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Evaluation of factors associated with resistance to sub-acute ruminal acidosis

by

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DEDICATION

I dedicate this thesis to my fiancé, Alex, for being there for me, supporting me, and encouraging me throughout this degree.

ABSTRACT

Sub-acute ruminal acidosis is a digestive disorder experienced by animals fed a high grain diet, and causes substantial economic loss to the dairy industry. The objective of this research was to evaluate novel approaches to manage and prevent sub-acute ruminal acidosis. In Study 1, I found that precision processing barley (processing based on kernel size) does not improve dairy cow productivity. In Study 2, I showed that the extent of ruminal acidosis varies greatly among animals fed a common diet, and that acidosis resistant steers have lower volatile fatty acid (VFA) concentrations compared with susceptible steers, suggesting that resistant steers absorb more VFA, produce less VFA, or both. Further, expression of Na+/H+ exchanger-3, which imports sodium from the cell and exports hydrogen to the ruminal lumen, was higher for resistant steers. These findings suggest that Na+/H+ exchanger-3 is involved in the physiology of VFA absorption, and may play a key role in acidosis resistance.

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TABLE OF CONTENTS

EXAMINING COMMITTEE..... i

DEDICATION..... ii

ABSTRACT..... iii

ACKNOWLEDGEMENTS…...……v

LIST OF TABLES x

LIST OF FIGURES xi

LIST OF ABBREVIATIONS xii

1.0 LITERATURE REVIEW 1

1.1 Ruminal acidosis 1

1.1.1 Acute ruminal acidosis 1

1.1.2 Sub-acute ruminal acidosis 2

1.1.3 Acute phase response to acidosis 2

1.2 Regulation of rumen pH 4

1.2.1 Diet fermentability 4

1.2.1.1 Type of grain 5

1.2.1.2 Processing method of barley grain 7

1.2.2 Physically effective fiber 10

1.2.3 Absorption of acid from the rumen 13

1.2.4 Other factors involved in regulation of rumen pH 16

1.3 Transport and Metabolism of VFA in the Ruminal Epithelium 17

1.3.1 Simple Diffusion 17

1.3.1.1 Sodium/Proton Exchangers 17

1.3.1.2 Role of Na^+/K^+ ATPase in function of NHE 19

1.3.2 Bicarbonate exchangers in the rumen epithelium 19

1.3.3 Regulation of ketogenesis 21

1.3.3.1 3-Hydroxy-3-methyl-glutaryl coenzyme A synthase 22

1.3.4 Monocarboxylate transporters 23

1.5 Summary 24

1.6 Literature Cited 26

2.0 STUDY 1: PRECISION PROCESSING BARLEY GRAIN DID NOT AFFECT RUMEN pH OR PRODUCTIVITY OF LACTATING DAIRY COWS 42

2.1 Introduction 42

2.2 Materials and Methods 43

2.2.1 Diets, Animals, and Experimental Design 43

2.2.2 Ruminal Measurements 46

2.2.3 Nutrient digestibility measurement 47

2.2.4 Milk Collection and Analysis 48

2.2.5 Statistical Analysis 48

2.3 Results 48

2.3.1 HB vs. LB 48

2.3.1.1 Intake, Digestibility and Sorting Index 48

2.3.1.2 Rumen pH and Metabolites 49

2.3.1.3 Milk Yield and Composition 49

2.3.2 PP vs. CON 49

2.3.2.1 Intake, digestibility and sorting index 49

2.3.2.2 Rumen pH and Metabolites 49

2.3.2.3 Milk Yield and Composition 50

2.4 Discussion 50

2.5 Conclusion 54

2.6 Acknowledgements 54

2.7 Literature Cited 61

3.0 STUDY 2: THE RELATIONSHIP BETWEEN RUMINAL ACIDOSIS RESISTANCE AND EXPRESSION OF GENES INVOLVED IN REGULATION OF INTRACELLULAR pH AND BUTYRATE METABOLISM OF RUMINAL EPITHELIAL CELLS IN STEERS..... 65

