Adaptive Response of the Bovine Rumen Epithelium to Dietary Changes

by

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This thesis studied the short-term and long-term adaptive response of the bovine rumen epithelium to dietary changes, and the impact of butyrate on the epithelium adaptive response. To study the short-term adaptive response, Holstein steers were switched from an ad libitum diet to either a subacute ruminal acidosis (SARA) challenge or a feed restriction treatment, and then acetate and butyrate fluxes through different short chain fatty acid (SCFA) transport mechanisms were measured in Ussing Chambers. Using immunofluorescence, we studied the long-term adaptive response by measuring temporal changes in SCFA transport capacity and intracellular pH regulation in rumen papillae over the transition period in Holstein dairy cows. Lastly, we investigated butyrate’s potential to influence the adaptive response by adding butyrate to the rumen during SARA in early lactating cows. In the first study, 5 d feed restriction increased butyrate flux via an upregulation of monocarboxylate co-transporter isoform 1, a basolateral SCFA transporter in the rumen epithelium. In the long-term response study, the transition period increased SCFA absorption capacity through epithelial remodeling and increased absorptive
surface area, evidenced by decreased abundance of carbonic anhydrase 2. Due to maintenance of SCFA transporters concurrent with an increase in absorptive surface area, SCFA absorption capacity increased. In the last study, butyrate augmented the SCFA absorption capacity during SARA by increasing monocarboxylate co-transporter 1 expression, and improving epithelial barrier integrity by increasing expression of genes associated with glycolysis and oxidative phosphorylation, while decreasing expression of lipogenic genes. Together, these studies show that the adaptive response of the rumen epithelium involves a short-term cellular response and a long-term morphological response that increase SCFA absorption capacity. The cellular response increases monocarboxylate co-transporter isoform 1 abundance, which can be augmented by supplying supplemental butyrate to the rumen. The morphological response increases absorptive surface area through epithelial remodeling. Epithelial remodeling can be improved by butyrate because butyrate induces a genomic shift to mobilize energy and thus help maintain epithelial barrier integrity. Both the cellular adaptive response and the morphological adaptive response are dependent on key molecular factors that serve to modulate SCFA absorption in dairy cows during dietary transitions.
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## Table of Contents

Abstract ........................................................................................................................................... ii  

Acknowledgments.......................................................................................................................... iv  

Table of Contents ........................................................................................................................... vi  

List of Tables .................................................................................................................................... xi  

List of Figures .................................................................................................................................... xii  

List of Symbols and Abbreviations............................................................................................... xv  

Chapter 1: Literature Review.......................................................................................................... 1  

1.1 General Introduction ................................................................................................................ 1  

1.2 Subacute Ruminal Acidosis ..................................................................................................... 3  

1.2.1 Causes of Subacute Ruminal Acidosis ........................................................................ 3  

1.2.2 Effects of Subacute Ruminal Acidosis on Barrier Function ........................................ 5  

1.2.3 Effects of SARA on Gut Health.................................................................................... 7  

1.3 Transport physiology .............................................................................................................. 9  

1.3.1 SCFA/proton Transport Mechanisms ......................................................................... 9  

1.3.2 Intracellular pH Homeostasis ..................................................................................... 13  

1.3.3 Subacute Ruminal Acidosis and Transport ............................................................... 14  

1.4 Epithelial plasticity ............................................................................................................... 16  

1.4.1 Butyrate and Epithelial Remodelling/Therapeutics .................................................. 16
Chapter 3: Changes in the rumen papillae during the periparturient transition in Holstein dairy cows are accompanied by changes in abundance of proteins involved in intracellular pH regulation, but not SCFA transport.

3.1 Abstract

3.2 Introduction

3.3 Materials and Methods

3.3.1 Husbandry and Sampling

3.3.2 Immunofluorescence

3.3.3 Statistics

3.4 Results

3.4.1 Rumen SCFA Profile and Rumen pH

3.4.2 Immunofluorescence

3.5 Discussion

3.5.1 Transport Capacity

3.5.2 Epithelial Remodelling

3.6 Conclusion
Chapter 4: Butyrate and subacute ruminal acidosis affect abundance of membrane proteins involved with proton and short chain fatty acid transport in the rumen epithelium of dairy cows

................................................................. 67

4.1 Abstract ................................................................. 67

4.2 Introduction ............................................................ 68

4.3 Materials and Methods.................................................... 70

4.3.1 Animals and Treatment ............................................... 70

4.3.2 TMR, Milk, Rumen and Blood Sampling .................................. 71

4.3.3 Histology and Immunofluorescence .................................... 72

4.3.4 Statistics ................................................................. 73

4.4 Results ...................................................................... 74

4.4.1 DMI, Milk, Rumen and Blood Responses .............................. 74

4.4.2 Immunofluorescence and Histochemistry ............................ 75

4.5 Discussion ................................................................. 81

4.5.1 Effect of Time ........................................................... 81

4.5.2 Effect of Treatment ..................................................... 83

4.6 Conclusion ................................................................. 84

Chapter 5: Butyrate supplementation affects mRNA abundance of genes involved in glycolysis, oxidative phosphorylation and lipogenesis in the rumen epithelium of Holstein dairy cows........ 86

5.1 Abstract ................................................................. 86
5.2 Introduction .................................................................................................................... 87

5.3 Materials and Methods .................................................................................................. 89
  5.3.1 Animals, Treatment and Sampling .......................................................................... 89
  5.3.2 Microarray and qRT-PCR ....................................................................................... 90

5.4 Results ............................................................................................................................ 92
  5.4.1 Milk Production, DMI, SCFA, Blood, Rumen pH ................................................. 92
  5.4.2 Microarray and qRT-PCR ....................................................................................... 92

5.5 Discussion ...................................................................................................................... 94
  5.5.1 Metabolic Stress and Barrier Integrity .................................................................... 94
  5.5.2 Butyrate and Metabolic Stress ................................................................................ 95

5.6 Conclusion ...................................................................................................................... 97

Chapter 6: General Discussion .......................................................................................... 99
  6.1 Study Summary .............................................................................................................. 99
  6.2 SCFA Transport Capacity ............................................................................................ 101
  6.3 Intracellular pH as an Important Factor in SCFA Transport ........................................ 102
  6.4 Future Research .......................................................................................................... 103

References .......................................................................................................................... 104
List of Tables

Table 2.1 Chemical composition of buffers used in Ussing Chambers to measure acetate and butyrate flux in Holstein steers (n=21) given ad libitum DMI, a ruminal acidosis challenge, or feed restriction. ................................................................. 43

Table 2.2 Tissue conductance and short-circuit current of caudal-dorsal ruminal tissue used in Ussing Chambers to measure acetate and butyrate flux in Holstein steers (n=21) given ad libitum DMI, a ruminal acidosis challenge, or feed restriction. ................................. 45

Table 2.3 Pearson correlation coefficients between ruminal pH and acetate/butyrate flux and transporters in the rumen epithelium of Holstein steers (n=21) given ad libitum DMI, a ruminal acidosis challenge, or feed restriction. .......................................................... 49

Table 4.1 Protein abundance of membrane transport proteins in stratum basale of rumen epithelium of Holstein Dairy cows dosed or not dosed with butyrate at 2.5% of DMI; n = 16. ........................................................................................................................................ 81

Table 5.1 Primer sequences for genes verified by PCR. ........................................................................ 91
List of Figures

Figure 1.1 Identified SCFA transport mechanisms in the rumen epithelium. Adapted from Laarman (2011). A more comprehensive diagram including apical nitrate-sensitive, bicarbonate-independent mechanism and a basolateral large anion channel for which transporters have not yet been identified can be found in Aschenbach et al (2010). AE2 – anion exchanger, isoform 2; NHE3 – sodium/proton exchanger, isoform 3; NHE1 – sodium/proton exchanger, isoform 1; NBC1 – sodium/bicarbonate co-transporter, isoform 1; MCT1 – monocarboxylate co-transporter, isoform 1; CA2 – carbonic anhydrase, isoform 2. 11

Figure 2.1 Net acetate flux via different SCFA transport mechanisms across rumen epithelium in Holstein steers (n=21) given ad libitum DMI (CTRL), a ruminal acidosis challenge (ACID), or feed restriction (FR). *denotes $P < 0.05$. PMF – protein-mediated flux; MCTF- monocarboxylate-transporter-mediated flux; PDF – passive diffusion flux. 46

Figure 2.2 Net butyrate flux via different SCFA transport mechanisms across rumen epithelium in Holstein steers (n=21) given ad libitum TMR (CTRL), a ruminal acidosis challenge (ACID), or feed restricted TMR (FR). *denotes $P < 0.05$. PMF – protein-mediated flux; MCTF- monocarboxylate-transporter-mediated flux; PDF – passive diffusion flux 47

Figure 2.3 Transporter abundance in rumen epithelium rumen epithelium in Holstein steers (n=21) given ad libitum TMR (CTRL), a ruminal acidosis challenge (ACID), or feed restricted TMR (FR). *denotes $P < 0.05$. AE2 – anion exchanger, isoform 2; NHE3 – sodium/proton exchanger, isoform 3; NHE1 – sodium/proton exchanger, isoform 1;
Figure 3.1 Protein abundance of membrane transport proteins in the rumen epithelium of Holstein dairy cows during the periparturient transition (PRE = 3 weeks prior to planned parturition; PERI = 1 week after parturition; POST = 6 weeks after parturition); n = 12; *Significantly different from PRE (P < 0.05).

Figure 3.2 Abundance of MCT1 in rumen epithelium of Holstein dairy cows during the periparturient period. A = PRE (3 weeks prior to parturition), B = PERI (1 week after parturition), C = POST (6 weeks after parturition), D = Negative control (secondary antibody only); n = 12; scale bar = 20.0 µm.

Figure 3.3 Abundance of NHE3 in rumen epithelium of Holstein dairy cows during the periparturient period. A = PRE (3 weeks prior to parturition), B = PERI (1 week after parturition), C = POST (6 weeks after parturition), D = Negative control (secondary antibody only); n = 12; scale bar = 20.0 µm.

Figure 3.4 Abundance of NBC1 in rumen epithelium of Holstein dairy cows during the periparturient period. A = PRE (3 weeks prior to parturition), B = PERI (1 week after parturition), C = POST (6 weeks after parturition), D = Negative control (secondary antibody on only); n = 12; scale bar = 20.0 µm.

Figure 3.5 Abundance of CA2 in rumen epithelium of Holstein dairy cows during the periparturient period. A = PRE (3 weeks prior to parturition), B = PERI (1 week after parturition), C = POST (6 weeks after parturition), D = Negative control (secondary antibody only); n = 12; scale bar = 20.0 µm.
Figure 3.6 Relationship between rumen pH and the abundance of NHE3 proteins in the rumen epithelium ($r = 0.39$, $P = 0.03$). ................................................................. 62

Figure 4.1 Model of membrane transport protons in the rumen epithelium involved in SCFA and proton transport. Adapted from Laarman (2011). ................................................................. 69

Figure 4.2 MCT1 protein expression in the rumen epithelium stratum basale of Holstein cows fed a high NFC TMR with and without exogenous butyrate dosed at 2.5% DMI; n = 16. ..................................................................................................................................... 76

Figure 4.3 NHE1 protein expression in the rumen epithelium stratum basale of Holstein cows fed a high NFC TMR with and without exogenous butyrate dosed at 2.5% DMI; n = 16. ..................................................................................................................................... 77

Figure 4.4 NHE3 protein expression in the rumen epithelium stratum basale of Holstein cows fed a high NFC TMR with and without exogenous butyrate dosed at 2.5% DMI; n = 16. ..................................................................................................................................... 78

Figure 4.5 NBC1 protein abundance in the rumen epithelium stratum basale of Holstein cows fed a high NFC TMR with and without exogenous butyrate dosed at 2.5% DMI; n = 16. ..................................................................................................................................... 79

Figure 4.6 Papillae sloughing score in the rumen epithelium stratum basale of Holstein cows fed a high NFC TMR with and without exogenous butyrate dosed at 2.5% DMI; n = 16. ..................................................................................................................................... 80
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADF</td>
<td>Acid detergent fibre</td>
</tr>
<tr>
<td>AE2</td>
<td>Anion exchanger isoform 2</td>
</tr>
<tr>
<td>AIC</td>
<td>Akaike information criterion</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
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<tr>
<td>AU</td>
<td>Arbitrary units</td>
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<td>Lipopolysaccharide binding protein</td>
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<tr>
<td>MCT1</td>
<td>Monocarboxylate cotransporter isoform 1</td>
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</tbody>
</table>
MMP.......................................................................................................................... Matrix metalloproteinase
NBC1 ........................................................................................................ Sodium/bicarbonate cotransporter isoform 1
NDF.......................................................................................................................... Neutral detergent fibre
NFC.......................................................................................................................... Non-fibre carbohydrates
NHE1 .................................................................................................................. Sodium/proton exchanger isoform 1
NHE3 .................................................................................................................. Sodium/proton exchanger isoform 3
NSAID ........................................................................................................ Non-steroidal anti-inflammatory drug
PAT1 .................................................................................................................. Putative anion transporter isoform 1
PBS......................................................................................................................... Phosphate-buffered saline
pCMB............................................................................................................... p-chloromercuribenzoic acid
peNDF........................................................................................................... Physically effective neutral detergent fibre
pH.......................................................................................................................... Intracellular pH
pHMB............................................................................................................... p-hydroxymercuribenzoic acid
qRT-PCR...................................................................................................... Quantitative reverse transcriptase polymerase chain reaction
SARA........................................................................................................ Subacute ruminal acidosis
SCFA............................................................................................................... Short chain fatty acid
TMR.................................................................................................................. Total mixed ration
Chapter 1: Literature Review

1.1 General Introduction

Modern dairy cows can produce over 45 kg of milk daily at the peak of lactation. Such milk production levels create a demand for energy, and it is a principal challenge of dairy nutrition to meet that demand. The fermentation of feedstuffs in the rumen produces short chain fatty acids (SCFA) which account for approximately 80% of the energy needs of the cow (Bergman, 1990). High energy diets that meet the demands of the cow are highly fermentable; as a result, they reduce ruminal pH. When ruminal pH decreases below 5.6, subacute ruminal acidosis (SARA) occurs (Schwartzkopf-Genswein et al., 2003), which has negative effects on fibre digestibility (Cerrato-Sanchez et al., 2007), and can lead to adverse health events such as liver abscesses and lameness (Plaizier et al., 2008), as well as reduced milk production. It was estimated that the daily losses to SARA are approximately $1.12/head (Stone, 2004). Thus, dairy nutrition must balance the high energy demands resulting from milk production with the ruminal acidotic pressure that results from rapid fermentation of feeds.

In dairy diets, energy is provided in two broad forms: fibre and non-fibre carbohydrates (NFC). Fibrous carbohydrates consist of hemicellulose, cellulose, and lignin (NRC, 2001). In applied ruminant nutrition, fibre is divided into neutral detergent fibre (NDF) and acid detergent fibre (ADF; Van Soest, 1967), based on their solubility in respective detergents. Since acid detergent fibre includes slowly degradable lignin, optimal forages and fibre sources have lower ADF content and higher NDF content. A higher NDF:ADF ratio implies fibre is more readily digestible and fermentable, thus is of more potential use to the ruminant.

1J. Dairy Sci. (Submitted)
Non-fibre carbohydrates primarily include sugars, starches, and pectins; all of them are readily fermentable. Sugars are primarily fermented by ruminal bacteria (Titus and Ahearn, 1992), although the ovine rumen epithelium does contain a sodium-linked glucose transporter, suggesting that some glucose may be absorbed directly by the rumen epithelium (Aschenbach et al., 2000a). Although starches are more complex carbohydrates, they too are readily fermentable in the rumen, and can lead to a rapid build-up of SCFAs (Titus and Ahearn, 1992). Because of its fermentability and energy density, starch is an important nutrient for meeting the energy requirements of lactating dairy cows and growing calves. In early lactation, when energy demands are highest, NFC inclusion can be as high as 42% (NRC, 2001), while in calf starters, starch inclusion often goes up to 30% (Laarman et al., 2012d).

The benefits of NFC lie in its high energy density. Through its rapid fermentation, NFC provide energy to bacteria, which produce SCFA for use by the cow. Increasing dietary NFC content results in a buildup of SCFAs in the rumen (Krause and Oetzel, 2006). Thus, NFCs increase the energy intake but also increase the buildup of acids in the rumen. The buildup of acids in the rumen is partly offset by fibre, through its slower fermentation as well as its ability to stimulate rumination (Stone, 2004). Indeed, roughage consumption in calves is a primary driver of the development of rumination and essential rumen function (Nocek et al., 1984). Further, adequate particle size in adult ruminants is essential for stimulating rumination and saliva production (Allen, 1997). Saliva production is an important component of ruminal buffering, and it is estimated that 40% of ruminal acids are neutralized by salivary buffers (Allen, 1997).

An optimal ruminant diet must meet the energy needs using an optimal NDF:NFC ratio. The higher the ratio, the greater the buffering capacity of the rumen, and the lower the energy content
of the diet. High energy diets have a lower NDF:NFC ratio, thus have a lower buffering capacity of the rumen. In circumstances where energy demands are high, such as early lactation in dairy cows, diets with a low NDF:NFC ratio are used (Krause and Oetzel, 2006). During this period, dairy cows are in a negative energy balance, thus energy intake must be maximized. Associated with this high energy intensity of the diet is a reduced ruminal buffering capacity, and thus an increased downward pressure on ruminal pH, risking the occurrence of subacute ruminal acidosis.

1.2 Subacute Ruminal Acidosis

1.2.1 Causes of Subacute Ruminal Acidosis

Ruminal pH and SCFA concentrations are determined by a balance of production and elimination. The production of $H^+$ and SCFA are entirely from the fermentation of feedstuffs, whereas the elimination of SCFAs and protons from the rumen can come from passage down the digestive tract, neutralization through buffers, primarily saliva, and through absorption by the rumen wall (Allen, 1997). These three fractions comprise approximately 15%, 30%, and 50% of $H^+$ removed from the rumen (Allen, 1997).

Improving $H^+$ buffering through salivary buffering is accomplished through optimal inclusion of physically effective NDF (peNDF) – NDF that promotes chewing and salivation – and managing forage to concentrate ratio (F:C). In a Penn State Particle Separator, peNDF is the fraction of a total mixed ration (TMR) that is retained by the sieves, and promotes salivation. In dairy cow diets, recommended peNDF inclusion is 21% (Zebeli et al., 2008). Increasing F:C ratio from 40:60 to 60:40 increases the salivation of feed from 2.84L/kg of dry matter (DM) to 3.45 L/kg
DM (Maekawa et al., 2002). In managing both NDF and peNDF, ruminal pH can be more effectively managed and kept above SARA thresholds (Allen, 1997).

As previously mentioned, high energy diets are commonly fed to meet the energy demands of high producing dairy cows, but carry an elevated risk of SARA owing to the low NDF:NFC ratio of the diet. With such diets, SCFA production in the rumen becomes elevated (Titus and Ahearn, 1992) and the rumen must absorb, neutralize or remove the associated protons from the rumen. When the rumen removes an insufficient amount of SCFA-associated protons, ruminal pH decreases. Once the ruminal pH decreases below a threshold of 5.6 SARA is defined as occurring (Schwartzkopf-Genswein et al., 2003). Since the rumen is a dynamic system, the pH changes continuously, making monitoring pH vital for studying SARA.

Measurement of ruminal pH is an important tool in diagnosis and monitoring of SARA. Several in-dwelling pH measurement systems have been developed (AlZahal et al., 2007; Penner et al., 2009a). These systems are useful in the continuous monitoring of ruminal pH over time, and allow scientists to elucidate the extent and duration of ruminal pH depressions due to dietary differences as well as diurnal variation (Wales et al., 2004). Further, by measuring continuously, ruminal pH becomes a more informative metric, and we can use a more liberal threshold for SARA, using 5.6 or 5.8 for continuous measurements rather than 5.5 (Aschenbach et al., 2011). Another benefit is that continuous pH measurements allow us to establish baseline ruminal pH values, which has been shown to influence susceptibility to SARA in that cows with lower baseline ruminal pH were more susceptible to SARA than cows with higher baseline ruminal pH (Dohme et al., 2008). By measuring ruminal pH continuously, the duration of SARA can be measured and we can distinguish between a diet that is associated with SARA for 2 min/d versus
a diet associated with SARA for 200 min/d. As a result, dietary causes of SARA can be more clearly identified and their impacts more clearly studied.

