	0.0	10.1	0.0
Triticale DDGS	0.0	0.0	18.6
Corn gluten meal, %DM	4.0	4.0	0.0
SoyPlus[3], %DM	9.2	9.2	4.0
Mill run, %DM	10.6	10.6	11.3
Soybean meal, %DM	13.7	13.7	13.7
Canola meal, %DM	5.5	5.5	5.5
Dehydrated alfalfa, %DM	3.1	3.1	3.1
Vitamins and trace minerals[3], %DM	2.4	2.4	2.4
Molasses, %DM	1.7	1.7	1.7
Deccox (Decoquinate), %DM	0.1	0.1	0.1
Chemical composition			
DM, %	89.5	89.8	92.8
OM, %DM	91.7	91.9	92.8
CP, %DM	27.1	25.0	24.7
Ether extract, %DM	1.9	1.6	3.1
NDF, %DM	17.8	18.8	21.8
Starch, %DM	35.3	33.4	31.4
in situ 2-h DM disappearance, %	48.0	48.9	56.3
in situ 8-h DM disappearance, %	54.4	53.0	60.3

[1]BP – Beet pulp
[2]DDGS – Triticale dried distillers' grains with solubles
[3]SoyPlus – Heat-processed soybean meal with 60% rumen bypass protein
[4]Included 0.07% Vitamin ADE mix, 0.02% vitamin B, 0.01% vitamin D, 0.02% vitamin E, 0.11% trace mineral mix, 0.12% custom trace mineral mix, 0.04% selenium, 0.75% dicalcium phosphate, 1.8% limestone, and 0.6% salt.

Calves were raised on an intensified nursing program using a commercial milk replacer containing 26% CP and 18% fat (Excel Calf Milk Replacer, Grober Nutrition, Cambridge, ON, Canada). The milk replacer was fed in 2 equal portions at 0600 and 1715 h. After arrival, amount of milk replacer fed to calves was gradually increased from 2 to 4L per feeding over a 7-d period to adapt calves to intensive liquid feeding. During the adaptation period, calves were offered no calf starter. From 4 until 6 weeks in age, calves were fed milk replacer at 1,200 g/d and were offered one of the calf starters ad libitum at 0615 h. In week 7, weaning was started by reducing milk replacer provided to 900 g/d. Amount of milk replacer offered was further reduced to 600 g/d in week 8. In week 9, no more milk replacer was provided and mixed legume hay was provided ad libitum.

Amount of starter offered was limited to 2.50 kg/d, to minimize fluctuations in intake. When calves consumed 2.45 kg/d for 3 consecutive days, a small ruminant rumen pH measuring system (SRS; 20.6 mm diameter, 138 mm length, 245 g mass; Dascor, Escondido, CA, USA was inserted orally and rumen pH was measured every 2 min continuously for 4 days as previously described (Penner et al., 2009a). To ensure placement of the SRS in the rumen, when the SRS was dosed, calves were held in a reclining position to avoid SRS entry to the reticulum; the SRS entered the dorsal sac of the rumen, and moved to the ventral sac when calves were allow to stand. After 4 d of rumen pH measurement period, calves were killed by captive bolt gun followed by exsanguination. The SRS was retrieved from the rumen and rumen contents were collected and strained through a perforated screen (Peetex, pore size = 355 μm; Sefar Canada Inc., Scarborough, Ontario, Canada) to collect rumen fluid and stored at -80^{o}C until analysis.

3.3.2 Sample Analysis

Samples of calf starter were collected weekly and dried at 55°C for 48 h. All samples were ground through a 1-mm screen using a Wiley Mill (Thomas-Wiley, Philadelphia, PA, USA) and composited monthly. Composite samples were analyzed for DM at 135°C for 2h and OM content was determined after 2-h combustion at 600°C (AOAC, 2002). Crude protein content was analyzed using flash combustion (TruSpec analyzer, Leco Corp., St. Joseph, MI, USA; Wrolstad et al., 2004). Starch content was analyzed using an enzymatic method (Karkalas, 1985), and NDF content was analyzed using a heat-stable amylase and sodium sulfite as described by Van Soest et al. (1991). Ether extract content was analyzed using a Goldfisch extractor (Labconco, Kansas City, MO; Wrolstad et al., 2004).

To determine in situ DM disappearance, composited feed samples were placed in nylon bags and the nylon bags were placed in the rumen of a lactating dairy cow for 2 and 8 h. Immediately after the removal of nylon bags, they were rinsed with water and frozen at -80°C overnight, then rinsed with water again. All samples were then dried at 105°C overnight and DM disappearance was determined.

Rumen fluid samples were thawed and centrifuged at 10 000 x g at 4°C for 20 min. In an autosampler vial, 200 µL of 25% phosphoric acid (v/v) and 200 µL of 0.05% isocaproic acid (v/v), which was used as an internal standard, were added to 800 µL of SCFA supernatant. Samples were analyzed using gas chromatography (Varian 3400GC, Varian, Walnut Creek, CA, USA) using a Stabilwax-DA 30-m column (Restek Corp., Bellefonte, PA, USA) with 7.5-psi head pressure and a 20-mL/min split vent flow. The injector temperature was 170°C, the column temperature was 170°C and the detector temperature was 190°C. The run time for each sample was 10 min; samples were run in duplicate. Chromatograms were integrated using Galaxie software (Varian, Walnut Creek, CA, USA).