3.1 Introduction 65

3.2 Materials and methods 66

3.2.1 Screening Study 66

3.2.2 Sample Collection and Analysis 67

3.2.2.1 Ruminal pH Measurement 67

3.2.2.2 Ruminal Fluid Collection 67

3.2.2.3 Blood Collection 68

3.2.2.4 Ruminal Papillae Collection 69

3.2.2.5 RNA Extraction 69

3.2.2.6 Reverse Transcription 69

3.2.2.7 Primer and Probe Design 69

3.2.2.8 Quantitative Real Time PCR 70

3.2.3 Statistical Analysis 70

3.3 Results and Discussion 71

3.3.1 Screening Study 71

3.3.2 Ruminal pH and VFA Profile 71

3.3.3 Gene Expression 74

3.3.3.1 Epithelial Intracellular pH Regulation 74

3.3.3.2 Epithelial Cell Metabolism 77

3.3.4 Plasma Metabolites and Hormones 78

3.3.5 Implications 78

3.4 Conclusion 79

3.5 Acknowledgements 79

3.6 Literature Cited 89

4.0 GENERAL DISCUSSION 93

4.1 Summary of research findings 93

4.2 Questions to be addressed in future studies 95

4.2.1 The role of VFA production and absorption on resistance to acidosis 95

4.2.2 NHE3: Cause or effect of ruminal acidosis 97

4.2.3 Is susceptibility to ruminal acidosis determined by animal or microbial factors? 98

4.2.4 Effects of acute phase proteins 99

4.3 Conclusions 103

4.4 Literature Cited 111

LIST OF TABLES

Table 2-1.Ingredients and chemical composition of experimental diets ……..55

Table 2-2. Particle size distribution and process index of rolled barley grains (Yang et al. 2012)……..56

Table 2-3. Effects of processing barley grain according to grain size on nutrient intake and digestibility.....57

Table 2-4. Effects of processing barley grain based on size on sorting index.....58

Table 2-5. Effects of processing barley grain based on size on rumen pH and metabolites 59

Table 2-6. Effects of processing barley grain based on size on milk yield, composition, body weight, and body condition score 60

Table 3-1. Ingredient and chemical composition of the diet.....81

Table 3-2. Primer and probe sequences and National Center for Biotechnology (NCBI) accession numbers for quantitative real time PCR analysis.....82

Table 3-3. DMI and pH measurements of acidosis susceptible (n = 3) and resistant (n = 3) steers in the screening study 82

Table 3-4. Comparison of BW, DMI, and pH measurements between acidosis resistant and susceptible steers.....83

Table 3-5. Comparison of ruminal VFA profile between acidosis resistant and susceptible steers 84

Table 3-6. Comparison of mRNA abundance of genes involved in intracellular pH regulation and VFA metabolism between acidosis resistant vs. susceptible steers. 85

Table 3-7. Comparison of plasma blood metabolite and hormone concentration between acidosis resistant vs. susceptible steers. 86

LIST OF FIGURES

Figure 3-1. Comparison of ruminal pH between acidosis resistant and acidosis susceptible steers after feeding. Group effect, $P < 0.05$; hour effect, $P < 0.01$; group × hour interaction, $P = 0.02$. . 87

Figure 3-2. Comparison of total rumen VFA concentration between acidosis resistant and acidosis susceptible animals after feeding. Group effect, $P = 0.005$; hour effect, $P < 0.001$; group × hour interaction, $P = 0.02$. 88

Figure 4-1. Comparison of molar butyrate proportion between acidosis resistant and susceptible steers after feeding. Group effect, $P < 0.01$, hour effect, $P < 0.05$, group × hour interaction, $P = 0.02$. 104

Figure 4-2. Relationship of PAT1 expression to VFA concentration in the rumen. Regression is PAT1 expression = -0.0081 × VFA concentration, mM + 1.8167 (n = 6, $P = .88$, $r^2 = -0.24$)..... 105

Figure 4-3. Relationship of DRA expression to VFA concentration in the rumen. Regression is DRA expression = -0.0215 × VFA concentration, mM + 5.0291 (n = 6, $P = 0.20$, $r^2 = 0.21$) 106

Figure 4-4. Relationship of NHE3 expression to molar butyrate proportion in the rumen. Regression is NHE3 expression = 0.0747 × butyrate proportion, % + 0.0148 (n = 6, $P = 0.02$, $r^2 = 0.74$) 107

Figure 4-5. Relationship of NHE3 expression to acidosis index. Regression was NHE3 expression = - 0.0351 × acidosis index, pH × min × kg^{-1} + 1.3873, (n = 6, $P < 0.01$, adjusted $r^2 = 0.82$)..... 108