1.2.2 Effects of Subacute Ruminal Acidosis on Barrier Function

One of the effects of SARA on the ruminant is to adversely affect barrier integrity of the rumen epithelium. Barrier function is one of the principal functions of the rumen epithelium; it is key in preventing translocation of bacteria and lipopolysaccharides (LPS) into the bloodstream; the translocation of bacteria or LPS into the bloodstream can cause significant adverse health effects such as rumenitis and laminitis (Plaizier et al., 2008). Managing and preventing SARA is therefore an important element in maintaining ruminant health through maintaining barrier integrity of the rumen epithelium.

Barrier integrity is achieved via the extracellular matrix of epithelial cells being physically anchored to each other, forming a barrier between the external and internal milieu that severely restricts paracellular movement of solutes, bacteria and LPS. There are three types of complexes that contribute to the creation of a barrier: tight junctions, adherens junctions and desmosomes (Camilleri et al., 2012). On the apical side of the epithelium, a nearly impenetrable tight junction is formed with tight junction proteins such as occludin and zonula occludens 1, with the cytoskeleton structure of epithelial cells. Moving basolaterally, adherens junctions are cell junctions where the cytoplasmic face is linked to the actin cytoskeleton (Camilleri et al., 2012). On the basolateral end of the epithelial cell membrane are the desmosomes, formed by interactions between desmoglein, desmocollin, and keratin filaments (Camilleri et al., 2012). Together, these 3 protein complexes keep epithelial cells anchored to each other and create a physical barrier that prevents solutes and bacteria from translocating across the epithelium.
In the rumen, the barrier integrity of the epithelium lies in the stratum granulosum, the second strata from the lumen (Graham and Simmons, 2005). There are four strata that comprise the rumen epithelium: stratum corneum, stratum granulosum, stratum spinosum and stratum basale, from lumen to bloodstream (Alonso and Fuchs, 2003). Together, these four strata comprise one stratified, squamous epithelium, with barrier integrity formed by the same mechanisms as in columnar epithelia.

With SARA, barrier integrity can be compromised. In dairy cows, a rapid switch from a high forage diet to a high concentrate diet caused sloughing of the stratum corneum (Steele et al., 2009). When goats were fed a high concentrate diet, barrier function in the colon was disrupted (Tao et al., 2014). In cows induced with SARA, LPS in the bloodstream spiked as a result, suggesting a disruption in barrier function (Khafipour et al., 2009a), although this disruption was only present with grain-induced acidosis, not alfalfa-induced SARA (Khafipour et al., 2009d). In all these examples, SARA resulted from a rapid dietary transition that drastically increased diet fermentability in a short period of time.

The impact of SARA on barrier function is somewhat nuanced. While a single 3h episode of SARA where ruminal pH decreases to 5.77 does not cause barrier deterioration, a drop in pH to 5.2 does (Penner et al., 2010), highlighting the important role of pH. During the hallmark ruminal pH decreases in SARA, rumen osmolarity increases; higher osmolarity depresses intracellular pH (pHi) of ruminal epithelial cells (Schweigel et al., 2005). Intracellular acidification of cardiac myocytes disrupts Connexin 43/Zonal Occludens 1 interactions, resulting in reduced intercellular coupling (Duffy et al., 2004). In renal MDCK epithelial cells, extracellular pH depressions cause a marked decrease in claudin-2 expression, contributing to a
compromise in barrier integrity (Balkovetz et al., 2009). In the rumen, SARA induces a cascade of events where rumen osmolarity increased, resulting in lower pH, in the rumen epithelium, which then causes dissociation of intercellular coupling complexes, leading to a loss of barrier integrity.

Another mechanism by which SARA can compromise barrier integrity is through energy depletion. In rat colon cells, increased SCFA production increases mitochondrial gene and protein expression, leading to increased mitochondrial proton pump activity, depleting cellular ATP reserves (Rodenburg et al., 2008). As a result of ATP depletion, barrier function becomes compromised. Namely, infection of Caco-2 cells with rotavirus results in ATP depletion, leading to zonal occludens 1 being located more in the cytoplasm (Dickman et al., 2000). Losing the link between occludin and the actin cytoskeleton is associated with decreased transepithelial resistance, demonstrating reduced barrier integrity (Dickman et al., 2000).

Whether through intracellular acidification or ATP depletion of rumen epithelial cells, SARA presents a real stress on the barrier integrity of the rumen epithelium. Severe ruminal pH decreases to 5.2 or sustained ruminal pH below 5.6 are both indicative of SARA, and can lead to a loss of barrier integrity. Losing barrier integrity of the rumen epithelium increases the ability of bacteria and bacterial toxins to translocate across the rumen epithelium, thus posing significant challenges for rumen health and systemic health.

### 1.2.3 Effects of SARA on Gut Health

The effect of SARA on gut health involves the stimulation of the ruminant immune system. During an ex vivo 4 hour bout of SARA in sheep epithelia exposed to pH 5.1, transepithelial permeability increased fourfold and increased non-catabolized histamine flux across the
epithelium threefold (Aschenbach et al., 2000b), which can be problematic for the immune system. Under healthy circumstances, histamine taken up by the epithelium is almost completely catabolized (Brent, 1976), rendering it unable to stimulate the immune system.

Other immune system factors also come into play as a result of SARA. When steers were switched from a chopped hay diet to a 61% concentrate diet over the course of 21 days, serum haptoglobin and serum amyloid A increased significantly, suggesting SARA is linked to serum amyloid A (Gozho et al., 2006). Furthermore, proteomic analysis revealed that SARA increases the abundance of 40 proteins involved with cellular stress, differentiation and inflammation (Bondzio et al., 2011). In all, these results show that SARA directly stimulates the immune system of the ruminant and can lead to an acute phase response.

Lastly, SARA affects gut health through an increase in the production of lipopolysaccharide (LPS) in the rumen from the death of Gram-negative bacteria. In bovine rumen epithelium, a 5-fold increase in the amount of LPS translocated across the membrane resulted from a drop in ruminal pH from 7.4 to 5.4 (Emmanuel et al., 2007). The LPS response to SARA occurs as a result of ruminal pH, but the immune response is feed-specific. In lactating dairy cows, replacing alfalfa hay with alfalfa pellets causes an increase in free LPS in the rumen but not an increase in serum amyloid A or haptoglobin (Khafipour et al., 2009a) while replacing alfalfa hay with wheat-barley pellets caused both an increase in free rumen LPS and an increase in serum amyloid A and haptoglobin (Khafipour et al., 2009d). In short, SARA causes a spike in rumen LPS, which can lead to immune stimulation through the activation of the acute phase response.

The acute phase response of the immune system is the immunological cascade resulting from an injury. It is stimulated by pro-inflammatory cytokines such as IL-1, IL-5 and TNF-alpha, and
results in the production of acute phase proteins (Carroll, 2008). During SARA, reduced barrier integrity leads to translocation of LPS across the epithelium (Emmanuel et al., 2007). Then, LPS binds to LPS binding protein (LPSBP) which catalyzes the transfer of LPS to membrane protein CD14. At this point, CD14-endotoxin complex associates with toll-like receptor 4 (TLR-4), leading to a stimulation of macrophages through myeloid differentiation isoform 2, causing a signalling cascade in macrophages (Beutler, 2004). Macrophage activation results in the release of pro-inflammatory cytokines, such as tumour necrosis factor alpha, interleukin (IL)-1, IL-5, IL-8 and nuclear factor kappa beta (Zebeli and Metzler-Zebeli, 2012). The inflammation that results can lead to rumenitis. Chronic rumenitis can reduce the absorptive capacity of the rumen epithelium (Krause and Oetzel, 2006), thus gut health is an important factor to maintain optimal SCFA absorptive capacity of the rumen.

1.3 Transport physiology

1.3.1 SCFA/proton Transport Mechanisms
The absorptive capacity of the rumen is dictated by the efficacy of two SCFA transport mechanisms: passive diffusion and facilitated transport. Together, these mechanisms account for approximately 88% of all SCFAs taken up by the ruminant (Allen, 1997). Between the two uptake mechanisms, facilitated transcellular transport is responsible for the majority of SCFA transport (Aschenbach et al., 2009). The movement of SCFAs across the rumen epithelium requires the translocation across all four strata of the rumen epithelium; from lumen to bloodstream: stratum corneum, stratum granulosum, stratum spinosum and stratum basale (Alonso and Fuchs, 2003). While the tight junctions and epithelial barrier are located primarily at the stratum granulosum (Graham and Simmons, 2005), most transporters are located in the
stratum basale (Graham et al., 2007), and thus SCFA transport is often discussed in a simplified form with all epithelial layers amalgamated into one layer.

The first SCFA uptake mechanism is passive diffusion, which refers to the movement of associated SCFAs across the epithelium. In the associated form, SCFAs are non-polar, and thus can diffuse freely across lipid membranes. The longer the chain length, the more lipophilic the SCFA; in other words, butyrate is more lipophilic than propionate, which is more lipophilic than acetate (Sehested et al., 1999). The pKₐ values of acetate, propionate and butyrate are 4.8 (Enemark et al., 2002). At a ruminal pH of 5.8 and 6.8, the proportions of SCFAs that exist in the associated form are 0.1 and 0.01, respectively. The positive relationship between SCFA lipophilicity and the mucosal-to-serosal flux rate is not log-linear, suggesting that passive diffusion does occur, but is not the sole transport mechanism (Sehested et al., 1999).

The majority of SCFA uptake across the rumen epithelium is facilitated transport through membrane transporters (Figure 1.1). To date, 2 principal SCFA transporters have been identified: anion exchanger isoform 2 (AE2) and monocarboxylate cotransporter isoform 1 (MCT1). On the apical membrane, AE2 is an antiporter that exports HCO₃⁻ and imports dissociated SCFA. Further, the monocarboxylate cotransporter isoform 4 (MCT4) has been identified on the apical membrane (Kirat et al., 2007), but its role is not entirely clear. On the basolateral membrane, MCT1 co-exports a dissociated SCFA and H⁺ (Graham et al., 2007). Together, these transporters constitute a primary pathway for facilitated SCFA transport.

Facilitated SCFA transport is intertwined with passive diffusion. Basolateral MCT1 exports SCFAs regardless of their apical source. Since intracellular pH is regulated at 7.4 (Muller et al., 2000), 99.7% of SCFAs exist in dissociated form inside the epithelial cell. This implies that
MCT1 exports SCFAs from both passive diffusion and AE2 facilitated transport. Immediately after weaning in dairy calves when SCFAs become the principal energy source, there is a rapid upregulation of MCT1 in the rumen epithelium (Laarman et al., 2012a). Whether such increases are due to an increase in facilitated transport or passive diffusion is not immediately clear, but MCT1 clearly plays an important role in facilitating SCFA uptake.

Figure 1.1 Identified SCFA transport mechanisms in the rumen epithelium. Adapted from Laarman (2011). A more comprehensive diagram including apical nitrate-sensitive, bicarbonate-independent mechanism and a basolateral large anion channel for which transporters have not yet been identified can be found in Aschenbach et al (2010). AE2 – anion exchanger, isoform 2; NHE3 – sodium/proton exchanger, isoform 3; NHE1 – sodium/proton exchanger, isoform 1; NBC1 – sodium/bicarbonate co-transporter,
isoform 1; MCT1 – monocarboxylate co-transporter, isoform 1; CA2 – carbonic anhydrase, isoform 2.

Although MCT1 is a monocarboxylate transporter, its role in acetate, propionate and butyrate transport are most heavily studied, owing to the prevalence of these SCFAs in the rumen. In ex vivo bovine rumen tissue exposed to a mixture of acetate, propionate, and butyrate, butyrate will have a mucosal-to-serosal flux that is $2.15 \pm 0.10 \mu\text{mol/cm}^2\text{h}$, as opposed to a butyrate flux of $1.53 \pm 0.12 \mu\text{mol/cm}^2\text{h}$ when butyrate is present as a monosolution (Sehested et al., 1999).

Inhibition of MCT1 expression in HT29 cells causes a 72% reduction in butyrate uptake (Cuff et al., 2005). Butyrate uptake through MCT1 is an important subject because of butyrate’s importance in regulating cell development in the digestive tract (Sakata, 1987).

Besides butyrate, MCT1 also transports acetate and propionate. Inhibition of MCT1 with 1mM p-chloromercuribenzoic acid (pCMB) reduced acetate uptake across the bovine caecum by 57.2% (Kirat and Kato, 2006). Acetate uptake was reduced by 40% when 100 mM propionate was added, suggesting that MCT1 is the principal transporter for both acetate and propionate as well (Kirat and Kato, 2006). These results further suggest that there is differential affinity for the different SCFAs to MCT1, dependent on SCFA chain length.

Regardless of which SCFA is being transported by MCT1, $H^+$ is being transported simultaneously. The same physiological function occurs with AE2 and with passive diffusion. While passive diffusion carries with it an associated $H^+$, AE2 exports a $\text{HCO}_3^-$ ion. The movement of $H^+$ and $\text{HCO}_3^-$ affects the $pH_i$ of the ruminal epithelial cell, implying that SCFA transport is intrinsically linked to $pH_i$ of the ruminal epithelial cell.
1.3.2 Intracellular pH Homeostasis

Homeostasis of pHi is an important component of the epithelial function. In rat colon, cells respond to low luminal pH by increasing the number of cells in S-phase, the DNA-synthesizing phase of cell growth (Lupton et al., 1985). Further, modest acidification from pH 7.42 to pH 6.79 of C7-MDCK cells results in a significant decrease in cell mortality (Akimova et al., 2006). In eukaryotic cells, pH\textsubscript{i} affects enzyme activity, cell cycle and cell division, thus its maintenance is critical for cell survival (Madshus, 1988). Maintaining pHi is done on a very rapid time scale. Rumen epithelial cells, when challenged acidotically by butyrate, recover their pHi within 15 minutes (Muller et al., 2000).

The principal proteins involved in regulating pHi, in addition to the SCFA transporters MCT1 and AE2, are sodium/proton exchanger isoforms 1, 2, and 3 (NHE1, NHE2, NHE3), sodium/bicarbonate cotransporter isoform 1 (NBC1), down-regulated in adenoma (DRA), putative anion transporter isoform 1 (PAT1), and a voltage-gated proton ATPase (vH\textsuperscript{+}-ATPase; Connor et al., 2010). Like the SCFA transporters, these pHi-regulating transporters are located primarily in the stratum basale and the stratum spinosum (Graham et al., 2007). Under normal circumstances, NHE1, NHE3 and vH\textsuperscript{+}-ATPase are responsible for 50%, 30% and 20%, respectively, of proton extrusion from the cytosol (Etschmann et al., 2006).

Under suboptimal environmental circumstances, such as SARA, several pHi-regulating transporters are affected. Despite NHE3 being responsible for only 30% of proton extrusion, it is highly sensitive to different environmental conditions. Because NHE3 contains a butyrate-binding element (Kiela et al., 2003), it is very sensitive to changes in SCFA concentrations. Furthermore, NHE3 is functionally coupled to apical Cl\textsuperscript{-}/HCO\textsubscript{3}\textsuperscript{-} exchangers, like PAT1 and
DRA, which together results in electroneutral NaCl absorption (Donowitz and Li, 2007). NHE2, on the other hand, is involved in barrier function recovery in porcine ileum (Moeser et al., 2006). In all, the ruminal epithelial cells are very adept at regulating pH for survival purposes, by transporting both H⁺ and HCO₃⁻.

The reason both HCO₃⁻ and protons are involved is because of the role of carbonic anhydrase isoform 2 (CA2), which catalyzes the reaction

\[ \text{H}_2\text{O} + \text{CO}_2 \leftrightarrow \text{HCO}_3^- + \text{H}^+ \]

By removing HCO₃⁻ from the cytosol, the equilibrium shifts to the right, thus releasing more H⁺ in the cytosol, lowering pH. Thus, the movement of HCO₃⁻ across the rumen epithelium affects the pH₁ significantly. Further, CA2 is also linked to SCFA transport because it enhances the activity of MCT1 (Klier et al., 2014) and AE2 (Imtaiyaz Hassan et al., 2013), forming a physical complex to the membrane protein, thus providing the transporters with a constant supply of H⁺ and HCO₃⁻. The transport of Cl⁻/HCO₃⁻ exchangers, such as DRA and PAT1, is activated by pH-sensitive regions on the intracellular modifier site of the HCO₃⁻ transporters (Hayashi et al., 2009). Cytosolic CA2 also forms complexes with NHE1 (Purkerson and Schwartz, 2007), thus providing a similar transport metabolon for H⁺ export.

### 1.3.3 Subacute Ruminal Acidosis and Transport

Transport of SCFA and pH₁ of the rumen epithelium are very responsive to SARA. During SARA, two main factors affect the epithelium change: ruminal SCFA concentration increases and ruminal pH decreases (Krause and Oetzel, 2006). As a result, both the H⁺ and SCFA
gradient across the rumen epithelium significantly increase. As the pH of the rumen decreases from an optimal pH of 6.8 to the near-SARA pH of 5.8, the proportion of SCFAs that exists in the associated form increases from 0.01 to 0.1, representing a ten-fold increase. Passive diffusion of associated SCFAs across the rumen epithelium increases as ruminal pH decreases in sheep and bovine rumen epithelium (Gabel and Sehested, 1997; Schurmann et al., 2014), although the increase in SCFA flux is much smaller than diffusion kinetics would expect (Sehested et al., 1999). Since MCT1 is linked to passive diffusion of associated SCFA, the response of SCFA transporters to SARA is also important.

Improving SCFA uptake through epithelial transporters is an important adaptation of the rumen epithelium to SARA. Following a rapid buildup of SCFAs, accomplished by feeding concentrate to lactating dairy cows once per day, there is an immediate increase in SCFA transport capacity (Sehested et al., 1997). In sheep with high resistance to SARA, acetate and butyrate flux rates are significantly higher than in sheep with low resistance to SARA (Penner et al., 2009b), demonstrating the importance of the SCFA and H⁺ transporters in the epithelial response to SARA. On an mRNA level, an increase in butyrate causes upregulation of MCT1, MCT4, PAT1, and DRA in the sheep rumen, suggesting the genomic response towards SCFA buildup is to increase SCFA transport capacity (Dengler et al., 2014). Surprisingly, AE2 does not appear to be regulated by diet (Martens et al., 2012).

Due to the linkage between SCFA transport and pHᵢ regulation, a response to SARA in pHᵢ regulators is also evident. For instance, NHE3 is responsible for cytosolic acidification recovery following SCFA acidification (Schweigel et al., 2005). Both NHE2 and NHE3 are increased in rat colon following metabolic acidosis in the bloodstream (Lucioni et al., 2002). The collective
response of H⁺ buildup is to increase H⁺ export from the cytosol. Reducing the H⁺ load in the cytosol is a key response to SARA then, concurrent with an increase in SCFA uptake.

In the long term, SARA results in structural adaptations of the rumen epithelium. Following a switch to an early lactation diet, ruminal pH in lactating dairy cows decreases (Dionissopoulos et al., 2014). As ruminal pH decreases, the rumen epithelium cells are exposed to an increased acid load, which puts acidotic pressure on the cytosol. Modest intracellular pH decreases in C7-MDCK and PAEC cell lines results in an inhibition of cell death signalling (Akimova et al., 2006). Through a reduction in cell death, the rumen epithelium can proliferate and remodel, leading to the observation of increased surface area of the rumen epithelium following dietary changes (Dirksen et al., 1985).