3.3.3 Rumen pH Data

Data were downloaded from the SRS and minimum, mean and maximum pH were calculated. Also, duration of pH below 5.8 and 5.2 (min/d) and area under pH 5.8 and pH 5.2 (min×pH/d) were calculated.

3.3.4 Statistics

Wither height gain, hip height gain, heart girth gain, ADG and rate of starter intake increase were calculated by regression analysis of weekly measurements using PROC REG in SAS (v.9.1; SAS Institute, Cary, NC, USA). Then, all data were analyzed using a randomized complete block design in PROC MIXED of SAS using the model

$Y = \mu + Treat_i + Block_j + e_{ij}$.

Pre-planned orthogonal contrasts were made to compare CORN and BP, and CORN and DDGS. Significance and tendency of treatment effects were declared at $P < 0.05$ and $P < 0.10$, respectively.

3.4 Results

3.4.1 Growth

There was no difference in overall ADG, wither height gain, hip height gain, or hearth girth gain between CORN and BP nor between CORN and DDGS (Table 3.2). During the weaning transition, from 1 week before start of weaning to 1 week after completion of weaning (6 to 9 weeks in age), ADG was not affected by treatment, but daily increases in starter intake was lower for calves fed

Table 3.2 Effect of partially replacing corn grain with beet pulp or triticale dried distillers' grains with solubles in calf starter pellet on growth performance of Holstein bull calves

	CORN	BP	DDGS	*P* CORN vs. BP	*P* CORN vs. DDGS
Overall growth rate					
ADG, kg/d	1.05 ± 0.04	0.98 ± 0.04	1.00 ± 0.04	0.22	0.42
Wither Height Gain, cm/d	0.23 ± 0.01	0.23 ± 0.01	0.24 ± 0.01	0.99	0.56
Hip Height Gain, cm/d	0.26 ± 0.02	0.24 ± 0.02	0.28 ± 0.02	0.47	0.41
Heart Girth Gain, cm/d	0.35 ± 0.02	0.38 ± 0.02	0.40 ± 0.02	0.34	0.13
Weaning transition[1]					
ADG, kg/d	1.02 ± 0.08	0.86 ± 0.08	0.93 ± 0.08	0.15	0.43
Rate of increase in starter DMI, g/d	77.5 ± 3.4	68.1 ± 3.1	87.7 ± 3.4	0.04	0.04
Age reaching maximum starter intake[2], d	65.9 ± 1.5	68.7 ± 1.4	64.3 ± 1.4	0.20	0.44

[1]Calculated from 1 week prior to weaning to 1 week after completion of weaning (6 – 9 weeks of age)
[2]Maximum starter intake refers to the 3rd consecutive day where starter intake was greater than 2450 g

BP than those fed CORN (68.1 vs. 77.5 g/d; $P = 0.04$), but higher for calves fed DDGS than those fed CORN (87.7 vs. 77.5 g/d; $P = 0.04$).

3.4.2 Rumen pH

Between CORN and BP treatments, there were no differences in minimum, mean, or maximum pH, nor the duration or area under pH 5.8 or 5.2 (Table 3.3). The BP group tended to have a greater water intake during the rumen pH measurement period than the CORN calves (6.6 vs. 5.8 L/d; $P < 0.07$).In addition, there was no difference in hay intake between BP and CORN treatments.

Between CORN and DDGS treatments, there was no difference in water intake or hay intake. Minimum pH was lower in DDGS than in CORN (4.77 vs. 4.91; $P < 0.01$), but mean pH did not differ. Standard deviation of mean pH tended to be greater for DDGS than CORN treatment (0.62 vs. 0.51; $P = 0.05$). The duration that rumen pH is below 5.8 did not differ between DDGS and CORN, but the area under pH 5.8 was greater in DDGS than CORN treatment (470 vs. 295 pH×min/d; $P = 0.03$). Both duration below pH 5.2 (403 vs. 205 min/d; $P = 0.03$) and area under pH 5.2 (72.7 vs. 16.4 pH×min/d; $P < 0.01$) were greater for DDGS than CORN treatment.

During the rumen pH measurement period, average daily hay intake was positively correlated to mean rumen pH ($r = 0.48$, $P < 0.01$) for dataset containing all treatments (Figure 3.1); however, water intake was not related to mean rumen pH ($r = 0.21$, $P = 0.28$; Figure 3.2). Inclusion of hay DMI as a covariate in statistical analysis of pH data did not affect significance of treatment effects.