Figure 4-6. Comparison of NHE3 expression between acidosis resistant and susceptible animals after feeding. Group effect $P < 0.005$, hour effect $P = 0.03$, group × hour interaction $P = 0.43$. 109

Figure 4-7. Comparison of plasma serum amyloid A concentration between AR and AS animals. Group effect $P = 0.18$, hour effect $P = 0.14$, group × hour interaction $P = 0.81$..... 110

LIST OF ABBREVIATIONS

ACTB	β-Actin
ADG	Average daily gain
AR	Acidosis resistant
AS	Acidosis susceptible
BCS	Body condition score
BW	Body weight
BHBA	β-hydroxy-butyric acid
CON	Control diet
CP	Crude protein
DRA	Down-regulated in adenoma
DM	Dry matter
DMI	Dry matter intake
FCM	Fat corrected milk
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HB	Heavy barley grain diet
HMGCS	3-hydroxy-3-methylglutaryl coenzyme A synthase
LB	Light barley grain diet
LRCpH	Lethbridge Research Centre Ruminal pH Measurement System
MCT	Monocarboxylate transporter
Na+/K+ ATPase	Sodium/Potassium ATPase alpha 1 polypeptide
NCBI	National Center for Biotechnology Information

NDF	Neutral detergent fiber
NEFA	Non-esterified fatty acids
NFFS	Non-forage fiber source
NHE	Sodium hydrogen exchanger
OM	Organic matter
PAT	Putative anion transporter
pH_i	Intracellular pH
PP	Precision processed diet
RPLP0	Ribosomal protein, large P0
SAA	Serum amyloid A
SARA	Sub-acute ruminal acidosis
SRS	Small ruminant pH measurement system
TLR	Toll-like receptor
VFA	Volatile fatty acid

1.0 LITERATURE REVIEW

1.1 Ruminal acidosis

Ruminal acidosis is a digestive disorder caused by production of acids in the rumen without sufficient removal (Allen, 1997). Dairy cattle have great energy demands due to their high milk production, which producers attempt to meet by feeding high grain diets. Unfortunately, these diets cause excess fermentation in the rumen which predisposes cattle to the disease. Researchers classify ruminal acidosis as acute or sub-acute, depending on the severity of the pH decline.

1.1.1 Acute ruminal acidosis

Acute acidosis is typically caused by a rapid increase in ruminal fermentation. This occurs when energy demands suddenly increase, such as after calving when dairy cows receive higher energy diets (Krause and Oetzel, 2006). Acute acidosis is associated with a rise in lactic acid, because lactate producing organisms are not sensitive to low pH while lactate users are. Ruminal lactic acid concentration rises as a result, further decreasing ruminal pH, causing acute ruminal acidosis (Owens et al., 1998).

Acute ruminal acidosis has major impacts on the industry, and is diagnosed by a dramatic drop in pH; thresholds used by researchers range between pH < 5.2 to pH < 5.0. It usually presents with obvious clinical signs, such as decreased or ceased feed intake, depressed milk fat, diarrhea, lethargy, and ultimately death if untreated (Kleen et al., 2003). It is also associated with liver abscesses (Nagaraja and Lechtenberg, 2007), laminitis (Nocek, 1997), and systemic acidosis. However, proper feed management practices, such as slowly

transitioning to high energy diets (Radiostits et al., 1994) are usually effective approaches to allow the ruminant to adapt to increased fermentation, and as a result, have greatly reduced the prevalence of acute acidosis in industry.

1.1.2 Sub-acute ruminal acidosis

Sub-acute ruminal acidosis (**SARA**) is caused by accumulation of volatile fatty acids (**VFA**) in the rumen without a simultaneous rise in lactate (Krause and Oetzel, 2006). Ruminants experiencing SARA do not always present with obvious clinical signs, making it difficult to detect (Slyter, 1976; Nocek, 1997; Nagaraja and Lechtenberg, 2007). Thresholds to diagnose SARA vary among researchers, ranging from ruminal pH below 5.8 but greater than 5.2 (Penner et al., 2006), to rumen pH below 5.5 but greater than 5.0 (Krause and Oetzel, 2006). Though incidences of SARA are more difficult to detect, symptoms are similar to acute acidosis, although they might not be as severe (Krause and Oetzel, 2006). Consequently, more cases of SARA occur unnoticed, and further, animal variability in susceptibility (Penner et al., 2009b; Brown et al., 2000) makes it difficult to formulate a common safe ration for a given physiological stage. As a result, SARA still has major impacts on both animal welfare and the economics of the dairy industry.