1.4 Epithelial plasticity

1.4.1 Butyrate and Epithelial Remodelling/Therapeutics

Epithelial remodelling is a key adaptive response of the rumen epithelium to SARA, and it is a response driven by butyrate. Butyrate is both a prime energy substrate for epithelial tissues (Bergman, 1990) and an inhibitor of the histone deacetylase complex (HDAC), which results in a hyperacetylation of histone proteins in cultured mammalian cells (Davie, 2003). In turn, the hyperacetylation drives a host of changes in gene expression that, among other effects, increase apoptosis in cancer cell lines such as RSB and Caco-2 (Avivi-Green et al., 2002). In young ruminants, butyrate is a main stimulant of rumen epithelial cell differentiation, driving the functional development of the rumen (Sander et al., 1959). The history of butyrate as a prime stimulant of epithelial remodelling is long established.
Butyrate can drive epithelial remodelling because it affects such a wide array of genes. Many of the genes affected by butyrate in normal mucosal tissue are involved with arresting cell cycle and differentiation (Daly and Shirazi-Beechey, 2006). Also, in Caco-2 cells, epithelial permeability is decreased and transepithelial resistance increased when butyrate is administered at low concentrations (Peng et al., 2007), showcasing butyrate’s effect on epithelial barrier integrity.

Butyrate increases expression of tight junction and claudin proteins in bovine rumen epithelium (Baldwin et al., 2012). Tight junctions are redistributed by butyrate through the activation of AMP-activated protein kinase (AMPK; Peng et al., 2009), which is required for maintaining cell polarity in a low energetic state (Mirouse et al., 2007).

Energy sources in epithelia are important because of the role they play in maintaining epithelial barrier integrity. While various levels of protein and lactose inclusion have no effect on barrier integrity, low energy state compromises the barrier integrity of the porcine small intestine (Spreeuwenberg et al., 2001). Recent evidence indicates that butyrate also aids in epithelial remodelling by acting as an energy source through butyrate metabolism (Donohoe et al., 2011). These studies demonstrate how butyrate improves epithelial function by providing energy for maintaining barrier integrity.

Butyrate is also used as a therapeutic agent in many instances, such as ulcerative colitis and Crohn’s disease. In human ulcerative colitis patients, the butyrate receptor SLC16A1 is down-regulated, suggesting the higher expression levels in inactive ulcerative colitis demonstrate the important role butyrate plays in minimizing colitis (Planell et al., 2013). Furthermore, in healthy humans, a 60 mL 100 mM butyrate enema administered for 14 days caused a significant increase in GSH in the colon epithelium and GSH/GSSG ratio in epithelial proteins, indicative of lower
oxidative stress, since higher GSSG concentrations are linked to ulcerative colitis (Hamer et al., 2009). In mild to moderate instances of Crohn’s disease, an oral dose of 4 g/d for 8 weeks reduced Crohn’s disease activity index in 69% of patients (Di Sabatino et al., 2007). As a therapeutic agent, butyrate has an established record and shows potential for a number of other disorders and diseases.

Butyrate also has therapeutic effects in cancer cell lines. In a cancerous state, cells become highly glycolytic as a means to support rapid cell growth, a phenomenon known as the “Warburg effect”. In HT29 colorectal cell lines, butyrate has been shown to alter the metabolic profile to counter the Warburg effect (Fung et al., 2009). Mechanistically, the shift away from the Warburg effect happens via a decreased abundance of glycolytic enzyme proteins, namely glucose-6-phosphatase, and increase abundance of proteins involved in the citric acid cycle and in fatty acid beta-oxidation (Fung et al., 2009; Janssen et al., 1997). Butyrate has the ability to positively influence the metabolic profile of cells, to increase energy mobilization, and can thus has therapeutic potential during epithelial remodelling, when ATP is needed in great quantities.

In agriculture, butyrate is already being used successfully as a therapeutic. In broiler chickens, 700 ppm protected sodium butyrate caused a significantly higher body weight gain and increased feed conversion ratios in chickens between 15 and 42 d of age (Chamba et al., 2014). Butyrate’s ability to remodel epithelia can translate into significant productivity gains in the poultry sector. Similarly, butyrate shows potential in the dairy sector to improve gut health through improving epithelial remodelling during SARA.
1.4.2 Dietary Changes

Epithelial remodelling is very pronounced following dietary changes in dairy cows. Diet changes are a regular occurrence, as different physiological stages of the dairy cow have vastly different dietary needs, and no one diet will encapsulate all physiological states. One of the principal dietary changes occurs during the weaning transition in young calves, when the calf starter fed increases SCFA concentrations in the rumen (Sander et al., 1959; Stobo et al., 1966). Another great dietary change is that revolving around the transition cow period, from 3 weeks before calving until 3-6 weeks post-calving (Drackley, 1999).

What dietary transitions have in common is a sudden rapid change in diet fermentability (Krause and Oetzel, 2006) and, therefore, SCFA load (Sutton et al., 2003). The increase in diet fermentability comes from an increase in concentrate inclusion in the diet (Bannink et al., 2012). Increased SCFA load in the rumen disrupts the epithelial SCFA absorption capacity from its equilibrium position. While dietary changes in dairy nutrition are inevitable and very beneficial for a dairy operation, they also demonstrate the plasticity of the rumen epithelium on a cellular level. Understanding and managing this plasticity is very important for maintaining gut health of dairy cows.

One of the main observed responses to dietary change is a change in cellular proliferation. Increasing energy content of the diet of young goats from $500 \text{ kJ/kg}^{0.75\text{*d}}$ to $1200 \text{ kJ/kg}^{0.75\text{*d}}$ causes rapid proliferation of papillae (Shen et al., 2004). In pre-weaned calves, the introduction and fermentation of calf starter results in a rapid proliferation of the rumen epithelium (Sander et al., 1959). Likewise, in lactating dairy cows, proliferation of the rumen epithelium occurs rapidly after parturition and papillae size increase over the next 6 to 8 weeks (Dirksen et al., 1985). This
rate of increase is enhanced when cows are fed increases in concentrate more rapidly, reducing the adaptation time from 8 weeks to 3 to 4 weeks (Bannink et al., 2008). The end result of increased cellular proliferation and papillae growth is an increase in absorptive surface area (Dirksen et al., 1985; Shen et al., 2004), allowing the epithelial absorption capacity to reach a new equilibrium with the SCFA production in the rumen.

The cellular adaptation of the rumen epithelium occurs rapidly after dietary transition and is predominant in the first week. When sheep were transitioned from a 100% hay diet to a 50/50 (400 g hay, 400 g concentrate) diet, 73% of the adaptation of sodium transport occurred in the first week (Etschmann et al., 2009). Changing sheep from a 75:25 F:C ratio to a 25:75 F:C ratio increased Na\(^+\)/K\(^+\) ATPase activity in the rumen epithelium, highlighting the increased metabolic activity as a result of increasing diet fermentability (McLeod and Baldwin, 2000). Changes in epithelial physiology following a dietary change are most likely driven by butyrate, which spikes in dairy cows after transitioning from a high forage diet to a high grain diet (Steele et al., 2011d). Dietary change to a high grain diet causes not only epithelial remodelling, but also increases the risk of SARA, meaning that epithelial remodelling and gut health often co-exist in dairy cows.

1.4.3 Epithelial Remodelling and Gut Health

Epithelial remodelling is a necessary response of the rumen epithelium to dietary changes. Since SARA often occurs as a result of dietary changes, epithelial remodelling also represents a risk to gut health. During epithelial remodelling, the absorptive surface area of the rumen increases (Dirksen et al., 1985), but is associated with a risk of increased epithelial permeability (Streuli, 1999). Humans with type 2 diabetes have increased levels of bacterial DNA in the bloodstream, pointing to increased gut epithelium permeability (Tremaroli and Bäckhed, 2012). During
SARA, LPS concentrations often increase (Khafipour et al., 2009a; d) as Gram-negative bacteria die in the rumen. When reduced barrier function during epithelial remodelling is combined with an increase in LPS concentrations, the likelihood of an LPS concentration spike in the bloodstream increases.

Epithelial remodelling can lead to LPS translocation when barrier integrity is compromised. As discussed, SARA poses a significant challenge for maintaining barrier integrity. For instance, desmoglein 1 is down-regulated when cows switch from a high forage diet to a high grain diet (Steele et al., 2011a). In mice with colitis, matrix metalloproteinases (MMP) isoforms 7 and 10 are highly expressed at the regenerative end of an epithelial ulcer, whereas MMP isoforms 1, 3, 9, 12, and 14 are involved in the breakdown of extracellular matrix proteins, contributing to reduced barrier function (Sengupta and MacDonald, 2007).

Reduced barrier function can lead to immune response. In cows, the induction of SARA increases translocation of LPS across the rumen epithelium (Emmanuel et al., 2007). In baboons, old baboons (18+ years old) had significantly reduced barrier integrity, which was associated with a downregulation of the tight junction proteins zonal occludens isoform 1, occludin, and junctional adhesion molecule and an upregulation of pro-inflammatory markers interferon-gamma and interleukin-6 (Tran and Greenwood-Van Meerveld, 2013). During inflammation, many pathways interplay at the same time with inflammation. Among these pathways, intestinal inflammation reduced Wnt/beta catenin signalling through PI3K/Akt activity, thus highlighting the importance of modulating various signalling pathways during epithelial inflammation (Koch and Nusrat, 2012). In kidney epithelial LLC-PK1 cells, TNF-alpha caused a significant increase in tight junction permeability (Mullin et al., 1992). Likewise, IFN-gamma increases permeability
in intestinal epithelial monolayers (Madara and Stafford, 1989). Dietary changes lead to an integrated cascade of events that includes both epithelial remodelling and gut health.

Some nutritional strategies exist that manage dietary transitions more effectively. For example, bovine colostrum helps to restore epithelial barrier integrity in rats (Playford et al., 1999). Since defatted milk failed to restore barrier function to NSAID-damaged gut epithelia, it is likely that colostrum-specific growth factors play an important role in restoring barrier function (Playford et al., 1999). Given the complexity of the integrated epithelial remodelling and gut health response to dietary changes, further nutritional strategies should be developed.

1.5 Knowledge Gap

Modern dairy practices include many dietary changes during different physiological stages of the cow. These dietary changes are important and speak to the variable needs of dairy cows. Many dietary changes involve a rapid increase in energy content and dietary fermentability, which leads to a rapid buildup of SCFA and H⁺ in the rumen. In response the rumen epithelium, responsible for 50% of SCFA removal from the rumen (Allen, 1997), must adapt its absorption capacity to reach an equilibrium with ruminal SCFA production.

The rumen epithelium is a squamous, stratified epithelium that is selectively permeable to solutes. In feeding high-energy diets common to modern production systems, the rumen epithelium is exposed to environments that are high in SCFA and H⁺. Rapid changes in the SCFA load and H⁺ imply that the epithelium must adapt either cellulary, through changes in SCFA and H⁺ transporters, or morphologically, through epithelial remodelling. If the rumen
epithelium does not adapt quickly enough to changes in the rumen milieu, SCFAs and H\(^+\) will accumulate, and ruminal pH will decrease.

Low ruminal pH is detrimental to the rumen epithelium because of its well-documented effects on barrier integrity, the maintenance of which is crucial for ruminant gut health. Therefore, it is vital to improve our understanding of SCFA and H\(^+\) transport within the context of dietary changes. Understanding how the epithelium adapts to changing ruminal environments is key to eventually develop nutritional strategies that help to maintain barrier integrity and improve gut health during dietary transitions. The role of SCFA transporters during dietary transitions is an understudied subject.

The objectives of this thesis are to investigate the short-term and long term effects of diet transitions on gut health and SCFA transport capacity. Recent work has suggested that the first week following a diet change is the time where the primary molecular adaptation happens (Steele et al., 2011d), while morphological changes happen over the course of 6-8 weeks (Dirksen et al., 1985). Our understanding of the cellular adaptation of the rumen epithelium to dietary changes is limited.

In addition, this thesis also investigates the morphological adaptation of the rumen epithelium to dietary changes. A major component of this process is the remodelling of the epithelium, part of which involves reducing barrier function as tight junctions are cleaved, cells migrate and tight junctions reform. This is a normal process part of both remodelling and wound healing, but becomes an issue when SARA is considered. With SARA, the insult to the rumen epithelium reduces barrier function while increasing the LPS concentrations in the rumen. Overall, this poses a significant health risk and is contributory to the transition period in cows being a period
marked by high health risks. While the gut health risks of SARA are well established, the role of morphological adaptation to dietary changes and SARA is complex and warrants further study.

Lastly, this thesis studies the therapeutic potential of butyrate to improve both SCFA transport capacity as well as gut health. There are 2 principal targets for butyrate during epithelial remodelling. The first is improving barrier function during the epithelial remodelling phase. This is especially important because SARA can increase LPS concentration in the rumen (Khafipour et al., 2009d), which can lead to inflammation and systemic health effects (Plaizier et al., 2008). Managing the rumen epithelium to minimize barrier function disruption will help improve gut health in cows during SARA. The other target of butyrate is to improve SCFA absorption. SCFA absorption is linked to SARA resistance (Penner et al., 2009b), so improving SCFA absorption is key in developing nutrition strategies to minimize SARA while optimizing energy balance. The potential of butyrate to augmenting barrier integrity and SCFA absorption capacity is key in improving the health and productivity of dairy cows following dietary transitions.

In short, this thesis has two objectives: to investigate short-term and long-term effects of dietary transitions on gut health and SCFA transport, and to investigate the therapeutic potential of butyrate to improve gut health and SCFA transport. The hypothesis is that dietary transitions cause a cellular-level adaptive response in the short-term and a morphology-level adaptive response in the long term. Further, we hypothesize that butyrate can be an effective therapeutic to mitigate the effects of SARA in order to promote gut health and SCFA absorption capacity.
Chapter 2: Increase in butyrate flux is mediated through monocarboxylate co-transporter 1

2.1 Abstract

The objective of this study was to investigate the role of MCT1 for SCFA flux across the rumen epithelium, using subacute ruminal acidosis and feed restriction as models. Twenty-one Holstein steers (216.8 ± 31.4 kg BW) were individually housed and fed a total mixed ration (TMR) with a 50:50 forage:concentrate ad libitum for 5 d. Then, calves were assigned one of three treatments: Control (CTRL) calves were fed the TMR ad libitum on day 1; subacute ruminal acidosis (ACID) calves were given 25% of their ad libitum DMI on day 1, then given a barley grain challenge at 30% of ad libitum DMI on day 2; feed restriction (FR) calves were given 25% of their ad libitum DMI for 5 days. Ruminal pH was continuously measured during the entire study. At the end of the study, rumen tissue was harvested and acetate and butyrate flux were measured. Selective inhibitors were used to differentiate total flux, protein-mediated flux (PMF), MCT-mediated flux (MCTF) and passive diffusion flux (PDF). Duration of subacute ruminal acidosis where rumen pH < 5.6 was greater in ACID compared to CTRL and FR (57 ± 90 vs. 519.71 ± 90 min/d vs. 30 ± 90 min/d for CTRL, ACID and FR; P < 0.01). Total acetate flux was greater in FR than in CTRL (421.1 ± 41.4 vs. 630.6 ± 38.9 nmol/cm2× h, P < 0.01), but no difference was observed between CTRL and ACID (421.1 ± 41.4 vs. 455.4 ± 38.9 nmol/cm2× h). Also, total butyrate flux was greater in FR than in CTRL (625.5 ± 86.3 vs. 1241.9 ± 94.8 nmol/cm2× h; P < 0.01), but no difference was detected between CTRL and ACID (625.5 ± 86.3 vs. 716.7 ± 81.0 nmol/cm2× h). For butyrate flux, PMF was greater for FR than in CTRL (99.9 ± 86.3 vs.

1J. Dairy Sci. (Submitted)
479.21 ± 103.9 nmol/cm²× h, \( P < 0.01 \), but no difference was observed between CTRL and ACID treatments (99.9 ± 86.3 vs. 90.2 ± 81.0 nmol/cm²× h). Immunofluorescence analysis showed an increase in MCT1 abundance in the FR treatment compared to ACID (4187 ± 1537 vs. 9250 ± 1648 A.U.; \( P = 0.03 \)) but not compared to CTRL (7241 ± 1648 A.U. vs. 9250 ± 1648 A.U.; \( P = 0.15 \)). The increased butyrate flux concurrent with increased MCT1 abundance demonstrate that transporter-mediated changes in SCFA flux are mediated through MCT1 in the rumen epithelium.

### 2.2 Introduction

In contemporary ruminant production systems, diets are formulated and fed to meet or exceed nutrient requirements. During the periparturient period, diet fermentability and dry matter intake (DMI) change rapidly. In the three weeks leading up to calving, DMI decreases as much as 32% (Hayirli et al., 2002), reducing rumen fermentation. After parturition, DMI increases dramatically (Krause and Oetzel, 2006), and the early lactation diet has a much greater non-fibre carbohydrate (NFC) content, increasing diet fermentability (AlZahal et al., 2014), and increasing production and concentration of short chain fatty acids (SCFA) in the rumen.

The rumen epithelium has been reported to respond to an abrupt increase in diet fermentability by upregulating Na\(^+\) transport (Etschmann et al., 2009; Schurmann et al., 2014) and SCFA transport (Schurmann et al., 2014). However, an abrupt increase in diet fermentability has also been reported to compromise barrier function of the rumen epithelium (Klevenhusen et al., 2013; Schurmann et al., 2014). Moreover, short-term feed deprivation for 48 h (Gabel et al., 1993) or low feed intake for 5 d (Zhang et al., 2013) have been reported to decrease SCFA uptake and compromise barrier function of the gastrointestinal tract. A reduction in barrier function has been
suggested to increase the risk for laminitis and liver abscesses and depress milk production (Plaizier et al., 2008).

A potential mitigation mechanism for subacute ruminal acidosis lies in reducing ruminal H⁺ abundance through increasing SCFA uptake. Higher uptake rates of acetate and butyrate correspond to a greater resistance to subacute ruminal acidosis (Penner et al., 2009b). Protein-mediated pathways for SCFA transport are responsible for absorbing approximately 50% of SCFAs produced in the rumen (Allen, 1997), which accounts for nearly 70% of systemic metabolizable energy needs (Bergman, 1990). As a result, the plasticity of SCFA transport mechanisms and their adaptability to diet changes are an on-going interest.

On the luminal side of the epithelium, SCFAs are absorbed through 3 principal mechanisms: passive diffusion, nitrate-sensitive uptake, and anion exchangers (Figure 1), principal of which is anion exchanger isoform 2 (AE2). These mechanisms contribute substantially to the uptake of SCFAs into the rumen epithelium (Aschenbach et al., 2009), but also contribute to a change in the rumen epithelial intracellular pH (pHi; Muller et al., 2000). The pHi is influenced by both H⁺ and HCO₃⁻ concentrations because of the carbonic acid/bicarbonate equilibrium, which is catalyzed by carbonic anhydrase isoform 2 (CA2). Both the import of protons and the export of HCO₃⁻ have an acidifying effect on pHi, thus SCFA transport is intrinsically linked to pHi.

Previously, rumen epithelial cells were shown to respond rapidly to pH challenges (Muller et al., 2000), highlighting that changes in SCFA and SCFA metabolites have a direct impact on pHi.

One of the principal basolateral SCFA transporters is monocarboxylate co-transporter 1 (MCT1), previously shown to be responsive to changes in ruminal butyrate concentrations (Laarman et al., 2013) in the short term, but not the long term (Laarman et al., 2015). While the epithelial
remodeling leading to morphological adaptation is well-documented (Dionissopoulos et al., 2013; Dirksen et al., 1985), the mechanisms involved in the short-term adaptation to dietary changes warrant further study. Previously, MCT1 has been shown to play an important role in SCFA uptake in the bovine caecum and Caco-2 cells (Kirat et al., 2006; Stein et al., 2000) and is critical for maintenance of pH (Muller et al., 2000). Despite the evidence of MCT1’s importance in SCFA transport, the extent to which MCT is involved in the short-term adaptive response of the rumen is unclear.

The objective of this study was to investigate the role of MCT in adapting to changes in SCFA flux across the rumen epithelium. The hypothesis was that MCT1 is a key determinant of acetate, butyrate, and butyrate metabolite flux, and that MCT1 abundance is related to SCFA flux.

2.3 Materials & Methods

This study was carried out at the University of Saskatchewan, was pre-approved by the University of Saskatchewan Animal Research Ethics Board (protocol 20100021), following the guidelines set out by the Canadian Council on Animal Care (Ottawa, Canada).