Table 3.3 Effect of partial replacement of corn grain in starter pellet with beet pulp or triticale dried distillers' grains with solubes on rumen pH profile of Holstein bull calves post-weaning

	CORN	BP	DDGS	*P* CORN vs. BP	*P* CORN vs. DDGS
Minimum pH	4.91 ± 0.04	4.90 ± 0.03	4.77 ± 0.03	0.69	< 0.01
Mean pH	5.79 ± 0.07	5.83 ± 0.05	5.72 ± 0.06	0.59	0.52
Maximum pH	6.88± 0.08	6.97 ± 0.07	7.11 ± 0.08	0.38	0.04
Standard deviation	0.51 ± 0.04	0.48 ± 0.03	0.62 ± 0.04	0.44	0.05
Duration pH < 5.8, min/d[1]	867 ± 67	784 ± 49	875 ± 56	0.32	0.93
Duration pH < 5.2, min/d[1]	205 ± 70	143 ± 67	403 ± 70	0.45	0.03
AUC[2] pH < 5.8, min×pH/d	295 ± 57	298 ± 47	470 ± 54	0.97	0.03
AUC[2] pH < 5.2, min×pH/d	16 ± 14	13 ± 13	73 ± 13	0.85	< 0.01
Starter, kg/d	2.50	2.50	2.50		
Hay DMI, kg/d	0.26 ± 0.04	0.27 ± 0.04	0.24 ± 0.04	0.83	0.72
Water intake, kg/d	5.82 ± 0.32	6.63 ± 0.24	5.95 ± 0.26	0.07	0.78

[1]pH was measured every 2 min. If rumen pH was below a threshold, rumen pH was considered to be below the threshold for the following 2 min.
[2]Area under the curve, calculated by multiplying the time pH was below pH threshold by the number of pH units that pH was below threshold at each sampling point.

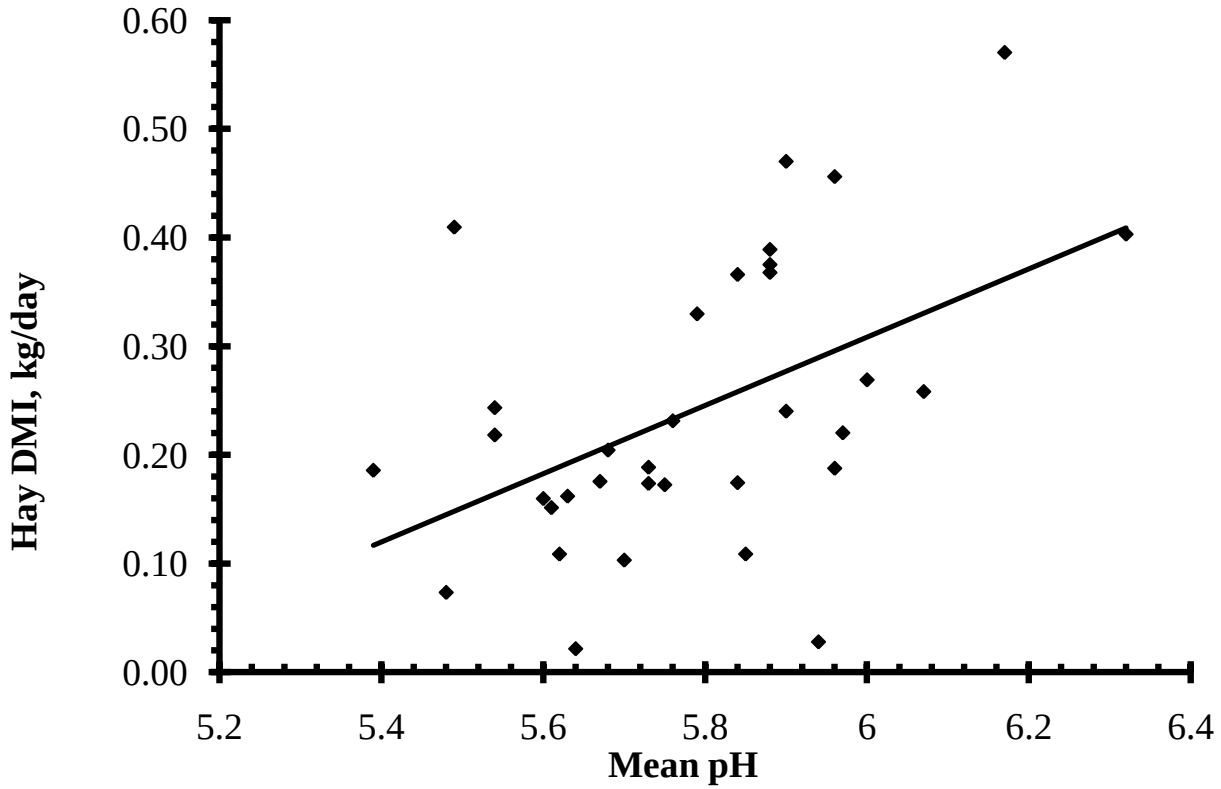

Figure 3.1 Relationship between hay DMI (kg/d) and mean rumen pH in Holstein bull calves after weaning (r = 0.48, P < 0.01). Linear line of best fit: y = 0.31x – 1.58; r^2 = 0.24