Due to the nature of the disorder, it is difficult to accurately measure the economic losses endured by the producer (Stone, 2003), but estimates range from about US$400 – US$475 per cow (Stone, 1999). Therefore, further research regarding methods to prevent and manage SARA is justified in order to improve feeding regimens for dairy cattle.

1.1.3 Acute phase response to acidosis

Both SARA and acute acidosis can be associated with an inflammatory response. Lipopolysaccharide (**LPS**) or endotoxin accumulates in the rumen when fermentation is increased, such as when ruminants receive a high grain diet (Khafipour et al., 2007a). Endotoxin is released when bacteria grow and divide, which increases when high starch diets are provided, or when they die, which increases during periods of low ruminal pH (Plaizier et al., 2012). Accumulation of endotoxin can result in local inflammation (i.e. rumenitis, Kleen et al. 2003) or compromise epithelial barriers, resulting in translocation of endotoxin into the circulation (Nocek, 1997). Both of these instances cause a rise of plasma acute phase proteins.

Lipopolysaccharide binding protein (**LBP**) is important to neutralization of trans-located endotoxin because it binds to LPS, beginning the transfer of LPS to lipoproteins, and thus, neutralizing them (Wright et al., 1990). The LPS-LBP complex also results in increased expression of cytokines, which drive the acute phase response (Plaizier et al., 2012). In cattle, serum amyloid A (**SAA**) and haptoglobin (**Hp**) are the most reactive to endotoxin challenge (Alsemgeest et al., 1994).

It is generally believed that laminitis and liver abscesses occur due to translocation of endotoxin during periods of acidosis (Nocek, 1997). This translocation may occur by paraketosis, or premature transition of cells into the keratinous layer which may occur due to sloughing of the epithelium (Steele et al., 2009). Acidosis is associated with increased ruminal osmolarity due to high concentrations of acid in the rumen. This can rupture the ruminal papillae due to

rapid entry of water into the rumen from blood circulation (Owens et al., 1998). However, recent evidence has indicated that in the case of SARA, translocation may not occur through the ruminal wall (Plaizier et al., 2012). When Khafipour et al. (2007a) induced SARA by a grain challenge, they found that it was associated with an increase in the acute phase proteins, SAA, Hp, and LBP, in plasma. However, inducing SARA by feeding insufficient physically effective fiber was not associated with an increase in plasma acute phase proteins, although in both cases rumen LPS increased (Khafipour et al., 2007b). The authors attributed the lack of an acute phase response when acidosis was induced using insufficient physically effective fiber to the fact that this type of acidosis does not likely increase starch fermentation in the large intestine. Therefore, it is possible that translocation of endotoxin actually occurs across the large intestinal epithelium, at least for SARA. This speculation was supported by greater LPS concentration in the feces of animals on the grain (Khafipour et al., 2007a) vs. the NDF challenge (Khafipour et al., 2007b).

1.2 Regulation of rumen pH

1.2.1 Diet fermentability

Much of previous research has focused on feeding regimens by evaluating impacts of grain source and processing method on rumen pH. The dairy industry in eastern North America primarily uses corn as a grain source, while western North America uses barley. The kernel uniformity and nutrient composition of barley is more variable than that of corn, making it more difficult for farmers to ensure they meet the dietary needs of their animals and avoid digestive disturbances (McAllister and Cheng, 1996). Further, the amount of physically

effective fiber has been shown to be an important factor in the diet to stimulate chewing behavior, promoting production of saliva to aid in buffering ruminal acid (Bailey and Balch, 1961).

1.2.1.1 Type of grain

In animals fed high grain diets, it is important to consider the effect of the grain source on ruminal fermentation. Corn starch is degraded more slowly in the rumen compared with that of oats, barley, and wheat, especially when the grain is not heat treated (Orskov, 1986). Further, barley grain has a lower starch concentration compared with that of corn, which results in decreased productivity unless grain is included as a greater portion of the ration (Yang et al., 1997). These two factors might put animals fed barley grain at a greater risk of digestive disturbances compared with that of corn.