2.3.1 Animals and Diets

Twenty-one individually housed Holstein bull calves (216.8 ± 31.4 kg BW) were blocked by body weight and fed a total mixed ration (TMR) consisting of 25.0% barley silage, 25.0% grass hay, 28.0% rolled barley grain, 5.0% pelleted barley grain, 9.0% canola meal and 8.0% vitamin and mineral supplement pellet providing 33 ppm monensin (DM basis). Calves were fed the TMR at 0800 h daily for 5 d and water was provided ad libitum. All calves were housed on rubber mats, and pens were cleaned twice daily.
After 5 d of ad libitum intake, calves were grouped into 7 groups (n=3 per group) and assigned to 1 of 3 treatments. The control treatment (CTRL) was fed TMR ad libitum for 1 additional day. The subacute ruminal acidosis treatment (ACID) was fed 25% of ad libitum TMR DMI for 1 d, followed by a grain overload (barley grain at a rate of 30% of their ad lib TMR DMI) at 0800 h. Following the challenge, calves were fed a full allotment of TMR at 1200 h. Lastly, the feed restriction treatment (FR) was fed the TMR at a rate of 25% of ad lib DMI for all 5 d.

2.3.2 Sampling

Throughout the study, reticular pH was measured continuously using an in-dwelling small ruminant pH logger system (Penner et al., 2009; Dascor Inc., Escandido, CA, USA). Reticular pH is consistently higher than rumen pH, but follows a similar circadian trajectory to rumen pH and the two pH values are highly correlated at $r = 0.83$ on a 93:7 F:C ratio and $r = 0.80$ on a 45:55 F:C ratio diet (Kimura et al., 2011), thus reticular pH measurements can be a useful indicator of subacute ruminal acidosis in cows. The pH logger was calibrated prior to oral dosing and after retrieval at the end of the study. Rumen pH was measured once per minute. Data were downloaded at the end of the study and a threshold of pH 5.6 was used for determination of daily incidence of subacute ruminal acidosis (Schwartzkopf-Genswein et al., 2003; Laarman et al., 2013). For the ACID group, only pH data from the second day where calves were fed a grain overload were analyzed. For the FR group, the average daily pH values for all 5 days were used for analysis.

At the end of the study, calves were killed via captive bolt and exsanguinated and the caudal-dorsal blind sac of the rumen was harvested for analysis, principally due to its homogeneity compared to the ventral or dorsal sac. Approximately 15 large papillae were harvested, washed
in PBS, and preserved in 10% formalin for 24 h, then transferred to a 70:30 ethanol:water mix at room temperature for immunofluorescence analysis. Further, epithelium from the caudal-dorsal blind sac was collected for SCFA flux analysis using Ussing chambers (described below).

### 2.3.3 Immunofluorescence

Papillae were processed and embedded in paraffin wax (Animal Health Laboratory, University of Guelph, Guelph, ON). Thereafter, 5 µm sections were mounted on charged microscope slides (Fisher, Mississauga, ON) and analyzed as described previously (Laarman et al., 2013). Briefly, samples were deparaffinized and rehydrated using xylene and isopropanol. Then, samples were incubated in a 10 mM sodium citrate solution at 95°C for 30 or 60 min for antigen retrieval and then cooled to room temperature, based on optimal fluorescent signal for each antibody. Once the samples had cooled to room temperature, they were washed with PBS and blocked for 30 or 60 min, based on optimal fluorescent signal for each antibody. The blocking buffer contained 10% goat serum (Sigma-Aldrich, Mississauga, ON) and 0.3% Triton-X100 in PBS. Samples were then incubated in primary antibody, dissolved in blocking buffer, for 90 min at room temperature or overnight at 4°C. Samples were washed in PBS, incubated in 1:200 fluorescent secondary antibody (Fisher-Pierce Antibodies, Mississauga, ON) for 40 min and mounted using ProLong Antifade with 4',6-Diamidino-2-Phenylindole (DAPI) nuclear stain (Life Technologies Inc., Burlington ON, Canada).

Slides were visualized using a SP5 upright confocal laser microscope (Leica Instruments, Hannover, Germany) at the Confocal and Imaging Facility at the Advanced Analytics Centre (University of Guelph, Guelph, ON). For each antibody, the same visualization settings were used for all samples. Quantification was conducted as described previously (Laarman et al., 2013).
2013) using an adapted technique from Gavet and Pines (2010). Specifically, cells from the stratum basale were identified and the whole cell signal was quantified. Then, an area beside the papilla was quantified as a background signal correction.

2.3.4 Flux Analysis

Rumen epithelium from the caudal-dorsal blind sac was washed in pre-heated (38.5°C) oxygenated transport buffer (Table 1) until clean. Subsequently, epithelial tissue was gently hand stripped to remove sub-mucosal tissues and placed in fresh buffer solution that was continuously oxygenated for transport to the laboratory. Six pieces of epithelial tissue were then mounted in Ussing chambers (Physiological Instruments, San Diego, CA) with a 1.34 cm² surface area exposure. The 6 chambers were used to partition SCFA flux into total flux (i.e. non-inhibited flux), flux where anion exchangers and the nitrate-sensitive pathways were inhibited, and MCT-mediated flux. This was accomplished by incubating tissues in buffers containing HCO₃⁻ buffer (HCO₃⁻; n = 2/calf), a HCO₃⁻-free buffer with 40 mM nitrate (NO₃⁻; n = 2/calf), and a HCO₃⁻-free buffer with 40 mM nitrate and 10 mM p-hydroxymercuribenzoic acid (pHMB; INH; n = 2/calf).

The mucosal and serosal buffers (Table 1) were prepared with chemicals from Sigma-Aldrich (Oakville ON, Canada). Buffers with HCO₃⁻ were gassed with a CO₂:O₂ ratio of 95:5 while buffers that did not contain HCO₃⁻ were gassed with O₂. Mannitol was used to adjust osmolality to a final buffer osmolality of 290 mOsmol/kg.

To more closely resemble in vivo rumen conditions, all mucosal buffers were adjusted to a final pH of 5.6 and all serosal buffers were adjusted to a pH of 7.4 using gluconic acid. When rumen pH decreases, SCFA flux increases, but only a small component of the measured increase is attributable to passive diffusion (Sehested et al., 1999), thus the increase in SCFA flux is likely
via protein-mediated mechanisms (Aschenbach et al., 2009). Further, MCT1 is upregulated after 7 d of subacute ruminal acidosis (Laarman et al., 2013), thus, at a lower pH, the flux through MCT1 was expected to be more apparent. For that reason, the mucosal buffer was adjusted to pH 5.6, representing conditions during an acidosis challenge.

After tissues were mounted, the transmembrane voltage was clamped to 0 mV using a computer-controlled VCC MC6 voltage-clamp system using Acquire and Analyze software (Physiologic Instruments, San Diego, CA). Transmembrane conductance ($G_t$) and short-circuit current ($I_{sc}$) were measured continuously every 5 s. Twenty minutes were provided for stabilization of $G_t$ and subsequently two chambers out of the 4 chambers that contained HCO$_3^-$-free buffers were assigned to INH, ensuring that initial $G_t$ was balanced within calf and across the course of the study. Then $^3$H-labelled acetate (100 kBq; Perkin-Elmer, Woodbridge, ON, CA) and $^{14}$C-labelled butyrate (72 kBq; Moravek Biochemicals Inc., Brea, CA) were added to the mucosal chamber and the system was allowed to equilibrate for 45 minutes. Two consecutive 1-h flux periods started immediately following equilibration. At the start and end of each flux period, 500 μL serosal buffer was sampled from the 10 mL column, and 100 μL mucosal buffer was sampled from the 10 mL column at the start of the first flux period and the end of the second flux period. Serosal buffer was added to the serosal column to replace sample volume and minimize changes in hydrostatic pressure. Buffer samples were diluted in 4.5 mL scintillation cocktail (ProGold, Perkin-Elmer, Woodbridge, ON, Canada) and analyzed on a scintillation counter (Perkin-Elmer, Woodbridge, ON, Canada).
Fluxes were calculated as follows:

\[ F = \left( \frac{\text{Activity}_{\text{apparent}}}{\text{Activity}_{\text{total}}} \times SCFA \right) \div (\text{SA}_{\text{epi}} \times t_{\text{flux}}) \]

Where:

\( F = \) Flux of SCFA

\( \text{Activity}_{\text{apparent}} = \) Activity of radioisotope appearing in serosal buffer column (DPM)

\( \text{Activity}_{\text{total}} = \) Average total radioactivity added to column (DPM)

\( \text{SCFA} = \) Unlabelled SCFA added to mucosal buffer column (nmol)

\( \text{SA}_{\text{epi}} = \) Surface area of epithelium exposed to buffer (cm\(^2\))

\( t_{\text{flux}} = \) Time of flux period (h)

Fluxes were partitioned as follows:

Total (TOTAL) = flux measured for tissues incubated in HCO\(_3\) containing buffer

Protein-mediated flux (PMF) = flux measured for tissues incubated in HCO\(_3\) containing buffer – flux measured for tissues incubated with HCO\(_3\) - free buffer containing 40 mM nitrate and 10 mM pHMB

MCT-mediated flux (MCTF) = flux measured for tissues incubated with HCO\(_3\) - free buffer containing 40 mM nitrate - flux measured for tissues incubated with HCO\(_3\) - free buffer containing 40 mM nitrate and 10 mM pHMB

Passive diffusion flux (PDF) = flux measured for tissues incubated with HCO\(_3\) - free buffer containing 40 mM nitrate and 10 mM pHMB
2.3.5 Statistics

Data were analyzed using the Mixed Procedure in SAS v. 9.4 (SAS Institute, Cary, NC). To validate the experimental model and affirm that the inhibition by nitrate and pHMB reduced SCFA flux, we used the model

\[ Y = \mu + D_i + B_j + D \times B_{ij} + \varepsilon_{ijk} \]

Where \( Y \) = Variable
\( \mu \) = Overall mean
\( D_i \) = Effect of diet
\( B_j \) = Effect of buffer
\( \varepsilon_{ijk} \) = Residual error

Flux partition data were analyzed using the model

\[ Y = \mu + D_i + P_j + D \times P_{ij} + \varepsilon_{ijk} \]

Where \( Y \) = Variable
\( \mu \) = Overall mean
\( D_i \) = Effect of diet
\( P_j \) = Effect of flux pathway
\( \varepsilon_{ijk} \) = Residual error

Where significant \( D \times P_{ij} \) interactions existed, orthogonal contrasts were used post-hoc to compare CTRL vs. ACID, CTRL vs. FR, and ACID vs. CTRL, for each type of flux. Correlation analysis was conducted using the PROC CORR statement in SAS 9.4 (SAS Institute, Cary, NC).
2.4 Results

In this model, fluxes were partitioned into 4 pathways. The use of the NO$_3$-containing buffer simultaneously inhibited HCO$_3^{-}$-dependent flux and nitrate-sensitive flux, which reduced flux from the HCO$_3$ buffer in both acetate (501.1 ± 22.5 Vs. 363.8 ± 22.1 nmol/cm$^2$ × h, $P < 0.05$) and butyrate (878.5 ± 67.1 Vs. 761.0 ± 63.7 nmol/cm$^2$ × h, $P < 0.05$). Further inhibition by pHMB then inhibits MCT functionality, allowing us to isolate MCT-transport from other protein-mediated SCFA transport. Our flux data, however, demonstrated addition of pHMB to NO3 buffer caused no difference in uptake of acetate (363.8 ± 22.1 Vs. 335.4 ± 22.1 nmol/cm$^2$ × h, $P = 0.44$) or butyrate (761.0 ± 63.7 Vs. 716.8 ± 63.7 nmol/cm$^2$ × h, $P = 0.73$). Additionally, MCTF was not significantly different from zero in CTRL, ACID and FR ($P = 0.38$, $P = 0.66$, $P = 0.88$, respectively). Thus, it seems that the MCTF was insufficiently isolated in this study, and flux through MCT isoforms is occurring primarily through PMF.

2.4.1 Ruminal pH

Ruminal pH was affected by diet. Daily incidence of subacute reticular acidosis was significantly higher in the ACID group compared to the CTRL and FR treatments (57 vs. 520 vs. 30 ± 90 min/d for CTRL, ACID and FR, respectively; $P < 0.01$; data not shown).

2.4.2 Flux

Total acetate flux was higher across the epithelium of calves exposed to FR (630.6 ± 38.9 nmol/cm$^2$ × h) than for CTRL (421.1 ± 41.4 nmol/cm$^2$ × h; $P < 0.01$) and ACID (455.4 ± 38.9 nmol/cm$^2$ × h; $P < 0.01$) treatments (Figure 2.1). There was no difference among the CTRL, ACID and FR treatments for acetate PMF (131.9 ± 41.4, 165.8 ± 38.9, and 192.3 ± 38.9 nmol/cm$^2$ × h for CTRL, ACID and FR, respectively) and acetate MCTF (78.4 ± 38.9, 5.8 ± 38.9,
19.9 ± 41.3 nmol/cm² × h for CTRL, ACID and FR, respectively). In the FR treatment, PDF was greater (438.31 ± 38.9 nmol/cm² × h) than in the CTRL (278.3 ± 38.9 nmol/cm² × h; \( P < 0.01 \)) and ACID treatments (289.7 ± 38.9 nmol/cm² × h; \( P < 0.01 \)).

Total butyrate flux was significantly higher in the FR (1241.9 ± 94.8 nmol/cm² × h) treatment than in the CTRL (625.5 ± 86.3 nmol/cm² × h; \( P < 0.01 \)) and ACID (716.7 ± 81.0 nmol/cm² × h; \( P < 0.01 \)) treatments (Figure 2.2). Butyrate flux through PMF was significantly higher in the FR treatment (479.2 ± 103.9 nmol/cm² × h) than in the CTRL treatment (99.9 ± 86.3 nmol/cm² × h; \( P < 0.01 \)) and the ACID treatment (90.2 ± 81.0 nmol/cm² × h; \( P < 0.01 \)). There was no difference in butyrate MCTF among the CTRL, ACID, and FR treatments (76.1 ± 86.1, 36.1 ± 81.0, 14.2 ± 94.8 nmol/cm² × h, respectively). Lastly, butyrate PDF was significantly higher in the FR treatment (838.1 ± 94.1 nmol/cm² × h) than in the CTRL treatment (555.4 ± 81.0 nmol/cm² × h; \( P = 0.03 \)) but not compared to the ACID treatment (626.5 ± 81.0 nmol/cm² × h; \( P = 0.09 \)).

2.4.3 Transporter Abundance

There was no difference in AE2 abundance among the CTRL, ACID, and FR treatments (96700 ± 1108, 10509 ± 1108, 9673 ± 1197 A.U., respectively; Figure 2.3). Likewise, there was no difference in CA2 abundance among the CTRL, ACID, and FR treatments (7272 ± 1090, 8901 ± 1090, and 6331 ± 1160 A.U., respectively). Abundance of MCT1 was significantly higher in the FR treatment (9250 ± 1648 A.U.) than in the ACID treatment (4187 ± 1537 A.U.; \( P = 0.03 \)), but was not significantly different from the CTRL treatment (7241 ± 1648 A.U.; \( P = 0.15 \)). Further, abundance of NBC1 was not significantly different among CTRL, ACID and FR treatments (7975 ± 1209, 7447 ± 1119, and 9996 ± 1119 A.U., respectively). Also, NHE1 protein abundance was not significantly different among CTRL, ACID, and FR treatments (9484 ± 2209,
11478 ± 2383, and 8605 ± 2383 A.U., respectively). Lastly, NHE3 was significantly higher in
the FR treatment (14874 ± 2027 A.U.) than in CTRL (5874 ± 2189 A.U.; \( P = 0.02 \)), but was not
significantly different from the ACID treatment (11878 ± 2189 A.U.; \( P = 0.34 \)).

2.4.4 Transporter Abundance and Flux Rate Correlation

Correlation analysis showed a positive correlation between NHE1 and acetate PMF flux \( (r = 0.67, P < 0.01; \text{Table 2.3}) \). NHE3 was positively correlated to acetate TOTAL \( (r = 0.52, P = 0.03) \), and acetate PMF \( (r = 0.48, P = 0.04) \). Further, NBC1 was correlated to total acetate flux \( (r = 0.60, P < 0.01) \), acetate PMF \( (r = 0.52, P = 0.02) \), butyrate TOTAL \( (r = 0.61, P < 0.01) \) and
butyrate PMF \( (r = 0.63, P < 0.01) \). Also, NBC1 was also correlated to mean ruminal pH \( (r = 0.52, P = 0.02) \) and duration of ruminal pH < 5.6 \( (r = -0.48, P = 0.03) \).

2.5 Discussion

The objective of this study was to investigate the role MCT1 plays in the short-term adaptation
of the rumen epithelium in response to 2 different dietary challenges (namely rumen acidosis and
feed restriction). The changes observed in PMF and the abundance of MCT1 suggests protein-
mediated butyrate uptake mechanisms are affected by dietary changes. The correlations between
pH\(_i\)-regulating transporters and SCFA flux, independent of diet, further supports the interrelation
between SCFA absorption and pH\(_i\) regulation.

2.5.1 Diet-Dependent Factors

During feed-restriction, total acetate flux was increased. Investigation into the pathway of
absorption revealed that only PDF was increased, suggesting non-facilitated mechanisms were
responsible. Likewise, total butyrate flux was increased during feed restriction with a significant
increase in PDF. These results stand in contrast to in vivo studies showing a reduction in SCFA flux during feed restriction (Albornoz et al., 2013; Gabel et al., 1993; Zhang et al., 2013). In the study by Albornoz et al. (2013), steers were feed restricted using the same model as our study, but disappearance from the rumen was measured using a washed reticulorumen technique (Care et al., 1984). The main reason for the discrepancy is the inherent differences between in vivo and in vitro models of measuring SCFA transport. While using a washed reticulorumen technique, blood flow to and from the rumen can be adjusted by the ruminant, thus affecting the flux. In an Ussing chamber, blood flow is not affected, so while the epithelium appears to have a greater ability to take up SCFA, it seems blood flow or other in vivo systemic factors (e.g. motility) may curtail that increase in SCFA flux observed in our ex vivo study.

Further, feed restriction also resulted in an increase in PMF, despite MCTF not being affected. When looking at the abundance of candidate transporters to explain the increase in PMF, only MCT1 was upregulated. Therefore, it is likely that the increased butyrate flux observed in FR was due to basolateral MCT1 upregulation. The involvement of apical SCFA transporters such as AE2 cannot be ruled out because this study measured flux, as opposed to basolateral efflux. Irrespective of which apical transporter may be involved, a basolateral transporter must also be involved for SCFA transport, and our evidence suggests that basolateral transporter is MCT1.

In past studies, MCT1 has been linked to SCFA uptake in the bovine caecum (Kirat et al., 2006); in calves, feeding calf starter increased both ruminal SCFA concentrations and MCT1 mRNA abundance (Laarman et al., 2012a). The present study confirms that MCT1 is important in SCFA uptake and the concurrent increase in MCT1 abundance and in butyrate PMF demonstrates a possible link between MCT1 protein abundance to flux rate of butyrate or butyrate metabolites.
Although MCTF was not affected by feed restriction, the changes in flux are likely still through MCT. In a past study, MCT1 can be upregulated by different feeding regimens, such as feed restriction in our study, and sustained SARA in lactating dairy cows (Laarman et al., 2013).

Expression of NHE3 was significantly increased by both ACID and FR treatments, cementing NHE3’s role as a pH$_3$-regulating protein highly responsive to diet. Already, NHE3 is known to be very responsive to butyrate (Kiela et al., 2003). In FR, butyrate mucosal-to-serosal flux increased, thus epithelial cells were exposed to more butyrate, despite SCFA concentrations typically being low during feed restriction (Zhang et al., 2013). In lactating dairy cows exposed to SARA for one week, NHE3 protein abundance increased (Laarman et al., 2013), in agreement with our results. In increasing the abundance of NHE3 in response to changes in fermentation, there is an increase in proton export capacity, which may be helping the rumen epithelium cope in the short-term. In response to an acid challenge in rumen epithelial cells, NHE-related transport is increased, and pH is re-equilibrated within 10 minutes (Muller et al., 2000). Thus, NHE3 could be involved in short-term adaptation to SARA.