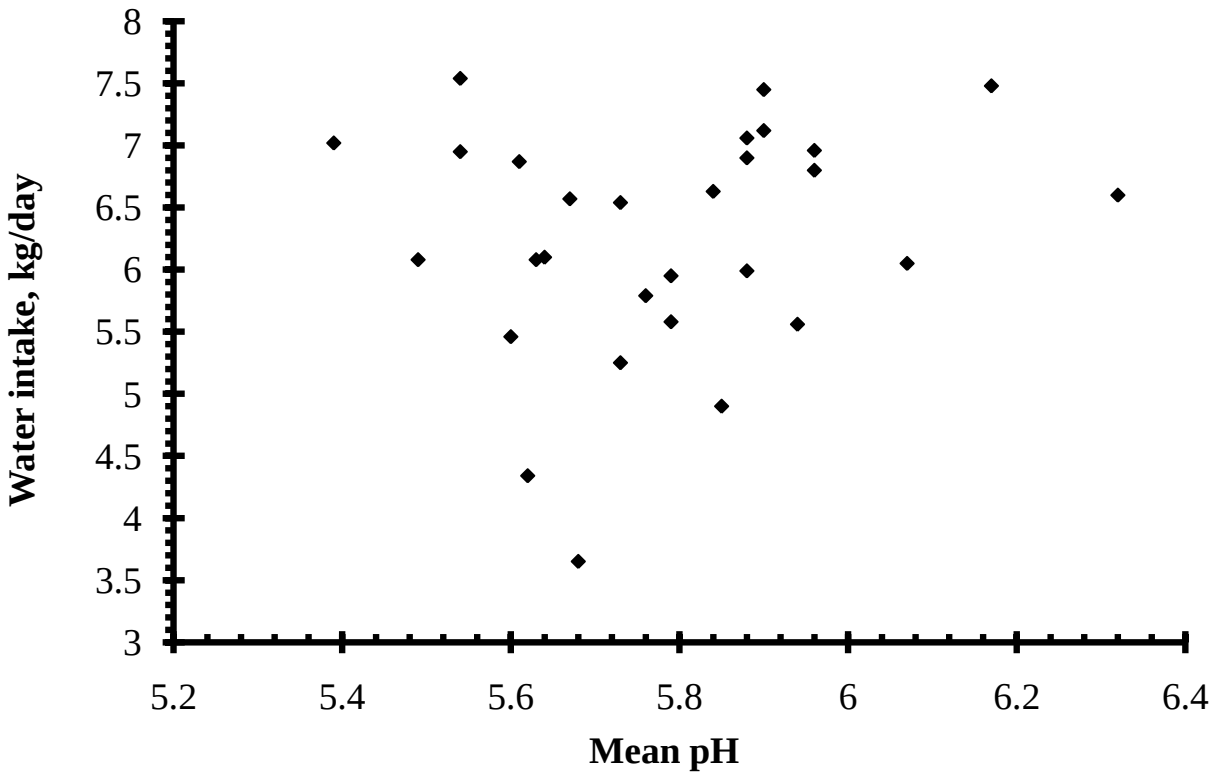

Figure 3.2 Relationship between water intake (kg/d) and mean rumen pH in Holstein bull calves after weaning (*r* = 0.21, *P* = 0.28).

3.4.3 SCFA Profile

Total SCFA concentrations in the rumen did not differ between BP and CORN treatments or between DDGS and CORN treatments (Table 3.4). Molar proportion of butyrate in rumen fluid tended to be lower in BP than in CORN (15.0 vs. 16.6 %; $P = 0.06$). Molar proportions of the other SCFA and the acetate-to-propionate ratio were not affected by treatment.

3.5 Discussion

In this study, treatment feedstuffs were included into pellets of texturized calf starters. Texturized calf starters are preferable over pelleted starters as calves fed texturized calf starters had greater starter intake both pre-weaning and during the weaning transition (Bach et al., 2007). Calves fed a completely pelleted calf starter started ruminating 2 weeks later than calves fed a coarse mash calf starter with average particle size over 2000 μm (Porter et al., 2007). Incorporating the treatment into the pellet in our study aimed to keep average particle size the same among all treatments and prevent calves from sorting feed.

3.5.1 Factors Influencing Rumen pH in Calves

Increasing dietary starch content depressed rumen pH in both goats (Wang et al., 2009) and lactating dairy cows (Silveira et al., 2007b). As such, we hypothesized that decreasing starch content of calf starter increase rumen pH. In the current study, to evaluate specific effect of starch content of calf starter on rumen pH, calves are limit-fed calf starters containing corn and barley grain as the common starch sources. In calves, lower solid feed intake has been shown to depress average rumen pH at

Table 3.4 Short chain fatty acid (SCFA) profile of weaned Holstein bull calves fed different high fibre by-products as partial replacement for corn grain in calf starter

	CORN	BP	DDGS	*P* CORN vs. BP	*P* CORN vs. DDGS
Total, m*M*	175 ± 6.5	175 ± 6.8	177 ± 6.3	0.98	0.84
Acetate, mol/100 mol SCFA	54.4 ± 1.7	57.8 ± 1.7	57.8 ± 1.7	0.14	0.14
Propionate, mol/100 mol SCFA	24.4 ± 1.4	22.5 ± 1.5	21.7 ± 1.6	0.24	0.14
Butyrate, mol/100 mol SCFA	16.6 ± 0.8	15.0 ± 0.7	16.8 ± 0.7	0.06	0.85
Valerate, mol/100 mol SCFA	4.3 ± 0.4	4.0 ± 0.3	3.6 ± 0.3	0.38	0.11

weaning because of lower protein intake and ruminal degradation (Khan et al., 2008). In our study, solid feed intake was limited to 2.50 kg/d, so it is unlikely that starter intake affected rumen pH since starter intake was constant during the rumen pH measuring period. It has been also suggested that, in weaned calves, higher starch degradation rates of wheat and barley may result in a lower rumen pH when wheat or barley are used as a calf starter starch source rather than oats or corn (Khan et al., 2008). In our study, the primary starch source was the same in all treatments, so it is unlikely that the difference in starch degradation rates among the treatments affected rumen pH.