The reason for different rates of starch digestion is due to the properties of the protein matrix surrounding the starch granules (McAllister and Cheng, 1996). The endosperm of barley is homogenous and loosely associated with the protein matrix, facilitating access by ruminal microbes to the endosperm (McAllister and Cheng, 1996). Additionally, the protein matrix of barley can be readily degraded by proteolytic bacteria in the rumen (Mcallister et al., 1990). In contrast, corn endosperm consists of two distinct regions known as the floury and horny endosperm (McAllister and Cheng, 1996). The composition of the floury endosperm is similar to that of barley, while the horny endosperm consists of starch granules more tightly bound to the protein matrix (Hoseney, 1986), making it difficult for rumen microbes to invade this region. Additionally, the protein matrix of corn is not as easily degraded in the rumen compared with that of barley

(McAllister et al., 1993), further contributing to slower ruminal starch degradation in corn. The horny to floury endosperm ratio influences digestibility of corn; a greater amount of horny endosperm reduced digestibility of starch and NDF (Lopes et al., 2009).

Previous researchers reported that feeding corn grain compared with that of barley does not affect mean ruminal pH in lactating cows (Depeters and Taylor, 1985; Khorasani et al., 1994; Casper et al., 1999; Khorasani et al., 2001). However, Overton et al. (1995) reported that ruminal pH decreases linearly as dietary inclusion of barley grain increases while Khorasani et al. (2001) found no effects on ruminal pH, but VFA concentration increased linearly as barley grain replaced corn grain in the diet . McCarthy et al. (1989) reported that there was no difference in ruminal pH between cows fed barley or corn, but ruminal VFA concentration was significantly higher in barley fed cows. Further, DMI and milk yield were higher for cows fed receiving corn treatments, but 4% fat corrected milk did not differ between grain treatments. Surber and Bowman (1998) found that ruminal pH was higher for steers fed a corn based diet compared with that of barley, which may be due to greater VFA concentration in the rumen of steers fed the barley based diet compared with that of corn.

Accumulation of acid in the rumen has negative effects on milk production and fat concentration (Bauman and Griinari, 2003). Khorasani et al. (1994) and Depeters and Taylor (1985) found that, although feeding corn vs. barley did not affect milk production, 4% fat corrected milk (**FCM**) yield was higher for cows fed diets with corn as the grain source compared with those fed

barley. Casper et al. (1999) reported a tendency for higher milk yield for cows fed corn compared with barley; additionally, 4% FCM was higher for cows fed corn, which was consistent with previous results from McCarthy et al. (1989). Overton et al. (1995) reported that milk yield increased linearly from cows fed barley vs. corn based diets, as did 3.5% FCM. Silveira et al. (2007) reported increased milk and 4% FCM yields for cows fed corn as opposed to barley, although efficiency for milk production was not different between corn and barley fed cows. In contrast, Khorasani et al. (2001) found no effect on milk production or 4% FCM yield between diets using corn or barley as the grain source. This suggests that the reason ruminal fermentation information varies across studies might be attributed to other dietary factors such as the inclusion level of grain in the diet, the source of grain, and the processing method of the grain, and thus the effect of the grain source is greatest when included at higher amounts in the diets or processed more extensively. Further, they may differ in the amount of physically effective fiber in the ration, which can counteract excessive fermentation in the rumen.

1.2.1.2 Processing method of barley grain

A purpose of mechanically processing grain is to damage the outer layer (hull and/or pericarp) of the grain, which is essential for the ruminal microbes to be able colonize the endosperm, particularly for barley (McAllister, 1990). Mastication is sufficient to damage these layers for corn grain (Beauchemin et al., 1994), although using mechanical processing of corn increases productivity. However, mastication alone is not sufficient to damage the hull and pericarp layers of barley grain (Beauchemin et al., 1994).

The extent of grain processing is important to the health of the ruminant, because smaller particle size increases the surface area of the endosperm available to rumen microbes; therefore, ultimately, fine particles increase the rate of fermentation in the rumen (Galyean et al., 1981). Beef animals are more affected by extensively processed grain as compared to dairy (Yang et al., 2000; Hironaka et al., 1992) which is explained by the higher levels of grain and low levels of forage in a typical beef ration compared to that of dairy rations.