In our study, ACID treatment caused no discernible difference in any aspect of acetate or butyrate flux. This is somewhat in disagreement with Schurmann et al. (2014), who found that adaptation to a moderately fermentable diet caused an increase in total acetate and butyrate flux after 3 weeks, predominantly through increases in passive diffusion. In heifers, after an episode of ruminal acidosis, SCFA remained unchanged after 2 d but increased after 8 d (Schwaiger et al., 2013). In our study, no increases were observed in either total flux or in passive diffusion, for either acetate or butyrate 1 d after the acidosis induction. One possible explanation for the absence of a flux response to ruminal acidosis is that our Ussing Chamber model fails to take
into account in vivo factors such as blood flow and rumen motility, whereas washed reticulorumen experimental approaches do include these factors (Schwaiger et al., 2013).

Another possibility is that the lack of difference in flux in ACID relative to the control may suggest a one-day challenge is less about adaptation to SARA and more about susceptibility to SARA. Past studies using a SARA challenge model have focused on the relative susceptibility to SARA of sheep (Penner et al., 2009b) and cows (Dohme et al., 2008). Therefore, our ACID results may indicate more about the existing transport capacity than about the adaptation to SARA. Further, functional (Etschmann et al., 2009; Schurmann et al., 2014) and molecular adaptations in the rumen epithelium occur primarily in the first week (Laarman et al., 2013; Steele et al., 2011d). It is possible that the adaptive response involving modulation of transporter abundance takes longer than one day, which explains why the 1 d ACID treatment failed to show a response whereas the 5 d FR treatment did show a response.

2.5.2 Diet-Independent Factors

Correlations between transporters and fluxes showed that, independent of treatment, acetate flux was positively related to NBC1, NHE1 and NHE3. Since acetate uptake is an important indicator of susceptibility to SARA (Penner et al., 2009b), understanding influences on flux is important. In this study, the pHᵢ-regulating transporters that were correlated to acetate flux do not physically transport SCFA. Rather, this study provides further evidence of linkages between pHᵢ-regulating transporters and SCFA transporters. Previous studies have shown CA2 to enhance the activity of MCT1 (Lecona et al., 2008) and NBC1 was found to be down-regulated in partnership with an increase in MCT1 (Laarman et al., 2013).
How pH$_i$ regulation could affect the flux rate of SCFAs is, then, the next focus. The major SCFA import mechanisms – AE2 and passive diffusion – are also pH$_i$ modulators. What these SCFA-transporting mechanisms have in common is that they increase the acidic pressure on the cytosol, through importing protons (passive diffusion) or exporting HCO$_3^-$ (AE2). The pH$_i$-modulating transporters positively correlated to SCFA flux (NHE3, NBC1, NHE1) all decrease the acidic pressure on the cytosol, through exporting protons (NHE1, NHE3) or importing HCO$_3^-$ (NBC1). Thus, the correlation may indicate that the removal of protons from the cytosol limits the SCFA flux. The faster protons can be removed from the cytosol, the faster SCFA can be transported across the epithelium.

This study provides evidence of linkage between pH$_i$ regulation and SCFA flux in the rumen epithelium. Further study is warranted to investigate any causal relationship between pH$_i$ modulators and SCFA transporters. Improving SCFA transport is key to increasing resistance to subacute ruminal acidosis (Penner et al., 2009b), thus a continuing dairy research interest.

### 2.6 Conclusion

This study examined the effects of a ruminal acidosis challenge and feed restriction on the uptake of acetate and butyrate across the rumen epithelium of Holstein steers. We found that feed restriction significantly increased passive diffusion of acetate and butyrate and we found increased butyrate flux through protein-mediated mechanisms, most likely MCT1, and we found acetate flux and several pH$_i$ transporters to be positively correlated. This study showed MCT1 to be an important transporter involved in the short-term adaptation of the ruminal epithelium. Strategies to increase MCT1 abundance and SCFA flux during dietary transitions are a potential mechanism to improve dietary transitions in ruminants. Further, the linkage of pH$_i$-regulating
transporters and SCFA transport was evident; more research is needed to understand this linkage and how optimal SCFA flux is achieved.
Table 2.1 Chemical composition of buffers used in Ussing Chambers to measure acetate and butyrate flux in Holstein steers (n=21) given ad libitum DMI, a ruminal acidosis challenge, or feed restriction.

<table>
<thead>
<tr>
<th>Substance (mM)</th>
<th>Wash/Transport</th>
<th>HCO$_3^-$</th>
<th>NO$_3^-$</th>
<th>INH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Serosal</td>
<td>Mucosal</td>
<td>Serosal</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>1.3</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NaCl</td>
<td>15.6</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>KCl</td>
<td>5.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ca-Gluconate</td>
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<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Mg-Gluconate</td>
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<td>50.6</td>
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</tr>
<tr>
<td>K-Gluconate</td>
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<td>5.5</td>
<td>5.5</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$</td>
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<td>0.6</td>
<td>0.6</td>
<td>0.6</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>2.4</td>
<td>2.4</td>
<td>2.4</td>
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<tr>
<td>Acetic acid</td>
<td>10.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Na-propionate</td>
<td>10.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Na-butyrate</td>
<td>10.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Na-D/L-lactate</td>
<td>5.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(60%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>1.0</td>
<td>1.0</td>
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43
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Table 2.2 Tissue conductance and short-circuit current of caudal-dorsal ruminal tissue used in Ussing Chambers to measure acetate and butyrate flux in Holstein steers (n=21) given ad libitum DMI, a ruminal acidosis challenge, or feed restriction.

<table>
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<td>ACID</td>
<td>FR</td>
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<tr>
<td>HCO₃</td>
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<td>4.69 ± 1.28</td>
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<tr>
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<tr>
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<td>-0.02 ± 0.11</td>
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1Superscripts in each column that are different denote significant difference (P < 0.05)
Figure 2.1 Net acetate flux via different SCFA transport mechanisms across rumen epithelium in Holstein steers (n=21) given ad libitum DMI (CTRL), a ruminal acidosis challenge (ACID), or feed restriction (FR). *denotes $P < 0.05$. PMF – protein-mediated flux; MCTF- monocarboxylate-transporter-mediated flux; PDF – passive diffusion flux.
Figure 2.2 Net butyrate flux via different SCFA transport mechanisms across rumen epithelium in Holstein steers (n=21) given ad libitum TMR (CTRL), a ruminal acidosis challenge (ACID), or feed restricted TMR (FR). *denotes $P < 0.05$. PMF – protein-mediated flux; MCTF- monocarboxylate-transporter-mediated flux; PDF – passive diffusion flux
Figure 2.3 Transporter abundance in rumen epithelium rumen epithelium in Holstein steers (n=21) given ad libitum TMR (CTRL), a ruminal acidosis challenge (ACID), or feed restricted TMR (FR). *denotes $P < 0.05$. AE2 – anion exchanger, isoform 2; NHE3 – sodium/proton exchanger, isoform 3; NHE1 – sodium/proton exchanger, isoform 1; NBC1 – sodium/bicarbonate co-transporter, isoform 1; MCT1 – monocarboxylate co-transporter, isoform 1; CA2 – carbonic anhydrase, isoform 2.
Table 2.3 Pearson correlation coefficients\(^1\) between ruminal pH and acetate/butyrate flux and transporters in the rumen epithelium of Holstein steers (n=21) given ad libitum DMI, a ruminal acidosis challenge, or feed restriction.

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<th>AE2(^2)</th>
<th>CA2(^6)</th>
<th>MCT1(^7)</th>
<th>NBC1(^8)</th>
<th>NHE1(^9)</th>
<th>NHE3(^10)</th>
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Acetate flux

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Butyrate flux

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\(^1\)Values only displayed where \(P < 0.05\).
\(^2\)PMF – protein-mediated flux
\(^3\)MCTF- monocarboxylate-transporter-mediated flux
\(^4\)PDF – passive diffusion flux
\(^5\)AE2 – Anion Exchanger isoform 2
\(^6\)CA2 – Carbonic Anhydrase isoform 2
\(^7\)MCT1 – Monocarboxylate Cotransporter isoform 1
\(^8\)NBC1 – Sodium/bicarbonate cotransporter isoform 1
\(^9\)NHE1 – Sodium/proton exchanger isoform 1
\(^10\)NHE3 – Sodium/proton exchanger isoform 3
Chapter 3: Changes in the rumen papillae during the periparturient transition in Holstein dairy cows are accompanied by changes in abundance of proteins involved in intracellular pH regulation, but not SCFA transport

3.1 Abstract

The objective of this study was to examine changes in SCFA transport capacity in the rumen epithelium during the parturition transition period. Twelve pregnant, cannulated primiparous and multiparous cows were fed a standard total mixed ration (TMR) for the duration of the dry period; after calving, all cows were switched to an early lactation TMR. Rumen pH, fluid and biopsies were taken 3 weeks before anticipated calving (PRE), 1 week after actual calving (PERI) and 6 weeks after calving (POST). Rumen biopsies were analyzed for transport protein abundance using immunofluorescence. Immunofluorescence analysis showed no significant changes in the abundance of monocarboxylate co-transporter, isoform 1 among the PRE, PERI and POST periods (16878 ± 1555 A.U., 15613 ± 1508 A.U. and 16886 ± 1555 A.U., respectively). Also, sodium/proton exchanger, isoform 3 was not significantly different among the PRE, PERI and POST periods (10467 ± 1066 A.U., 9465 ± 1066 A.U., 8552 ± 1109 A.U., respectively). Sodium/bicarbonate co-transporter, isoform 1 was likewise unaffected by time, with the protein abundance among PRE, PERI and POST parturition periods changing from 16467 ± 1274 A.U., 13983 ± 1318 A.U. and 15047 ± 1324 A.U., respectively. Abundance of

carbonic anhydrase, isoform 2 (CA2) decreased from 12332 ± 1580 A.U (PRE) to 7235 ± 1580 (PERI) and 8121 ± 1643 (POST) and this decrease was significant ($P = 0.01$ PRE v. PERI). The observed changes in protein abundance suggest that long-term adaptation of the rumen epithelium occurs to increase transport capacity. Decreased CA2 abundance suggests that intracellular acidification is occurring as part of epithelial remodelling. Taken together, this implies that, during the periparturient transition, a decrease in rumen pH leads to a decrease in intracellular pH in the rumen epithelium, promoting epithelial remodeling resulting in increased SCFA transport capacity.

### 3.2 Introduction

The transition period in dairy cows, the time surrounding parturition, is marked by large dietary changes. Prior to parturition, dairy cows in North America are typically fed a high forage, low energy diet, aimed at avoiding excessive build-up of non-esterified fatty acid reserves, which can cause adverse health events after calving (Drackley, 1999). After calving, dairy cows are typically fed high energy, rapidly fermentable diets that substantially increase ruminal fermentation due to greater dry matter intake (Reynolds et al., 2004). Increased ruminal fermentation places greater demand on absorption of short chain fatty acids (SCFA) across the rumen epithelium, resulting in morphological adaptations in the rumen wall. High energy diets have been documented by an increase in papillae length, width and surface area in the rumen of goats (Shen et al., 2004). The adaptation process results in major proliferation of the rumen epithelium, the integrity of which is important in preventing ruminal bacteria from entering the bloodstream (Plaizier et al., 2008). Thus, the transition period and its
encompassing dietary and physiological changes put tremendous adaptive pressure on the rumen epithelium.

Structurally, the rumen epithelium is organized into four distinct strata: The stratum corneum, stratum granulosum, stratum spinosum and stratum basale (Graham and Simmons, 2005). Previous studies have confirmed that most of the metabolic activity and most of the transporters are located in the strata spinosum and basale (Graham and Simmons, 2005). One of the major transporters on the basolateral membrane are monocarboxylate cotransporter isoform 1 (MCT1; Graham et al., 2007) and sodium/bicarbonate cotransporter isoform 1 (NBC1; Connor et al., 2010). On the apical side of the epithelium are several transport proteins, such as anion exchanger isoform 2 (AE2), putative anion transporter, sodium/proton exchanger isoform 3 (NHE3) and monocarboxylate cotransporter 4 (MCT4; Connor et al., 2010). Together, these transport proteins are responsible for the facilitated uptake of SCFAs. Alongside this facilitated SCFA uptake is passive diffusion of associated SCFAs. Since the non-acidotic, optimal pH of the rumen is between 1 to 2 pH units above the SCFAs’ pKₐ value of 4.7 (Enemark et al., 2002), approximately 90-99% of SCFAs in the rumen exist in the dissociated state. The total uptake of SCFAs from the rumen into the epithelial cells depends on both passive diffusion and facilitated uptake mechanisms (Aschenbach et al., 2009).

Once inside the epithelial cells, the SCFA exists almost purely in the dissociated form, due to a cellular pH of 7.4. Acidotic challenges to the intracellular pH result in a rapid recovery that aims to restore pH homeostasis in the cell within 10 min (Muller et al., 2000). Several mechanisms exist to regulate intracellular pH; prominent intracellular pH-regulating transporters include sodium proton exchangers (Kiela et al., 2003; Muller et al., 2000), which
export protons from the cytosol and carbonic anhydrase isoform 2 (CA2), which reversibly catalyzes the carbonic acid/bicarbonate equilibrium in the cytosol, thereby eliminating protons from the cytosol. Further, CA2 has been found to be integral to the transport of AE1, with AE1 and several other transport proteins like down-regulated in adenoma, containing a CA2-attachment site in their carboxy-terminus (Sterling et al., 2001). Further, MCT activity is enhanced by physically binding with CA2 (Klier et al., 2014). This highlights how SCFA transport mechanisms are very much intertwined with intracellular pH (pHi)-regulation mechanisms, thus the study of SCFA transport naturally includes pHi regulation.

Currently, our understanding of the adaptability of the rumen epithelium to dietary changes remains limited. While our understanding of morphological changes is well-established, the molecular changes on SCFA transport capacity are less understood. The objective of this study was to study changes in protein abundance of important SCFA-transporters in the rumen epithelium as well as proteins involved in pHi regulation, focusing specifically on the transition period. The hypothesis was that the increase in diet fermentability would cause increases in SCFA transporters and modulate the abundance of proteins involved in pHi regulation.

3.3 Materials and Methods

3.3.1 Husbandry and Sampling

This experiment was approved by the Animal Care Committee of the University of Guelph under the guidelines of the Canadian Council of Animal Care (Ottawa, ON) and is a partner study using the same animals as Dionissopoulos et al (2014). Twelve cannulated primiparous (n = 6;
mean wt. 644 ± 13 kg) and multiparous (n = 6; mean wt. 760 ± 10 kg) Holstein cows were selected for this study; one was removed for health reasons (Dionissopoulos et al., 2014). All cows were fed a standard dry-cow ration with 46.0% neutral detergent fiber (NDF) and 34.0% non-fibre carbohydrates (NFC) before parturition; immediately following parturition, all cows were fed an early lactation total mixed ration (TMR) with 34.0% NDF and 43.0% NFC (Dionissopoulos et al., 2014). All cows were fed at 08h00 and 14h00 throughout the study. Once per sampling week at 11h00, rumen fluid and biopsy samples were taken for analysis at three weeks prior to estimated parturition (PRE), one week after actual parturition (PERI), and six weeks after actual parturition (POST). Rumen fluid was collected from the ventral sac of the rumen and squeezed through 4 layers of cheesecloth and frozen at -20°C for SCFA analysis (Dionissopoulos et al., 2013), principally acetate, propionate, and butyrate. Rumen pH was spot-sampled using a portable pH meter (pH310, Oakton Instruments, Vernon Hills, IL).

For rumen biopsies, the rumen was partly evacuated to gain access to the ventral sac of the rumen. Approximately 150 mg of rumen papillae were harvested, rinsed with PBS, and stored in 4% formalin for 24 hours, after which papillae were processed and embedded in paraffin wax (Animal Health Laboratories, University of Guelph, Guelph, ON, Canada) for immunofluorescence analysis.

### 3.3.2 Immunofluorescence

Protein abundance of MCT1, NHE3, NBC1 and CA2 were analyzed as described previously (Laarman et al., 2013). Longitudinal sections of rumen papillae, 5 μm in thickness, were mounted on charged microscope slides (Fisher Sci, Burlington, CA). After deparaffinisation, antigens were retrieved using a 10 mM sodium citrate buffer at 95°C for 30 min then
blocked and permeabilized using 10% goat serum and 0.3% Triton-X 100 blocking buffer.
Samples were then incubated with a rabbit polyclonal primary antibody and a goat anti-rabbit fluorescent secondary antibody (Fisher Sciences, Burlington, CA). After each antibody incubation, samples were rinsed three times with PBS. Slides were mounted with ProLong AntiFade reagent that included DAPI nuclear stains (Life Sciences, Burlington, ON, Canada). One negative control free of primary antibody was included for each protein analyzed.

Samples were then visualized under a TCS SP5 confocal fluorescent microscope (Leica Microsystems, Mannheim, Germany). Smart gain and Offset settings were adjusted for each protein so that the range in signal strength of the samples was matched to the range in detection of the microscope. As a result, saturated pixels were minimal in all images. Signals were then quantified in ImageJ (NIH, Bathesda, MD, USA) using previously established formulas adapted for use in ruminal epithelia (Gavet and Pines, 2010; Laarman et al., 2013).

3.3.3 Statistics
Data were analyzed using PROC MIXED in SAS 9.2 (SAS Institute, Cary, NC), using the statistical model:

\[ Y = \mu + T_i + \varepsilon_{ij} \]

Where:

\( Y \) = Dependant variable
\( \mu \) = Overall mean
\( T_i \) = Effect of time
\( \varepsilon_{ij} \) = Residual error
Cow was used as a repeated measure with 5 variance/covariance structures. The variance/covariance structure with the lowest Bayesian Information Criteria was used for results. Orthogonal contrasts were done on PRE Vs. PERI, PRE Vs. POST and PERI Vs. POST. Correlations between rumen pH, SCFA measurements and protein abundance were done using PROC CORR.

3.4 Results

3.4.1 Rumen SCFA Profile and Rumen pH

Total SCFA concentrations did not change significantly over time (Dionissopoulos et al., 2014). Acetate concentrations also did not change between 3 weeks pre-parturition, 1 week post-parturition and 6 weeks post-parturition, measuring 40.75 ± 1.32, 40.53 ± 1.32, and 43.20 ± 1.32 mM (P = 0.91, PRE v PERI; P = 0.24, PRE v POST; P = 0.20, PERI v POST; Dionissopoulos et al., 2014). Propionate increased during the parturition period, from 17.57 ± 1.25 mM at PRE to 22.94 ± 1.25 mM at PERI and 22.26 ± 1.25 mM at POST (P < 0.01, P < 0.01, P = 0.643; Dionissopoulos et al., 2014). Similarly, butyrate concentrations increased over time, going from 6.97 ± 0.52 mM at PRE to 9.85 ± 0.52 mM at PERI and 9.40 ± 0.52 mM at POST (P < 0.01, P < 0.01, P = 0.51; Dionissopoulos et al., 2014). Mean rumen pH decreased over the parturition period, dropping from 6.38 ± 0.08 at PRE, to 5.81 ± 0.08 and 5.85 ± 0.08 at PERI and POST, respectively (P < 0.01, P < 0.01, P = 0.73; Dionissopoulos et al., 2014).
3.4.2 Immunofluorescence

From pre-parturition, through the parturition period and until 6 weeks post-parturition, there was no significant change in abundance in MCT1 protein, measuring 16878 ± 1555 A.U., 15613 ± 1508 A.U. and 16886 ± 1555 A.U., chronologically ($P = 0.64$, $P = 1.00$ and $P = 0.64$, respectively; Figure 3.1 and 3.2). Abundance of NHE3 protein also was unaffected by the parturition period.