In the current study, the greatest in situ 2-h DM disappearance was found in DDGS, the calf starter with the lowest dietary starch concentration. In addition, in spite of lower starch concentration of BP compared with CORN, in situ 2-h DM disappearance was not different. As such, the difference in 2-h DM disappearance cannot be attributed to the difference in starch content among calf starters. Severity of SARA was greater for DDGS compared with CORN, suggesting that in situ 2-h DM disappearance may be a good parameter that influences rumen pH in calves. The typical rumen pH profile showed a large drop in pH within 3 h of morning feeding followed by a recovery that lasted most of the day, highlighting the importance of the first few hours of digestion on rumen pH in calves.

Hay intake was positively correlated to mean rumen pH in the current study, which confirms the previous finding that hay intake was negatively correlated to the area below pH 5.8 (Chapter 2).. When compared to a calf-starter-only diet, inclusion of forages alongside the calf starter has been shown to increase rumen pH in pre-weaned calves (Suárez et al., 2007) . Inclusion of dietary forage NDF in mature cows promotes chewing, which stimulates salivary buffer flow (Allen, 1997). Since hay intake was negatively correlated to rumen pH, it is possible that hay intake may also stimulate chewing and salivary buffer flow in calves.

Greater water intake can increase rate of digesta passage (McDowell et al., 1969, Pagan et al., 1998), so it is possible that increased rate of passage would decrease fermentation in the rumen, which increases rumen pH. However, water intake was not correlated with any rumen pH variables. Water consumption tended to be greater for BP compared to CORN treatment, but this may be due to the hygroscopic nature of beet pulp (Phatak et al., 1988, Wen et al., 1988). Beet pulp may retain more water than corn or DDGS, so more water may have been needed to maintain the liquid environment for bacteria in the rumen, and did not appear to affect rumen pH.

3.5.2 Rumen pH and Overall Growth

Standard deviation of mean rumen pH for DDGS treatment was greater than CORN treatment, but there were no significant differences in standard deviation of mean rumen pH between BP and CORN treatments. Greater variability in rumen pH, especially at average pH below 6.0, has been shown to decrease digestion of DM, fibre and non-structural carbohydrate (de Veth and Kolver, 2001, Wales et al., 2004). Low rumen pH decreased digestion of OM and NDF (Cerrato-Sánchez et al., 2007, 2008). Based on these previous findings, it is speculated that calves fed DDGS decreased fibre digestion. Negative effects of depressed ruminal pH also include increased rumen epithelial permeability in steers (Emmanuel et al., 2007) and depressed DMI (Kleen et al., 2003). In calves under 6 weeks old, DMI is positively correlated to ADG (r^2 = 0.73; Lammers et al., 1998). As such, we hypothesized that SARA negatively affects ADG of calves during the weaning transition. However, although DDGS had greater incidence and severity of SARA in the current study, calves fed DDGS also had a faster rate of increase of starter intake during the weaning transition, providing no evidence to support the idea that SARA depresses DMI of calves. Growth rates also did not differ between DDGS and CORN treatments, indicating that SARA may not adversely affect growth in calves prior to

weaning or during the weaning transition.

During the weaning transition, calves fed DDGS had faster rate of increase in starter intake than those fed CORN, so the DDGS treatment would be expected to have a higher ADG than CORN during the weaning transition. In spite of the faster rate of increase in starter intake, ADG in calves fed DDGS did not differ from CORN during the weaning transition. Rate of increase in starter intake during the weaning transition was lower in BP than in CORN, which agrees with findings in mature cows, where inclusion of beet pulp in the diet caused a linear decrease in DMI, likely because beet pulp increased rumen fill through water retention (Voelker and Allen, 2003a). Despite lower rate of increase in calf starter intake, ADG during the weaning transition between BP and CORN was not different.

From our data, SARA does not appear to negatively affect feed intake and growth performance of calves during the weaning transition. It has previously been suggested that low rumen pH may be advantageous during rumen epithelial development (Stobo et al., 1966) as decreased luminal pH can depress epithelial intracellular pH (pH_i) in vitro (Müller et al., 2000). Modest acidification of pH_i to 7.0 prevented cellular apoptosis in kidney cells (Akimova et al 2006), and activated mitogen-activated protein kinase signalling pathways, leading to cell proliferation (Sarosi et al., 2005). The possibility of pH_i acidification stimulating rumen epithelial cell proliferation warrants further investigation. However, the data from the current study need to be interpreted with caution. Because calves were terminated shortly after weaning, the impacts of SARA on long term growth performance and animal health could not be evaluated in this study. In mature cows, SARA is detrimental to health (Krause and Oetzel, 2006, Nocek, 1997), so it is important to investigate the effects of SARA on animal health and growth at different stages of rumen development.