![Figure 3.1 Protein abundance of membrane transport proteins in the rumen epithelium of Holstein dairy cows during the periparturient transition (PRE = 3 weeks prior to planned parturition; PERI = 1 week after parturition; POST = 6 weeks after parturition); $n = 12$; *Significantly different from PRE ($P < 0.05$).]
Figure 3.2 Abundance of MCT1 in rumen epithelium of Holstein dairy cows during the periparturient period. A = PRE (3 weeks prior to parturition), B = PERI (1 week after parturition), C = POST (6 weeks after parturition), D = Negative control (secondary antibody only); n = 12; scale bar = 20.0 µm.
Figure 3.3 Abundance of NHE3 in rumen epithelium of Holstein dairy cows during the periparturient period. A = PRE (3 weeks prior to parturition), B = PERI (1 week after parturition), C = POST (6 weeks after parturition), D = Negative control (secondary antibody only); n = 12; scale bar = 20.0 µm.
Figure 3.4 Abundance of NBC1 in rumen epithelium of Holstein dairy cows during the periparturient period. A = PRE (3 weeks prior to parturition), B = PERI (1 week after parturition), C = POST (6 weeks after parturition), D = Negative control (secondary antibody on only); n = 12; scale bar = 20.0 µm.
Figure 3.5 Abundance of CA2 in rumen epithelium of Holstein dairy cows during the periparturient period. A = PRE (3 weeks prior to parturition), B = PERI (1 week after parturition), C = POST (6 weeks after parturition), D = Negative control (secondary antibody only); n = 12; scale bar = 20.0 µm.
Although there was a numerical decrease in protein abundance from PRE (10467 ± 1066 A.U.), to PERI (9465 ± 1066 A.U.) and POST (8552 ± 1109 A.U.), none of the differences were significant ($P = 0.57$, $P = 0.20$, $P = 0.61$; Figure 3.3). Further, NBC1 abundance did not differ significantly in chronological sequence from 3 weeks prior to parturition to 6 weeks post-parturition (16467 ± 1274 A.U., 13983 ± 1318 A.U., 15047 ± 1324 A.U.; $P = 0.26$, $P = 0.42$, $P = 0.63$; Figure 3.4). Lastly, CA2 abundance did change over time, decreasing from 12332 ± 1580 A.U. PRE to 7235 ± 1580 A.U. at PERI and 8121 ± 1643 A.U. at POST ($P = 0.01$, $P = 0.06$, $P = 0.64$; Figure 3.5). Abundance of NHE3 was positively correlated to rumen pH ($r = 0.39$, $P = 0.03$; Figure 3.6), and negatively correlated to acetate concentration ($r = -0.44$, $P = 0.03$).
propionate concentration ($r = -0.55, P < 0.01$) and butyrate concentration ($r = -0.55, P < 0.01$).

### 3.5 Discussion

The purpose of this experiment was to investigate changes in transport protein abundance during the parturition period. The parturition transition period is marked by a rapid onset of negative energy balance due to energetic demands of milk production (Drackley, 1999) resulting in a mobilization of energy reserves. In the rumen, early lactation TMRs contain considerably higher NFC than dry-off TMRs, making the diet more rapidly fermentable. Approximately 50% of ruminally-produced SCFAs are translocated across the rumen epithelium (Allen, 1997), thus the rumen epithelium’s adaptation during the parturition transition warrant further investigation. How a more rapidly fermentable diet combined with increased energy demands affects the transport capacity of the rumen epithelium is unclear.

#### 3.5.1 Transport Capacity

Moving SCFA across the ruminal epithelium is determined by several factors. While passive diffusion across the rumen epithelium certainly occurs, protein-mediated transport is also an important mechanism by which SCFA are transported from the lumen into the bloodstream (Aschenbach et al., 2009). During the periparturient period, both rumen pH and epithelial carbonic anhydrase 2 decreased. Carbonic anhydrase bi-directionally catalyzes the reaction between $\text{HCO}_3^-$ and carbon dioxide, associating with $\text{HCO}_3^-$ transporters such as AE1 and NBC1 to increase $\text{HCO}_3^-$ transport (Purkerson and Schwartz, 2007). The presence of CA2 increases MCT1 transport activity (Klier et al., 2014), which makes the decreased CA2 expression in our study puzzling from a transport capacity perspective.
In addition, the lack of response of MCT1 and NHE3 to changes in pregnancy is somewhat surprising, but may be due to morphological changes in rumen papillae that occur concurrently. In goats fed 90 g of concentrate per kilo of metabolic body weight, rumen papillae were significantly longer and wider, with a resulting increase in surface area, than goats fed only 20 g of concentrate per kilo of metabolic weight (Shen et al., 2004). In cows, the parturition transition is characterized with well-documented increases in rumen papillae surface area (Martens et al., 2012); these changes in papillae size were visually confirmed in our study. The most likely explanation for our findings, in light of past studies, is that the morphological changes in the rumen are the main drivers of increases in transport capacity. That is to say that the same transport protein abundance per cell over the time period is still resulting in increased transport capacity because the number of cells increased drastically over the course of our study. In sheep, a transition from a hay diet to a mixed hay/concentrate diet, similar to dairy cow periparturient transition, results in a marked increase in mucosal to serosal sodium flux in the first week post-transition and this increase is sustained for 12 weeks (Etschmann et al., 2009). Taken together, this likely means that transport-protein-mediated adaptation in the rumen likely takes place in the first week following dietary changes. Gene expression changes associated with a switch from a high forage diet to a high concentrate diet occurred primarily in the first weeks after the dietary change (Steele et al., 2011a), so the first several weeks following diet transition is most important in adaptations in cellular transport capacity.
3.5.2 Epithelial Remodelling

Epithelial remodelling may be the principal route for long-term changes in transport capacity. In the rumen epithelium, expression of genes involved in epithelial remodelling is modulated within one week of exposure to subacute ruminal acidosis (Dionissopoulos et al., 2014), so epithelial remodelling begins rather quickly after a dietary change. Recently, Schurmann et al. (2014) found part of the adaptive process in the rumen epithelium is an increase in passive diffusion and in the size of large papillae in the first 21 days of adaptation from a high forage diet to a moderately fermentable diet. The increase in passive diffusion is likely due to epithelial integrity disruption, pointing to evidence of epithelial remodelling, which is corroborated by the increase in papillae size in that study.

During epithelial remodelling, one of the first and important steps is the promotion of cell migration, which involves the cleaving of anchoring extracellular matrix proteins (Streuli, 1999). One of the ways the extracellular matrix proteins can be modulated is through intracellular acidification. Intracellular acidification disrupts the Connexin 43/Zonal-Occludens 1 interactions in cardiac myocytes and results in reduced intercellular coupling, promoting remodelling of junctions (Duffy et al., 2004).

In our study, the decreased abundance of CA2 in the PERI and POST and the positive correlation of NHE3 abundance to rumen pH could actually be related to epithelial remodelling by causing intracellular acidification of the epithelium. Decreased CA2 abundance results in less elimination of protons from the cytosol through the HCO$_3$/CO$_2$ equilibrium, while a lower NHE3 abundance means fewer protons are being expelled from the epithelial cell cytosol into the rumen. After parturition, when acetate, propionate and
butyrate concentrations increase and ruminal pH decreases, as was the case in our study, the increased proton influx from the rumen would presumably result in intracellular acidification in an environment with less CA2 and less NHE3 because fewer protons are being removed from the cytosol through CA2 catalysis and NHE3 efflux. From this perspective, the results in this study indicate that intracellular acidification might be occurring as part of the remodelling of the epithelium in the periparturient transition period.

3.6 Conclusion

During the parturition transition, morphological changes in the rumen wall were not accompanied by changes in the abundance of MCT1, NHE3 and NBC1. Protein abundance of CA2 decreased during the PERI and POST periods, suggesting that intracellular acidification may be occurring as fewer protons are being converted into CO$_2$ and H$_2$O. More evidence of intracellular acidification came from the positive correlation between NHE3 abundance and rumen pH. As rumen pH decreased, proton expulsion from the epithelial cell cytosol was decreased, furthering intracellular acidotic pressure. Intracellular acidification aids in breaking down cell adhesion to extracellular matrix components and thus contributes to epithelial remodelling in the periparturient transition.

Future research should focus on the role of epithelial remodelling in optimizing transport capacity of SCFAs during major dietary transitions, to balance disease risk with the need for changes in transport capacity across the rumen epithelium. Changes in the rumen epithelium affects disease risk as well as transport capacity of SCFAs and an effective balance between the two must be pursued for optimal health and productivity of dairy cows during the periparturient transition period.
Chapter 4: Butyrate and subacute ruminal acidosis affect abundance of membrane proteins involved with proton and short chain fatty acid transport in the rumen epithelium of dairy cows

4.1 Abstract

The objective of this study was to elucidate the effects of butyrate on the short chain fatty acids membrane transport proteins and proton membrane transport proteins in the rumen epithelium. Sixteen mid-lactation cows were fed a mid-lactation total mixed ration with an added grain supplement introduced over the course of 2 days, increasing the dietary non-fibre carbohydrate content from 40% to 44%. After the 2 day ramp-up of grain supplement, cows were divided into a control treatment and a butyrate treatment. For 7 days, the cows on the control treatment received a carrier treatment and the cows on the butyrate treatment received a ruminal butyrate dose at the rate of 2.5% of dry matter intake. Rumen pH was measured on days 6 and 7 and rumen biopsies were taken on days 1 and 7. Rumen pH measurements confirmed the occurrence of ruminal acidosis in both treatment groups, defined as a rumen pH of 5.6 for at least 3 h per day. Between the control and butyrate treatment, there was no difference in rumen pH profile. Immunofluorescence analysis performed on longitudinal ruminal papillae cross-sections showed that for the duration of the study, protein abundance in the stratum basale increased for monocarboxylate cotransporter isoform 1 (MCT1), sodium/proton exchanger isoform 3 and sodium/bicarbonate cotransporter isoform 1 (NBC1). There was a time*treatment interaction for MCT1 and NBC1, with the butyrate treatment group showing a higher abundance of MCT1 and NBC1.

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a lower abundance of NBC1 at day 7. Luminal butyrate appears to increase SCFA uptake
capacity by increasing the abundance of MCT1 transport proteins on the basolateral membrane
and decreasing basolateral HCO$_3^-$ uptake capacity through decreased NBC1 protein expression.
These effects decrease HCO$_3^-$ uptake capacity through NBC1 and help to offset the increased
MCT1, since MCT also creates alkalotic pressure by expelling protons from the cytosol.

4.2 Introduction

Short chain fatty acids (SCFA) are a major source of energy for rumen epithelial cells (Bergman, 1990). Historically, SCFA were thought to passively diffuse through the rumen epithelium, but
more recently, evidence has emerged that uptake of SCFA is primarily achieved through
facilitated exchange using a number of uptake mechanisms (Aschenbach et al., 2009). In human
adenocarcinoma cells, the two major mechanisms are a high affinity/low capacity
monocarboxylate co-transporter isoform 1 (MCT1) and a low affinity/high capacity
butyrate/bicarbonate antiporter (Lecona et al., 2008). Several models of rumen epithelial
transport have been developed in recent years (Connor et al., 2010; Gabel et al., 2002), which
include anion exchanger isoform 2 (AE2), sodium/proton exchanger isoform 3 (NHE3) on the
apical side and MCT1, sodium/proton exchanger isoform 1 (NHE1) and sodium/bicarbonate
cotransporter isoform 1 (NBC1) on the basolateral side (Figure 4.1).
Facilitated uptake of SCFA highlights the importance of rumen epithelium functionality and physiology. Higher uptake rates of acetate, for instance, have been linked to an increased resistance to subacute ruminal acidosis (SARA; Penner et al., 2009b). When the accumulation of protons from the fermentation of rapidly fermentable Non-Fibre Carbohydrates (NFC) overwhelms the ability of the rumen to buffer or neutralize the protons, the pH decreases and SARA can occur (Allen, 1997). In light of these recent findings and the continuing importance of mitigating the costly impacts of SARA (Krause and Oetzel, 2006), the importance of epithelial transport mechanisms has become apparent.

One principal candidate for modulating rumen epithelial transport mechanisms is butyrate, a bioactive SCFA. Treatment with butyrate increased cellular uptake of butyrate in butyrate-
sensitive BCS-TC2 colon adenocarcinoma cells (Lecona et al., 2008). Also, within the promoter region of NHE3, there exists a butyrate- response element (Kiela et al., 2007). Most of the bioactivity of butyrate studied to this point has primarily been attributed to its ability to hyperacetylate histone proteins through its action on the histone deacetylase complex (HDAC; Davie, 2003; Mathew et al., 2010), which potentially affects the expression of multiple genes. Although butyrate’s effects on gene expression and transport rates are well-studied, it is unclear which membrane transport mechanisms butyrate affects at the protein level.

The objective of this study was to investigate the effects of butyrate on ruminal epithelial transport proteins in cows with SARA. We hypothesized that butyrate would serve to increase the abundance of membrane transport proteins such as MCT1, closely associated with SCFA uptake. Since the effects of butyrate on transport rates have been established, identifying the role of protein expression is an important step in solidifying our understanding of SCFA uptake in ruminants.

4.3 Materials and Methods

4.3.1 Animals and Treatment

The experiment was carried out as described by Dionissopoulos et al. (Dionissopoulos et al., 2013) and approved by the University of Guelph Animal Care Committee using guidelines of the Canadian Council for Animal Care. Sixteen mid-lactation, rumen-cannulated cows were fed a mid-lactation total mixed ration (TMR) that included a concentrate mix formulated to
elevate the NFC content of the diet from 40.0 to 44.0% of dry matter over the course of 2 days immediately preceding the start of the study (Dionissopoulos et al., 2013). Cows were blocked by days in milk (DIM) and assigned either a control or a butyrate treatment. On the butyrate treatment, cows were dosed with butyrate (Proformix; Probiotech Inc., Saint Hyacinthe, QC) at a rate of 2.5% of their average daily dry matter intake (DMI); on the control treatment, cows were dosed with a carrier, in this case a paper bag. Butyrate was dosed at 10.00 h and 13.30 h daily, to correspond with feeding times.

4.3.2 TMR, Milk, Rumen and Blood Sampling

Milk production and DMI were measured daily and milking occurred at 5.30 and 16.00 h daily. Rumen pH was measured on day 6 and 7 of the experiment using an indwelling rumen pH system (AlZahal et al., 2007). Rumen fluid and blood were sampled on days 1 and 7 at 16.30 h. Rumen fluid was harvested from the rumen ventral sac and squeezed through 4 layers of cheesecloth, then snap frozen in liquid nitrogen. Blood was harvested from the tail vein and processed as previously described (Dionissopoulos et al., 2013).

Milk samples were pooled and analyzed for milk components (CanWest DHI Laboratory, Guelph, ON, Canada). Blood samples were processed and analyzed for serum β-Hydroxybutyrate (BHBA) by the Animal Health Laboratory (Guelph, ON, Canada) using established methods (Williamson et al., 1962). Rumen fluid was analyzed for SCFA by gas chromatography (Steele et al., 2011a).
4.3.3  **Histology and Immunofluorescence**

Approximately 20 papillae were harvested from the rumen of each animal and washed 3 times in ice-cold PBS, then stored in 4% formalin solution for 24 h. At that point, papillae were transferred to 70% ethanol solution and mounted in paraffin wax by Animal Health Laboratories (Guelph, ON, Canada). In each sample, an H and E stain was done according to established protocols and the images were taken using an Olympus BX60 light microscope mounted with an Olympus DP71 camera (Richmond Hill, ON, Canada). Images were analyzed for papillae sloughing on a scale of 1-5 using a scoring rubric previously developed in our lab (Steele et al., 2015). Briefly, papillae images were assessed a score of 1 for a completely intact epithelium; 2, 3, or 4 for increasing degrees of sloughing and papillae showing extensive sloughing were assessed a 5. Scoring was blind and involved multiple technical replicates per biological sample. To further control for bias, scoring was done simultaneously by 2 people and person-to-person variation was analyzed.

For immunofluorescence analysis, mounted samples were sectioned longitudinally (5 µm thick) and were mounted on charged microscope slides (Fisher, Whitby, ON, Canada). After paraffin removal, samples were incubated in 10 mM sodium citrate buffer (Fisher, Whitby, ON, Canada) at 95°C for antigen retrieval, then blocked and permeabilized with 10% goat serum and 0.3% Triton-X100 blocking buffer. Sections were incubated with primary antibody (1:50 dilution) at room temperature for 90 min, washed 3 times with PBS and then incubated with fluorescent secondary antibody (Fisher, Whitby, ON, Canada) for 30 min at room temperature. Slides were mounted with ProLong AntiFade reagent, including DAPI nuclear stain (Life Sciences, Burlington, ON, Canada). For each primary antibody, a negative control without
primary antibody was also stained. Immunofluorescence was detected using a Leica TCS SP5 Upright confocal fluorescent microscope (Leica MicroSystems, Mannheim, Germany). Smart Gain and Offset were adjusted for each target protein so that the strongest signal did not show extensive pixel saturation. For each blinded sample, 3 images were taken of 3 papillae, totaling 9 images per biological sample. Signal intensity was analyzed using ImageJ (National Center for Biotechnology Information, Bethesda, MD, USA), using a modified approach described by Gavet and Pines (2010). Specifically, similar-sized cells from the stratum basale were visually identified in each image and the whole cell signal quantified. Then, an area beside the papilla was quantified for background signal correction.

The corrected whole cell signal was calculated using the formula (Gavet and Pines, 2010):

\[ WCS = \text{ID}_{\text{cell}} \times (A_{\text{cell}} \times M_{\text{background}}) \]

Where:

- \( WCS \) = The Whole Cell Signal
- \( \text{ID}_{\text{cell}} \) = The integrated density of the cell
- \( A_{\text{cell}} \) = The surface area of the cell
- \( M_{\text{background}} \) = The mean background signal

4.3.4 Statistics

Data were analyzed using PROC MIXED of SAS 9.2 (SAS Institute, Cary, NC), using the model:
\[
Y = \mu + D_i + T_j + D*T_{ij} + \varepsilon_{ijk}
\]

Where:

\(Y\) = The dependant variable

\(\mu\) = Overall mean

\(D_i\) = The fixed effect of treatment

\(T_j\) = The fixed effect of time

\(D*T_{ij}\) = The interaction of time and treatment

\(\varepsilon_{ijk}\) = The residual error

Cow within treatment was used as a repeated variable, along with 5 variance/covariance structures. The variance/covariance structure with the lowest AIC and BIC values were used for statistical analysis.

### 4.4 Results

#### 4.4.1 DMI, Milk, Rumen and Blood Responses

The effects of butyrate supplementation on DMI, milk and rumen responses have been described previously (Dionissopoulos et al., 2013). Butyrate treatment lowered DMI compared to the control treatment (24.40 ± 0.77 Vs. 17.41 ± 0.77 kg/d; \(P < 0.01\)). Milk production, however, was not significantly different between control treatment (41.93 ± 2.50 kg/d) and butyrate treatment (34.74 ± 2.50 kg/d; \(P = 0.06\); Dionissopoulos et al., 2013). Actual NFC content was 44.0% of DMI; serum BHBA was elevated fourfold in the butyrate treatment compared to control on both day 1 and day 7 (Dionissopoulos et al., 2013). Rumen pH traces confirmed the occurrence of SARA in both treatments, with no significant difference in pH.
measurements between control and butyrate treatments (Dionissopoulos et al., 2013). Total SCFA was higher in butyrate than in control in day 1 (92.76 ± 4.51 Vs. 78.87 ± 4.51 mM) but not on day 7 (87.59 ± 4.51 Vs. 81.82 ± 4.51 mM). Butyrate concentrations were higher in the butyrate treatment than in the control treatment on both day 1 (22.60 ± 0.94 Vs. 9.88 ± 0.94 mM) and on day 7 (21.60 ± 0.94 Vs. 8.60 ± 0.94 mM; Dionissopoulos et al., 2013).

4.4.2 Immunofluorescence and Histochemistry

Exogenous butyrate addition resulted in increased abundance of MCT-1 over the 7 days of the experiment, with both control treatment (11043 ± 953 Vs. 11275 ± 953 A.U.; $P < 0.01$) and the butyrate treatment (8996 ± 1018 Vs. 14747 ± 953 A.U.; $P = 0.01$) increasing between days 1 and 7, respectively (Figure 4.2 and Table 4.1). Analysis of MCT-1 protein abundance also showed a treatment*time interaction ($P = 0.01$), although no effect of treatment was reported ($P = 0.48$). For NHE-1, the treatment effect ($P = 0.85$), time effect ($P = 0.26$) and diet*time interaction ($P = 0.98$) were not statistically significant (Figure 4.3).

Abundance of NHE-3 was significantly affected by time (Figure 4.4), increasing from day 1 to day 7 in the control treatment (9536 ± 1619 vs. 13598 ± 1461 A.U.; $P = 0.03$) and in the butyrate treatment (10023 ± 1698 Vs. 11828 ± 1400 A.U.; $P = 0.03$). No significant effects of treatment or treatment*time were observed. Protein abundance of NBC-1 was increased in both control (8897 ± 878 Vs. 15065 ± 992) and butyrate treatments (9458 ± 878 Vs. 11122 ± 992) between days 1 and 7, respectively ($P < 0.01$; Figure 4.5). In the NBC1 protein abundance, there was also a significant time*treatment interaction ($P = 0.01$). Papillae sloughing was not significantly different between treatments (Figure 4.6).
Figure 4.2 MCT1 protein expression in the rumen epithelium stratum basale of Holstein cows fed a high NFC TMR with and without exogenous butyrate dosed at 2.5% DMI; n = 16.
Figure 4.3 NHE1 protein expression in the rumen epithelium stratum basale of Holstein cows fed a high NFC TMR with and without exogenous butyrate dosed at 2.5% DMI; n = 16.
Figure 4.4 NHE3 protein expression in the rumen epithelium stratum basale of Holstein cows fed a high NFC TMR with and without exogenous butyrate dosed at 2.5% DMI; n = 16.
Figure 4.5 NBC1 protein abundance in the rumen epithelium stratum basale of Holstein cows fed a high NFC TMR with and without exogenous butyrate dosed at 2.5% DMI; n = 16.
Figure 4.6 Papillae sloughing score in the rumen epithelium stratum basale of Holstein cows fed a high NFC TMR with and without exogenous butyrate dosed at 2.5% DMI; n = 16.
Table 4.1 Protein abundance of membrane transport proteins in stratum basale of rumen epithelium of Holstein Dairy cows dosed or not dosed with butyrate at 2.5% of DMI; n = 16.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Day 1</th>
<th>Day 7</th>
<th>P Value</th>
<th>Treatment</th>
<th>Time</th>
<th>Trt*Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCT1</td>
<td>Control 11043 ± 953 8996 ± 1018</td>
<td>Butyrate 11275 ± 953 14747 ± 953</td>
<td>0.48</td>
<td>0.01</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>NHE1</td>
<td>Control 8286 ± 1212 9720 ± 1243</td>
<td>Butyrate 12448 ± 2463 10241 ± 2463</td>
<td>0.82</td>
<td>0.19</td>
<td>0.30</td>
<td></td>
</tr>
<tr>
<td>NHE3</td>
<td>Control 9692 ± 1170 10035 ± 1243</td>
<td>Butyrate 124313329 ± 124313049 ± 1243</td>
<td>0.97</td>
<td>0.02</td>
<td>0.79</td>
<td></td>
</tr>
<tr>
<td>NBC1</td>
<td>Control 8897 ± 878 9458 ± 878</td>
<td>Butyrate 15065 ± 992 11122 ± 992</td>
<td>0.15</td>
<td>&lt;0.01</td>
<td>0.01</td>
<td></td>
</tr>
</tbody>
</table>

4.5 Discussion

The existence of MCT-1, NHE-1, NHE-3 and NBC-1 proteins in the rumen epithelium has been previously characterized (Graham and Simmons, 2005). Our aim was to quantify protein abundance and study differences in protein abundance as affected by exogenous butyrate.

Previous research has shown a bioactive effect of butyrate on the mRNA expression on NHE3 (Kiela et al., 2003) and MCT-1 (Laarman et al., 2012a).

The ruminal butyrate dosing reported in this study is reflected in the pronounced difference in BHBA levels detected and was not due to endogenous factors. Specifically, butyrate is taken up from the rumen and largely metabolized into ketone bodies and CO₂ (Bergman, 1990).

Serum BHBA concentration increased in the butyrate treatment, suggesting an enhancement of butyrate transport across the rumen epithelium.

4.5.1 Effect of Time

Two days prior to the start of the experiment, a grain supplement was added to the TMR in stages so that by the day prior to the start of the experiment, NFC in the diet had been elevated to 44.0%. Increasing the NFC of the diet increases readily-available rapidly-
fermentable carbohydrates and can pose a considerable acidotic challenge. Indeed, both the Control and Butyrate treatments experienced an average of 536 min/d and 598 min/d of acidosis respectively, equivalent to 9-10 h per day. Thus, the effect of time, as measured in our experiment, is likely a physiological response to acidosis.

In other studies, higher inclusion of dietary concentrates increased MCT1 protein abundance, both when compared to an all-forage diet (Kuzinski et al., 2012) and in unrestricted access as compared to restricted access, despite similar SCFA concentrations (Koho et al., 2011). In our study, the increase in MCT1 and NHE3 on epithelial cells appears to be primarily aimed at maintaining intracellular pH. Both NHE3 and MCT1 are proton exporters (Aschenbach et al., 2011), while NBC1 is a HCO$_3^-$ importer (Connor et al., 2010). These points suggest that the rumen epithelium is adapting to the acidosis by increasing proton export, decreasing acidotic pressure and increasing HCO$_3^-$ import and thus increasing acid buffering capacity. Intracellular monocarboxylates present a considerable challenge for ruminal epithelial cells (Muller et al., 2002) as they can significantly affect acid/base homeostasis in the rumen.

Decreased mucosal pH induces an increase in acetate uptake that is driven primarily by intracellular HCO$_3^-$; this HCO$_3^-$ is primarily derived from exogenous sources (Aschenbach et al., 2009). In our case, the upregulation of NBC1 protein likely contributes to the HCO$_3^-$-uptake capacity of the rumen epithelium. Uptake of HCO$_3^-$ through NBC was previously shown to be, along with apical NHE activity, an important regulator of intracellular pH in rumen epithelial cells (Huhn et al., 2003). Combining the increased abundance of NBC1, MCT1 and NHE3 proteins, there is thus evidence that the rumen epithelium increases its capacity for intracellular pH regulation in response to a sustained ruminal acidotic challenge.
4.5.2 Effect of Treatment

Increased MCT1 expression not only increases monocarboxylate export, it also increases proton export from the cytosol. This proton expulsion puts an alkalotic pressure on the intracellular pH. Intracellular SCFA are preferentially shuttled into the bloodstream by MCT1; this process is HCO₃-dependent (Dengler et al., 2014). Since MCT1 activity also expels a proton from the cytosol for every monocarboxylate transported, HCO₃ import via NBC1 may not be needed for maintenance of cellular pH (Aschenbach et al., 2011). Instead, basolateral MCT1 may be primarily used for SCFA export.

To increase basolateral export of SCFA, more apical SCFA import is needed to replenish the intracellular supply of SCFA. Under normal physiological conditions, low intracellular pH induces global histone de-acetylation through the HDAC in order to provide an important source of acetate for basolateral MCT1 activity, resulting in acetate and proton export (McBrian et al., 2013). However, butyrate is an HDAC inhibitor (Davie, 2003), hence SCFA-mediated MCT1 activity is likely due to exogenous butyrate.

One of the principal SCFA uptake mechanisms is apical SCFA/HCO₃ exchange. Apical HCO₃ extrusion, important for SCFA uptake and most of the HCO₃, is likely to come from basolateral import rather than intracellular Carbonic Anhydrase 2 (CA2) activity, given the former’s much greater ability to drive acetate uptake at low ruminal pH (Aschenbach et al., 2009). However, since the expression of NBC1 was down-regulated in this study, it appears that the increased NHE3 abundance may counter alkalization from apical SCFA/HCO₃ exchange by recycling a proton into the rumen.
NHE3 may play an important role in intracellular pH regulation as well, as apical exchangers have been shown to be more essential in intracellular pH recovery after an acidotic challenge (Sellin and De Soignie, 1998). That NHE3 was not affected by the butyrate treatment may be because of the fact that NHE3 activity and protein abundance are strongly influenced by local concentrations of SCFA and not by SCFA metabolites (Musch et al., 2001). Further, salivary sodium is also an important modulator of apical NHE activity (Sehested et al., 1996), so factors other than butyrate may have negated any effect by butyrate. Together, this suggests that the levels of exogenous butyrate used in our trial may have been insufficient to trigger an additional upregulatory response in NHE3.

### 4.6 Conclusion

This study focused on the protein abundance of membrane transport proteins involved in SCFA shuttling across the epithelial barrier as well as membrane transport proteins involved in intracellular pH homeostasis. Our findings indicate that a sustained acidotic challenge for a period of one week will induce changes in the protein abundance of NHE3 on the apical membrane and NBC1 and MCT1 on the basolateral membrane. Collectively, these changes indicate that the rumen epithelium adapts to increase proton expulsion and increase HCO$_3^-$ uptake. The time*treatment interactions suggest that butyrate leads to downregulation of NBC1 and upregulation of MCT1, suggesting an increased capacity for transepithelial SCFA transport through MCT1. Adding exogenous butyrate to cows with SARA clearly highlights the plasticity of the membrane transport proteins in the rumen epithelium.

While this study exhibited the effects of butyrate on key membrane transport proteins, a dose response could not be elucidated because only one concentration of butyrate was used.
Further, while the membrane protein abundance certainly indicated transport capacity of the rumen epithelium, it does not necessarily imply transport rates. With an abundance of literature on SCFA transport rates in the rumen epithelium, some of which is cited elsewhere in this article, future studies ought to focus on linking protein abundance to transport rates, given the importance of the latter on susceptibility to SARA. Such studies will aid in our understanding of which SCFA proteins are most important and which have the biggest effect on transport rates.
Chapter 5: Butyrate supplementation affects mRNA abundance of genes involved in glycolysis, oxidative phosphorylation and lipogenesis in the rumen epithelium of Holstein dairy cows

5.1 Abstract

Energy availability in epithelial cells is a crucial link for maintaining epithelial barrier integrity; energy depletion is linked to impaired barrier function in several epithelia. This study aimed to elucidate the effects of exogenous butyrate on mRNA abundance of genes indirectly involved in rumen epithelial barrier integrity. Sixteen mid-lactation Holstein cows fed a total mixed ration received a concentrate mix introduced over the course of 2 days immediately prior to the start of the experiment, with the aim to induce subacute ruminal acidosis. For 7 days, while being fed the concentrate mix, cows were assigned either a control treatment or a butyrate treatment, in which cows were fed butyrate at 2.5% daily dry matter intake in the form of a calcium salt. On days 6 and 7, rumen pH was measured continuously and on day 7, rumen biopsies took place. Rumen pH fell below 5.6 for more than 3 hours per day in both treatments, confirming the occurrence of SARA. Microarray and pathway analysis, confirmed by real time PCR, showed that exogenous butyrate significantly increased the mRNA abundance of hexokinase 2 (fold change: 2.07), pyruvate kinase (1.19), cytochrome B-complex 3 (1.18) and ATP synthase, F0 subunit (1.66), which encode important glycolytic enzymes. Meanwhile, butyrate decreased mRNA abundance of pyruvate dehydrogenase kinase 2(-2.38), ATP citrate lyase (-2.00) and mitochondrial CoA

transporter (-2.27), which encode enzymes involved in lipogenesis. These data suggest exogenous butyrate induces a shift towards energy mobilization in the rumen epithelium, which may aid barrier function in the rumen epithelium during SARA.

### 5.2 Introduction

In cows fed a high grain diet for milk production purposes, subacute ruminal acidosis (SARA) continues to be a costly problem, with one estimate of $400 USD in lost milk production per cow per lactation (Plaizier et al., 2008). The accumulation of short chain fatty acids (SCFA) leads to SARA and strains the integrity of the rumen epithelium (Zebeli and Metzler-Zebeli, 2012). In cases where SARA compromises epithelial integrity, many detrimental health effects can result, such as liver abscesses, lesions and even laminitis (Krause and Oetzel, 2006). Adverse health events are caused by translocation of bacteria across the rumen epithelium, underscoring the importance of epithelial integrity.

Like other epithelia, ruminal barrier function is attained by tight junction proteins such as tight junction protein isoform 3, zonal occludins isoform 1 and claudin isoform 1. These proteins physically anchor the cells of the stratum granulosum (Graham and Simmons, 2005), effectively eliminating paracellular bacterial translocation. In so doing, the epithelium can facilitate SCFA and proton transport across the rumen epithelium using multiple transport mechanisms, in addition to a smaller fraction of SCFA that passively diffuses across the epithelium (Aschenbach et al., 2009). Maintaining epithelial integrity thus plays a crucial role in health maintenance in dairy cows.
One possible nutraceutical implicated in maintaining barrier function is butyrate, which is shown to genomically regulate several cellular processes and modulate gene expression through histone deacetylase complex (HDAC) inhibition (Ploger et al., 2012). In cows, butyrate increases expression of critical barrier function genes, including tight junction proteins and claudins (Baldwin et al., 2012). Another example is in Caco-2 cells, where butyrate decreases epithelial permeability and increases transepithelial resistance at low concentrations (Peng et al., 2007). On a protein level, butyrate promotes redistribution of tight junction proteins through activation of AMP-activated protein kinase (AMPK; Peng et al., 2009). Activity of AMPK is required for maintaining epithelial cell polarity, but only in a low energetic state, highlighting the importance of energy state in epithelial function (Mirouse et al., 2007). In freshly weaned piglets, low energy state adversely affects barrier function in the small intestine, whereas varying dietary inclusions of protein and lactose do not (Spreeuwenberg et al., 2001). How butyrate might affect energy status in epithelial cells is unclear.

The objective of this study was to determine how butyrate affects genes involved with energy mobilization in the rumen epithelium. Butyrate’s importance in the homeostasis of many processes can clearly be seen in previous studies. Whether genes involved in energy status dynamics are affected by butyrate, however, is unclear. We therefore hypothesized that butyrate would alter gene expression for the purpose of energy mobilization in the rumen epithelium to prevent energetic stress caused by SARA.
5.3 Materials and Methods

5.3.1 Animals, Treatment and Sampling

This study was carried out as described previously (Dionissopoulos et al., 2013). All animal procedures were approved by the Animal Care Committee at the University of Guelph under the guidelines of the Canadian Council for Animal Care (Ottawa, ON, CA). Sixteen fistulated cows on a mid-lactation total mixed ration (TMR) were fed a grain supplement that increased dietary non-fibre carbohydrate (NFC) content to 44.0% on a dry matter basis. Two days before the start of the study, only half the supplement was fed and on the day before the study, the full amount of supplement was fed. Thereafter, cows were divided into a control treatment and a butyrate treatment. Cows on the control treatment received a carrier only, while cows on the butyrate treatment received a carrier containing a butyrate dose (Proformix; Probiotech Inc., Saint Hyacinthe, QC) at the rate of 2.5% of Dry Matter Intake (DMI). Daily, DMI and milk production were measured. On days 1 and 7, blood was sampled and analyzed for serum β-hydroxybutyrate (BHBA) by the Animal Health Laboratory (Guelph, ON, Canada) using established protocols (Williamson et al., 1962). Also on the same day, rumen fluid from the ventral sac of the rumen was strained through 4 layers of cheesecloth and analyzed for SCFA profile by gas chromatography as described previously (Mutsvangwa et al., 2002). On days 6 and 7, rumen pH was continuously measured using an in-dwelling pH monitoring system (AlZahal et al., 2007). Rumen biopsies were done on day 7 by partial evacuation of the rumen and processed for microarray and quantitative real-time PCR (qRT-PCR).
5.3.2 Microarray and qRT-PCR

Rumen papillae samples were analyzed by microarray as previously described (Dionissopoulos et al., 2013). Samples from the control treatment were pooled and the butyrate samples were compared to the pooled control samples using student’s t-test (Xue et al., 2010). Significance was determined using a pre-screen of 95% confidence and a false discovery rate of 0.1. Through Ingenuity Pathway Analysis (Ingenuity Inc., Redwood City, CA, USA), networks of interconnected differentially expressed genes were created and a subset of differentially expressed genes were identified for confirmation by PCR. Primers were designed using Primer-BLAST (NCBI, Bethesda, MD, USA); most primers were intron-spanning (Table 5.1).

Primers were analyzed by Basic Local Alignment Search Tool (NCBI, Bethesda, MD, USA) to confirm specificity to the target gene and to confirm a low risk of non-specific binding. Primers were then further analyzed by OligoAnalyzer (Integrated DNA Technologies, Coralville, IA, USA) for primer-primer heterodimers, self-dimers and hairpins. After ordering the primers (Sigma-Aldrich, Oakville, ON, Canada), primer fidelity was empirically confirmed through dissociation curves. GAPDH was used as an internal control gene for quantification using previous methods (Pfaffl, 2001).
<table>
<thead>
<tr>
<th>Gene</th>
<th>Name</th>
<th>Accession No.</th>
<th>Primer sequence (F/P)</th>
<th>E (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HK2</td>
<td>Hexokinase 2</td>
<td>XM_002691189.2</td>
<td>F AGTGCAGAAGGGTTGACCAGT P CCAAGGCACACGGAAGTTGG</td>
<td>106</td>
</tr>
<tr>
<td>PKM2</td>
<td>Pyruvate kinase, muscle</td>
<td>BT030503.1</td>
<td>F GCCATGAAATGTCGGAAGGC P GATGTTGCGGGAAGAGGG</td>
<td>93</td>
</tr>
<tr>
<td>LDHA</td>
<td>Lactate dehydrogenase A</td>
<td>NM_174099</td>
<td>F GTGACGAGCTGCGAGCTAT P TAACCAGCCTGGAGTTTCT</td>
<td>101</td>
</tr>
<tr>
<td>LDHB</td>
<td>Lactate dehydrogenase B</td>
<td>NM_174100</td>
<td>F TCGTGAGCCCTTATCTACTC P CAGTCAGAGACTTCCCA</td>
<td>85</td>
</tr>
<tr>
<td>PFKFB4</td>
<td>6-phosphofructo-2-2,6-biphosphatase 4</td>
<td>NM_001192835</td>
<td>F ATGACCAACTGTCCAACGCT P GACCAACTGTCCAACGCT</td>
<td>99</td>
</tr>
<tr>
<td>ACAD9</td>
<td>Acyl-CoA dehydrogenase 9</td>
<td>NM_001078076</td>
<td>F GTCTGGGTCAACAAATGAGG P GACGCAACAAAGTCTCT</td>
<td>93</td>
</tr>
<tr>
<td>CYBASC3</td>
<td>Cytochrome B561, member A3</td>
<td>NM_001099149</td>
<td>F GTGTGGCGAGAGTCTGCAC P CTGACCAGGCCCTTTAT</td>
<td>83</td>
</tr>
<tr>
<td>ATP5G1</td>
<td>ATP synthase, F0 subunit</td>
<td>NM_176649</td>
<td>F CTATGCAAGGAAACCGTTC P AGTGTAGCACACTCCAC</td>
<td>88</td>
</tr>
<tr>
<td>ACLY</td>
<td>ATP citrate lyase</td>
<td>NM_001037457</td>
<td>F TGGGAGAGATAGGGGACAC P TGGAACCCTGGAGAGACAT</td>
<td>94</td>
</tr>
<tr>
<td>FASN</td>
<td>Fatty acid synthase</td>
<td>NM_001012669</td>
<td>F CTCACTGAGGTGTACCTCC</td>
<td>P TGCCTCAGGGCTTGTCTTGGT</td>
</tr>
<tr>
<td>PDK2</td>
<td>Pyruvate dehydrogenase kinase 2</td>
<td>NM_001159481</td>
<td>F CCCGAGTCCTAGAGTGGTC P GCCATACACGGCTGATCCA</td>
<td>93</td>
</tr>
<tr>
<td>SLC25A42</td>
<td>Mitochondrial CoA transporter</td>
<td>NM_001192032</td>
<td>F GACGTCCTCCTCAAGAGTG P GCACGTCTGCTCAAGAGTG</td>
<td>80</td>
</tr>
<tr>
<td>ATP1B1</td>
<td>Na/K ATPase</td>
<td>NM_001035334</td>
<td>F GCCCCACAGGATTAACACA P TGGGATCGTTAGGACGAAAGG</td>
<td>96</td>
</tr>
</tbody>
</table>
5.4 Results

5.4.1 Milk Production, DMI, SCFA, Blood, Rumen pH

Cows on the butyrate treatment had lower DMI than the control treatment (24.40 ± 0.77 Vs. 17.41 ± 0.77 kg/d; \(P < 0.01\)), but milk production was not significantly different between control treatment (41.93 ± 2.50 kg/d) and butyrate treatment (34.74 ± 2.50 kg/d; \(P = 0.06\); Dionissopoulos et al., 2013). Total SCFA concentrations were higher on day 1 in the butyrate treatment (92.76 ± 4.51 Vs. 78.87 ± 4.51 mM) but not on day 7 (87.59 ± 4.51 Vs. 81.82 ± 4.51 mM). Butyrate concentrations on both days 1 and 7 were higher in butyrate than in control (22.60 ± 0.94 Vs. 9.88 ± 0.94 mM and 21.60 ± 0.94 Vs. 8.60 ± 0.94 mM; Dionissopoulos et al., 2013). Serum BHBA was higher in the butyrate treatment than the control treatment on both days 1 (4201 ± 265 Vs. 910 ± 265 µM) and 7 (3262 ± 265 Vs. 800 ± 265 µM; Dionissopoulos et al., 2013). Rumen pH measurements confirmed the occurrence of acidosis in both butyrate and control treatments, as indicated by the time rumen pH was less than 5.6 (536 ± 89 and 598 ± 97 min/day, respectively; Dionissopoulos et al., 2013).