3.6 Conclusion

Dietary starch concentration in calf starter may not be the primary factor affecting rumen pH. Greater in situ DM disappearance and lower starch concentration in DDGS compared to CORN was accompanied with greater severity of SARA and ARA, suggesting that decreased starch concentration of calf starter does not necessarily means less fermentation in the rumen and higher rumen pH. Despite greater duration and severity of SARA for calves fed DDGS compared with those fed CORN, growth performance of calves was not affected during the weaning transition. The long-term effects of SARA on feed intake, growth performance, and general health of calves warrant further investigation.

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4.0 GENERAL DISCUSSION

4.1. Study Summary

Study 1 evaluated the effects of calf starter consumption on the expression of genes involved in ketogenesis and pH regulation in the rumen epithelium as well as rumen pH and short chain fatty acid (SCFA) profile. In this study, animals are fed diets that are similar in metabolizable energy concentration according to NRC (2001) so that results were not confounded by the difference in energy intake. For enzymes involved in ketogenesis, abundance of 3-hydroxy-3-methylglutaryl-CoA synthase (HMGS) mRNA decreased with provision of calf starter, while mRNA abundance of 3-hydroxy-3-methylglutaryl-CoA lyase (HMGL), β-hydroxybutyrate dehydrogenase -1 (BDH-1), β -hydroxybutyrate dehydrogenase-2 (BDH-2) and acetoacetyl-CoA thiolase (ACAT) were not affected by treatment. For transporter protein responsible for intracellular pH regulation, abundance of monocarboxylate transporter-1 (MCT-1) mRNA increased with calf starter, while sodium proton exchanger (NHE)-3 expression decreased and NHE-2 expression was not affected. Rumen pH measurements were not affected by consumption of calf starter in spite of higher SCFA concentrations in the rumen. Further, calf starter increased the molar proportion of butyrate and decreased the molar proportion of acetate and tended to decrease the acetate: propionate ratio. Although hay intake was not affected by calf starter feeding, hay intake was negatively correlated to severity of ruminal acidosis. Study 1 demonstrated that calf starter affects expression of genes involved in ketogenesis and intracellular pH regulation.

In study 2, we evaluated the effects of partially replacing ground corn (CORN) with beet pulp (BP) or triticale dried distillers' grains with soluble (DDGS) in calf starter to lower dietary starch content. We hypothesized that decreased starch concentration would decrease incidence and severity of

sub-acute ruminal acidosis (SARA), accelerate rate of increase in starter intake and therefore increase ADG. The study focused on growth, rumen pH, and SCFA profile during and immediately after the weaning transition. In the DDGS calf starter, in situ dry matter (DM) disappearance after 2h and 8h was greater than CORN, but in situ DM disappearance did not differ between CORN and BP. Growth rates in hip height, wither height and heart girth, and ADG were not affected by treatment. Although ADG during the weaning transition did not differ between treatments, DDGS treatment had a greater rate of increase in calf starter intake than CORN treatment, and CORN treatment had a greater rate of increase in calf starter intake than BP treatment. The BP treatment tended to have greater water intake post-weaning than CORN, although rumen pH measurements were not affected. The DDGS treatment had a lower minimum pH, tended to have greater variation in pH, and had greater severity of acidosis as well as a greater duration of acute acidosis (pH < 5.2). Other than a tendency for decreased molar proportion of butyrate in rumen fluid for the BP as compared to CORN treatment, there were no differences in SCFA profile between BP and CORN or between DDGS and CORN. Hay intake was positively correlated to mean rumen pH. Study 2 demonstrated the potential influence of hay intake and in situ DM disappearance of calf starter on rumen pH in dairy calves during the weaning transition.

4.2 New Contributions to Body of Knowledge

Study 1 demonstrated that consumption of calf starter affects gene expression of epithelia in the ventral sac of the rumen at the weaning transition. Specifically, genes involved in both ketogenesis and in intracellular pH regulation in ruminal epithelia were affected by calf starter fermentation. While mRNA abundance does not necessarily imply activity, up-regulation or down-regulation in gene expressions as a result of dietary changes can indicate a metabolic response. Study 2 demonstrated that decreasing dietary starch concentration of calf starter does not necessarily decrease its in situ DM disappearance of calf starter and increase rumen pH.

4.2.1 Calf Starter May Decrease Ketogenesis Capacity

In adult cows, ketogenesis in ruminal epithelia is an important process in systemic energy homeostasis. During the weaning transition, the ruminant liver transitions from being glucolytic, when glucose from milk is the primary metabolic fuel, to being glucogenic, when SCFA are the primary metabolic fuels (Baldwin et al., 2004). Propionate, in particular, is thought to account for as much as 80% of glucose produced from gluconeogenesis (Allen et al., 2009), so the hepatic metabolic adaptation is substantial. Since hepatic gluconeogenesis from propionate is significantly reduced by butyrate (Faulkner and Pollock, 1986), ketogenesis in ruminal epithelia may contribute to gluconeogenesis by reducing butyrate supply to the liver. Therefore, ketogenesis in the rumen epithelium is considered an important indicator of metabolic adaptation(Lane et al., 2002).

Study 1 evaluated the effect of starter feed on metabolic adaptation of ruminal epithelia without confounding effects of age or difference in energy intake. Our study found that under isocaloric conditions, ketogenesis efficiency in ruminal epithelia may be lower when starter feed partially replaces milk replacer. In mature rumen epithelium of sheep, HMGS activity parallels ketogenic rates *in vivo* (Leighton et al., 1983), meaning that HMGS is the rate-limiting enzyme; HMGS activity is regulated long-term by HMGS mRNA abundance (Thumelin et al., 1993). As high fat intake promotes hepatic ketogenesis (Quant et al., 1991), the low HMGS abundance for calves fed starter might be attributed to reduced fat intake. However, long chain fatty acids (LCFA) have been documented to undergo ruminal ketogenesis only during fasting when SCFA are limiting (Jesse et al., 1992), so it is not clear if the LCFA were used as substrates for ketogenesis in the MR calves. More research could be done to investigate whether LCFA are involved in ruminal ketogenesis prior to weaning and how ketogenesis affects growth and development of calves.

4.2.2 Calf Starter Alters Expression of Genes Involved in Ruminal Epithelial pH Regulation

In study 1, consumption of calf starter influenced expression of genes involved in intracellular pH regulation without affecting ruminal pH itself. The decrease in NHE-3 and the increase in MCT-1 mRNA abundance suggest that consumption of starter feed changed intracellular pH (pH_i) regulation mechanisms in ruminal epithelia. It is not clear at this point what specific factors are causing the differences in gene expression. Since SCFA enhance rumen papillae growth (Lane and Jesse, 1997), it is possible that SCFA concentration in the rumen is responsible for altering gene expression. Previous studies showed that incubating colonic cells in butyrate affects cell proliferation (Davie, 2003). Butyrate has been shown to up-regulate NHE-3 expression in colon cells, increasing proton expulsion from the cytosol (Kiela et al., 2007) while down-regulating Na^+-K^+-$2Cl^-$ cotransporter in human intestinal epithelial cells, decreasing Cl^- secretion (Matthews et al., 1998), thus indicating butyrate can affect trans-membrane ion transport. Further research should focus on both identifying other genes affected by starter consumption as well as the abundance and activity of these transport proteins.

4.2.3 Rumen pH Appears to be Affected by DM-disappearance Rates

Study 2 suggested that dietary starch content may not affect rumen pH. Rumen pH in goats has been shown to decrease when dietary starch concentration is increased from 35% to 46%, but not when it is raised from 28% to 35% (Wang et al., 2009). In our study, starch concentration of calf starters ranged from 31 to 35 % DM; the calf starter with lowest dietary starch concentration had a greater severity of ruminal acidosis, suggesting that starch content may not be the primary factor affecting rumen pH. However, the calf starter that had the highest in situ DM disappearance decreased rumen pH; DDGS treatment had the greatest severity of acidosis and greatest standard deviation of rumen pH.

4.3 Conclusion

We hypothesized that calf starter fermentation would decrease rumen pH and up-regulate expression of genes involved in pH_i regulation and ketogenesis in the rumen epithelium. We further hypothesized that decreased pH and increased severity of SARA would adversely affect growth. Our results demonstrated that calf starter fermentation did appear to increase overall fermentation in the rumen, but did not affect rumen pH. Although ruminal SCFA concentrations were used as an indicator of fermentation, it is by no means an exclusive indicator of SCFA production as ruminal SCFA concentrations are a balance between production from fermentation and removal via absorption and passage. Therefore, SCFA concentration data must be interpreted with caution. Furthermore, our results indicated that calf starter fermentation changes metabolic adaptations in rumen epithelium at the transcription level. Our results do not de facto translate to protein abundance or its activity, since many post-transcriptional regulatory mechanisms exist, so our results should be interpreted only as a transcription-level response to treatment.

Lastly, our results indicated that increased severity of SARA and ARA does not adversely affect growth during the weaning transition or during the pre-ruminant period of life. These results are limited by the short-term observational period of our study and do not give an indication of SARA and ARA on long-term growth performance. Although increased SARA was associated with a higher rate of increase of calf starter intake, that did not contribute to increased ADG, so it is not clear if increased SARA is economically adverse in a commercial setting.

4.4 Future Research

Study 1 also demonstrated the ability of calf starter to alter expression of genes regulating pH_i in ruminal epithelia. Other genes have also been implicated in a recent review (Connor et al., 2010). Specifically, downregulated in adenoma (DRA), an HCO_3^-/Cl^- antiporter, and alcohol dehydrogenase

were shown to be upregulated 142-fold and 91-fold, respectively, in calves fed milk replacer and calf starter or milk replacer and hay compared to calves fed milk replacer only (Connor et al., 2010). Increased expression of HCO_3^- exporters and alcohol metabolizers indicate the rumen adapts metabolically to increased ruminal fermentation by up-regulating genes involved in managing luminal and intracellular proton concentrations and metabolizing fermentation end-products. Although mRNA abundance is being analyzed, more protein abundance and activity studies are needed to facilitate a more complete understanding of molecular responses to calf starter fermentation. A better understanding of metabolic adaptation is needed to fully understand the physiology of pH_i regulation over the course of development.