5.4.2 Microarray and qRT-PCR

Pathway analysis showed arrays of glycolysis, lipogenesis and oxidative phosphorylation genes as being differentially expressed. Of the 31 genes identified by pathway analysis, 13 genes in these 3 processes were confirmed by qRT-PCR (Table 5.2). In cows on the butyrate treatment, glycolytic genes such as hexokinase 2 (fold change: 2.07), pyruvate kinase 2 (FC:1.19), lactate dehydrogenase A (FC:1.45), lactate dehydrogenase B (FC:1.18) were upregulated while the phosphofructokinase inhibitor 6-phosphofructo-2-kinase/fructose-2,6-
Table 5.2 Microarray and quantitative RT-PCR analysis of genes differentially expressed by microarray.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Name</th>
<th>Role</th>
<th>Microarray fold change</th>
<th>PCR Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>HK2</td>
<td>Hexokinase 2</td>
<td>Glycolysis</td>
<td>1.51</td>
<td>2.07</td>
</tr>
<tr>
<td>PKM2</td>
<td>Pyruvate kinase, muscle</td>
<td>Glycolysis</td>
<td>1.34</td>
<td>1.19</td>
</tr>
<tr>
<td>LDHA</td>
<td>Lactate dehydrogenase A</td>
<td>Glycolysis</td>
<td>1.33</td>
<td>1.45</td>
</tr>
<tr>
<td>LDHB</td>
<td>Lactate dehydrogenase B</td>
<td>Glycolysis</td>
<td>1.23</td>
<td>1.18</td>
</tr>
<tr>
<td>PFKFB4</td>
<td>6-phosphofructo-2-kinase/fructose-Glycolysis</td>
<td></td>
<td>1.37</td>
<td>-2.22</td>
</tr>
<tr>
<td></td>
<td>2,6-biphosphatase 4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GPI</td>
<td>Glucose-6-phosphate isomerase</td>
<td>Glycolysis</td>
<td>1.18</td>
<td>N/A</td>
</tr>
<tr>
<td>PFKL</td>
<td>Phosphofructokinase, liver</td>
<td>Glycolysis</td>
<td>1.18</td>
<td>N/A</td>
</tr>
<tr>
<td>ACAD9</td>
<td>Acyl-CoA dehydrogenase 9</td>
<td>Oxidative phosphorylation</td>
<td>1.18</td>
<td>-1.30</td>
</tr>
<tr>
<td>CYBASC3</td>
<td>Cytochrome B561, member A3</td>
<td>Oxidative phosphorylation</td>
<td>1.18</td>
<td>1.11</td>
</tr>
<tr>
<td>ATP5G1</td>
<td>ATP synthase, F0 subunit</td>
<td>Oxidative phosphorylation</td>
<td>1.15</td>
<td>1.66</td>
</tr>
<tr>
<td>ACLY</td>
<td>ATP citrate lyase</td>
<td>Lipogenesis</td>
<td>-1.31</td>
<td>-2.00</td>
</tr>
<tr>
<td>FASN</td>
<td>Fatty acid synthase</td>
<td>Lipogenesis</td>
<td>1.27</td>
<td>1.48</td>
</tr>
<tr>
<td>PDK2</td>
<td>Pyruvate dehydrogenase kinase 2</td>
<td>Lipogenesis</td>
<td>-1.37</td>
<td>-2.38</td>
</tr>
<tr>
<td>SLC25A42</td>
<td>Mitochondrial CoA transporter</td>
<td>Lipogenesis</td>
<td>-1.39</td>
<td>-2.27</td>
</tr>
<tr>
<td>ACSS2</td>
<td>Acyl-CoA synthetase short chain</td>
<td>Lipogenesis</td>
<td>-1.47</td>
<td>N/A</td>
</tr>
<tr>
<td>SCD</td>
<td>Stearoyl-CoA desaturase (delta9)</td>
<td>Lipogenesis</td>
<td>1.35</td>
<td>N/A</td>
</tr>
<tr>
<td>SLC27A2</td>
<td>Long chain fatty acid transporter</td>
<td>Lipogenesis</td>
<td>-2.21</td>
<td>N/A</td>
</tr>
<tr>
<td>ATP1B1</td>
<td>Na/K ATPase</td>
<td>Metabolic activity</td>
<td>1.75</td>
<td>1.50</td>
</tr>
</tbody>
</table>
biphosphatase 4 (FC:-2.22) was down-regulated. Critical oxidative phosphorylation genes were upregulated, including acyl-CoA dehydrogenase 9 (FC:-1.30), cytochrome B561, member A3 (FC: 1.11) and ATP synthase F0 subunit (FC: 1.66).

Also, genes involved in lipogenesis were mostly down-regulated, including ATP citrate lyase (FC: -2.00), fatty acid synthase (FC: 1.48), pyruvate dehydrogenase kinase 2 (FC: -2.38) and mitochondrial CoA transporter (FC: -2.27). Also, sodium/potassium ATPase was upregulated (FC: 1.50) by butyrate treatment.

5.5 Discussion

5.5.1 Metabolic Stress and Barrier Integrity

Energy demands in epithelial tissues can be quite high and potentially very indicative of epithelial barrier integrity status. Infection by rotavirus of Caco-2 cells causes concurrent decreases in trans-epithelial resistance and epithelial ATP concentrations (Dickman et al., 2000). In rumen epithelia, low mucosal pH and local ATP depletion are directly linked to increased epithelial permeability (Aschenbach et al., 2000b). Thus, generating adequate ATP in the epithelium is vital for the maintenance of epithelial integrity. Increased sodium/potassium ATPase mRNA in the butyrate treatment highlights a genomic shift to increased ATP demand. In previous research (McBride and Kelly, 1990), increased sodium/potassium ATPase (ATP1B1) activity was directly associated with increased ATP demand in rumen epithelium. Together, these studies highlight the vital role of ATP in maintaining epithelial integrity.
Energy substrate availability and ATP generation capacity in epithelial tissues are vital for maintaining ATP levels. For example, impairment of glucose transport dynamics through the glucose transporter GLUT1, causes significant impairment of blood-brain barrier function by reducing the expression of occludin in brain microvessels (Abdul Muneer et al., 2011). In rat pulmonary epithelium, impaired mitochondrial complex I function leads to ATP-depletion and increased endothelial permeability in lung tissue (Bongard et al., 2013). In T84 cell lines, inhibition of ATP synthesis through 2,4- dinitrophenol, coupled with \textit{E. coli} exposure, significantly reduces barrier function (Lewis and McKay, 2009). Since the bovine rumen typically has high \textit{E. coli} content, decreased electron transport chain function would likely have an adverse effect on barrier function. In summary, energy substrates must be abundant and metabolizable to ATP for maintenance of barrier function. Impairing the barrier integrity appears to be a direct function of ATP depletion, highlighting the importance of ATP levels in maintaining barrier integrity.

5.5.2 Butyrate and Metabolic Stress

Stressed epithelia, such as those in ulcerative colitis and Crohn’s disease, are positively impacted by butyrate (Leonel and Alvarez-Leite, 2012; Ploger et al., 2012), through several possible cellular mechanisms, such as HDAC inhibition (Davie, 2003). A recent study, however, suggests butyrate acts as an energy substrate rather than an HDAC inhibitor (Donohoe et al., 2011). This may be due to butyrate’s extensive metabolism in the epithelium, where 75-90% of absorbed butyrate is metabolized (Bergman, 1990). When mouse embryonic stem cells are exposed to sodium butyrate, however, glycolysis and glucose consumption rates increase (Sharma et al., 2006). While butyrate oxidation certainly provides energy to epithelial cells...
(Bergman, 1990), this energy is made available through its oxidation via the Kreb’s Cycle, not through glycolysis. Although increased use of butyrate as an energy substrate cannot be ruled out by our results, the mRNA abundance increases in glycolytic genes in our study, show butyrate acts at the genome level.

Genomically, butyrate inhibits HDAC, resulting in lower de-acetylation of lysine residues of histone proteins. Normally, deacetylation of histone proteins is an important and quick-acting buffer of intracellular pH because the released acetate drives proton export through monocarboxylate co-transporters (McBrian et al., 2013). With inhibition of HDAC, this source of acetate is unavailable and intracellular pH can drop accordingly. Changes in intracellular pH have pleiotropic consequences, from changes in ion transport, to changes in cell proliferation, to apoptosis (Casey et al., 2010), highlighting the importance of HDAC activity.

In glioma cells, HDAC inhibition by butyrate enhances apoptosis, but only when glycolysis is also blocked (Egler et al., 2008). Glycolysis, therefore, appears to be a crucial compensatory mechanism for HDAC inhibition. In mice, butyrate decreased HDAC activity and caused a concurrent increase in systemic energy expenditure (Gao et al., 2009). With increased systemic energy expenditures comes an increased energy demand, which could be filled by ATP-generating processes like glycolysis. In our study, butyrate increased relative mRNA abundance of hexokinase 2, pyruvate kinase 2, lactate dehydrogenases A and B and decreased mRNA expression of 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4, a phosphofructokinase inhibitor. Together, this suggests butyrate increases mRNA abundance of glycolysis genes, which may lead to a shift towards greater glycolytic activity in the rumen epithelium.
Other than glycolysis, oxidative phosphorylation is another cellular ATP-generator and a far more effective one. Oxidative phosphorylation is increased by butyrate in energy-depleted germ-free mouse colon cells (Donohoe et al., 2011), further supporting the concept of butyrate increasing energy supply in epithelial cells. In our study, butyrate increased mRNA expression of acyl-CoA dehydrogenase 9, cytochrome B and ATP synthase, involved in complexes 1, 3 and 5 of the electron transport chain, respectively. The increased mRNA expression shows butyrate induces a genomic shift towards increased oxidative phosphorylation in rumen epithelium.

Coupled with decreased gene expression of lipogenesis genes such as ATP citrate lyase and mitochondrial CoA transporter, our study shows butyrate genomically shifts epithelial metabolism towards energy mobilization in rumen epithelia in SARA cows, in agreement with our hypothesis. Previous studies have highlighted the role of epithelial energy mobilization in preserving barrier integrity and our results show butyrate induces this mobilization, thus may be involved in maintenance of barrier integrity at the genome level during SARA.

5.6 Conclusion

In this study, we found exogenous butyrate modulates epithelial expression of genes involved in glycolysis, oxidative phosphorylation and lipogenesis. Genomically, glycolysis and oxidative phosphorylation appear to be upregulated while lipogenesis is largely down-regulated. Overall, the changes in gene expression found in this study indicate butyrate induces a genomic shift towards energy production. As energy production is vital for maintaining
proper barrier function, butyrate may be beneficial for barrier function in cows experiencing SARA.

While this study found genomic shifts, the corresponding protein abundance and activity was not studied. In the future, a comprehensive study evaluating mRNA abundance, protein abundance and protein activity would add greatly to our understanding of butyrate’s mode of action in the rumen. Further, as this study lasted for 7 days only, we could only focus on the short-term effects of butyrate on gene expression. While short-term effects are certainly useful in studying adaptation to high-grain diets, the long-term effects were outside of this study’s scope and could be an appropriate focus of future studies.
Chapter 6: General Discussion

6.1 Study Summary

The two objectives of this thesis were to investigate the short-term and long-term effects of dietary transitions on the rumen epithelium in terms of short chain fatty acid (SCFA) transport capacity and epithelial integrity, and to investigate the therapeutic potential of butyrate in improving SCFA transport capacity and maintaining epithelial integrity. The hypothesis was that the adaptation of the rumen epithelium to dietary transitions would be to increase SCFA absorption capacity on a cellular level in the short-term and morphological level in the long term. A further hypothesis was that butyrate would improve SCFA absorption capacity and improve barrier integrity in rumen epithelium.

In the first study, we showed that a 5-day feed restriction resulted in increased acetate and butyrate flux via passive diffusion, indicative of compromised barrier integrity. Furthermore, we observed a transporter-mediated cellular response to a 5-day feed restriction that increased butyrate flux through basolateral monocarboxylate cotransporter isoform 1 (MCT1). A cellular response in both feed restricted steers and steers subjected to a ruminal acidosis challenge showed elevated abundance of sodium/proton exchanger isoform 3 (NHE3), an apical H⁺ exporter.

In the second study, we showed that cows following parturition demonstrated no significant increase in SCFA absorption capacity on a cellular level. In the first week after parturition, carbonic anhydrase isoform 2 (CA2) was significantly down-regulated, showing evidence of intracellular acidification. Considerable evidence links intracellular acidification to a dissociation
of the cytoskeleton from tight junction proteins, leading to a breakdown in tight junctions and overall barrier integrity as part of the epithelial remodelling process, a principal component of morphological adaptation. The depression in CA2 abundance persisted from the first week post-parturition until 6 weeks post-parturition, suggesting that morphological adaptation begins in the first week after the parturition dietary transition.

In the third study, butyrate significantly increased SCFA transport capacity via increased MCT1 protein abundance and decrease sodium/bicarbonate cotransporter isoform 1 (NBC1) protein abundance. The depression in NBC1 indicates a functional partnership with MCT1 to maintain intracellular pH (pH$_i$) while increasing SCFA transport capacity. Furthermore, we found in a microarray analysis that butyrate increased the gene expression of key glycolysis and oxidative phosphorylation genes and decreased expression of key lipogenesis genes. Through the microarray analysis, we detected a genomic shift towards increased energy availability in the ruminal epithelium which is beneficial for increasing the available energy to maintain epithelial integrity during subacute ruminal acidosis (SARA). In this study, we saw the beneficial effects of butyrate on both SCFA transport capacity as well as gut health.

Together, these three studies provided insight into two major themes: the functional response of the rumen epithelium to dietary transitions, as well as the importance of pH$_i$ in modulating the cellular and morphological adaptations of the rumen epithelium. In both themes, butyrate plays a role in improving the adaptation of the rumen epithelium to enhance SCFA transport capacity and gut health during ruminal epithelium adaptation.
6.2 SCFA Transport Capacity

SCFA transport capacity is an integral response to dietary transitions because many dietary transitions involve a rapid increase in SCFA production in the rumen (Krause and Oetzel, 2006). Changes in SCFA production can disrupt the equilibrium that exists between SCFA production and removal from the rumen, in which the rumen epithelium plays a major part (Allen, 1997). The response of the rumen epithelium to the disruption of the production/removal equilibrium involves two distinct phases: cellular adaptation and morphological adaptation.

Through a cellular adaptation response, SCFA transport can be increased in the short-term. The primary method for this adaptation is through an increase in MCT1 abundance. In past studies, MCT1 presence was uncovered in the bovine rumen (Connor et al., 2010) and found to play an important role in SCFA uptake in the bovine caecum (Kirat and Kato, 2006). In study 1, increased protein-mediated butyrate flux was associated with increased MCT1 expression, highlighting the importance of MCT1 in cellular adaptation.

The morphological adaptation of the rumen epithelium also increases SCFA transport capacity. After an increase in diet fermentability, the absorptive surface area of the rumen epithelium increases (Dirksen et al., 1985). In study 2, we found that one of the early stages of that increase is decreased abundance of CA2 in rumen epithelial cells, which likely results in intracellular acidification (Purkerson and Schwartz, 2007). Intracellular acidification dissociates the cytoskeleton from tight junction proteins such as zonula occludens 1 and connexin 43 (Duffy et al., 2004), and this disruption of tight junctions is an important step in epithelial remodelling (Streuli, 1999). In study 2, differences in SCFA capacity were absent on the cellular level, as indicated by the lack of significant difference in MCT1 abundance. Since CA2 downregulation...
indicated a morphological adaptation, the increase in SCFA transport capacity in the rumen epithelium comes from an increase in absorptive surface area with a consistent SCFA transport capacity per cell. In other words, an increase in epithelial cell count with a consistent cellular SCFA transport capacity implies that the systemic SCFA transport capacity was increased through morphological adaptation.

The adaptation of the rumen epithelium to dietary changes involves two distinct phases. The short-term response is cellular in nature and involves MCT1 upregulation, while the long-term response is morphological in nature, beginning with intracellular acidification to drive remodelling. In study 3, we showed that butyrate can augment the cellular adaptation of the rumen epithelium. Whether butyrate augments the morphological adaptation is not clear from this thesis; however, given butyrate’s well-documented effects on epithelial remodelling (Baldwin et al., 2012) and cell proliferation (Sander et al., 1959), butyrate does have potential to benefit the long-term adaptive response of the rumen epithelium.

6.3 Intracellular pH as an Important Factor in SCFA Transport

In this thesis, the adaptive response of the rumen epithelium was demonstrated to be heavily integrated with pHᵢ regulation. The data accumulated in this thesis show proton removal from the epithelial cytosol to be an important factor in SCFA flux. In studies 1 and 3, we found both treatment-dependent and treatment-independent relationships of pHᵢ and SCFA transport. In study 2, pHᵢ regulation was demonstrated to be involved in the morphological adaptive response of the rumen epithelium.
The interdependence of pH\textsubscript{i} and SCFA flux were demonstrated most clearly in study 1. There, the diet-independent correlations of sodium/proton exchanger isoform 1 (NHE1), NHE3 and NBC1 to total acetate flux imply that SCFA flux is dependent on H\textsuperscript{+} removal from the cytosol, not just SCFA flux through SCFA transport mechanisms. This correlation was corroborated in study 3, where butyrate induced a decrease in NBC1 abundance as an apparent compensatory mechanism for the increase in H\textsuperscript{+} export through MCT1. Combining studies 1 and 3 provides strong evidence to investigate pH\textsubscript{i} regulation more strongly within the context of SCFA transport.

\section*{6.4 Future Research}

Future research in this area includes the fundamental investigation of the role of pH\textsubscript{i} in the cellular and morphological adaptive responses of the rumen epithelium to dietary changes. Each study in this thesis demonstrated the role of pH\textsubscript{i} in SCFA flux across the rumen epithelium. Future research should be directed at furthering our understanding of which pH\textsubscript{i}-modulating transporters are involved in the cellular and morphological adaptive responses of the rumen epithelium.

Further research should also be made concerning the increase of butyrate in the rumen. In study 3, the benefits of butyrate to both SCFA transport capacity and to epithelial barrier integrity were highlighted, and contribute to the larger dynamic of butyrate as a positive influence on gut health. Feeding strategies to increase butyrate production in the rumen and the timing of said strategies are a promising area of future research.
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