Further research is also needed on the amount of hay that should be provided to calves. Inclusion of ground hay in calf starter enhanced ruminal papillae development over concentrate alone (Nocek et al., 1984), but there is regression of ruminal papillae when weaned calves switch from a high-concentrate to an all-hay diet (Stobo et al., 1966). To limit abnormal papillae growth, hyperkeratinisation (McGavin and Morrill, 1976) and reduced SCFA transport (Nocek et al., 1984), inclusion of 10% ground or chopped hay is recommended for young calves fed starter (Davis and Drackley, 1998). However, roughage inclusion at 10% has been associated with decreased ADG (Hill et al., 2008), so dietary roughage inclusion cannot recommended unless its benefits to calves are clearly demonstrated.

In study 1, we found that rumen pH < 5.2 was experienced by only 3 calves, all of whom consumed hay at less than 100 g/d (Figure 4.1). Based on a breakpoint analysis, calves that consumed more than 0.064 kg/d of hay did not appear to experience rumen pH < 5.2. Rumen epithelial barrier integrity is compromised at pH 5.1 (Aschenbach et al., 2000) and rumen permeability can be increased

5-fold at pH 5.5 in the presence of LPS (Emmanuel et al., 2007), thus low rumen pH is detrimental to proper epithelial barrier function. It is possible that providing a limited amount of hay will help prevent epithelial barrier function from being impaired, although we cannot conclude, from these data, that hay intake of 0.064 kg/d is sufficient to prevent ARA in all calves. More research is needed to investigate the effects of limit feeding hay on the incidence and severity of rumen acidosis.

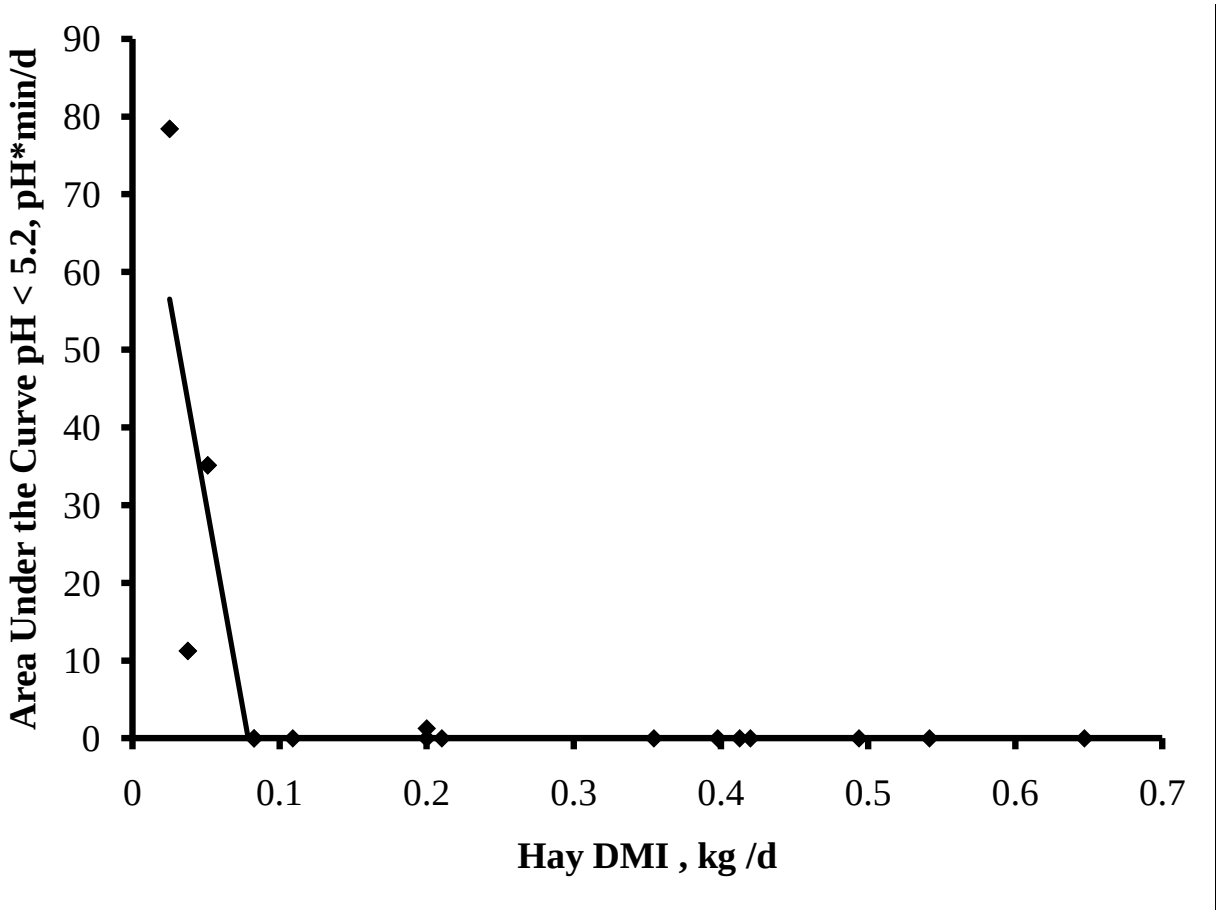

Figure 4.1 Relationship between hay DMI (kg/d) and area under pH 5.2 (r = -0.71; P < 0.01). The breakpoint analysis showed that area under pH 5.2 becomes zero at hay DMI of 0.064 kg/d.

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