The industry standard for barley weight is 48 lb/bu. Feed efficiency decreases below 43 lb/bu (Anderson et al., 2012). In order to avoid penalties on low quality grain, farmers often mix heavy and light barley to create a more marketable grain (Wang and McAllister, 2000). However, this creates difficulty with optimally processing grain using a roller mill. Wide roller settings optimally process heavier barley but result in undamaged small particles, limiting their digestibility, while narrow roller settings will optimally process light barley but shatter the heavy barley, which may increase fermentation in the rumen and result in digestive disturbances (McAllister et al., 2011). Previous research has shown that precision processing barley (processing grain based on size) improves nutrient digestibility for beef steers without affecting ruminal fermentation (McAllister et al., 2011), and therefore may ultimately improve feed efficiency.

Recently, the need for a quantitative method to evaluate extent of grain processing has come to light, because past research has defined extent of rolling using subjective terms such as “coarse”, “medium”, or “flat”. As a result, the settings for “coarse” in one study might be “medium” for another. To address this

issue, a processing index (**PI**) has been developed, which expresses the extent of grain processing as the volume weight of grain after it is processed as a percent of the volume weight of grain prior to processing (Yang et al., 2000). Higher PIs reflect less extensive processing, and subsequently, optimal PI differs between dairy and beef cattle. The optimal PI for beef cattle varies from approximately 70% (Beauchemin et al., 2001) to 85% (Hironaka et al., 1992), while optimal PI for dairy cows is approximately 65% (Yang et al., 2000). The variation seen in the literature for beef animals may be due to differences in processing techniques (e.g., dry-rolling vs. tempering or steam-rolling), grain source, or the level of grain inclusion in the experimental diet, which illustrates that defining an optimal PI alone may not be enough to determine the fermentability of grain in the ration. Further, PI doesn't allow for comparison of effects between processing methods, or take into account other factors such as grain quality (e.g. chemical composition, bushel weight; Dehghan-banadaky et al., 2007), and variation in kernel uniformity makes it extremely difficult to roll grain to a specific PI (McAllister et al., 2011). In fact, when barley grain varies severely in kernel uniformity, rolling grain to an optimal PI for a specific kernel size compromises overall nutrient digestibility in beef cattle (McAllister et al., 2011).

In addition to considering the method and extensity of processing, the type of grain is an important consideration when choosing a processing method. The association between the protein matrix and starch affects degradation in the rumen (McAllister et al., 1993), and how the grain succumbs to processing (Svihus et al., 2005). Offner et al. (2003) reported that ruminal break down of starch is increased

by grinding in corn to a greater extent than in barley, which may be explained by differences in the association of starch with the protein matrix. Starch granules are more likely to shatter when the protein matrix is more tightly associated with endosperm (Hoseney, 1986). Heat treating corn grain, particularly steam flaking, increases digestibility because the protein matrix is denatured, allowing greater access of hydrolytic enzymes to the starch (Corona et al., 2006); additionally, steaming decreases the likelihood that grains will shatter (Owens et al., 1997). Exposing grains to heat also results in gelatinization of starch granules which further increases ruminal fermentation (Waldo, 1973). Steam flaking corn grain has more benefits to ruminants compared with steam flaking barley grain because it helps to degrade the protein matrix, making the starch more accessible to the rumen microbes (Rowe et al., 1999).

However, dry rolling barley produces excessive fines and also makes it more difficult to control kernel thickness compared with steam rolling (Yang et al., 2000). Steam rolling moisturizes the pericarp of the grain, which reduces shattering and production of fines. Thus it is possible to roll to a finer thickness without shattering the grain for steam rolling compared with dry rolling.

Further research is necessary to develop a standard indicator of grain fermentability that considers the extent of grain processing, kernel uniformity, and grain quality (chemical composition and kernel size) to compare grains across different sources and processing methods.

1.2.2 Physically effective fiber

Neutral detergent fiber (**NDF**) is a measurement of the cell wall components (Schroeder, 1994), which is an important quality parameter for

ruminant diets. Neutral detergent fiber is associated with increased chewing activity (Welch and Smith, 1969), promoting secretion of saliva, which contains bicarbonate and phosphate that aid in buffering rumen contents (Bailey and Balch, 1961). Further, fiber increases gut fill, encouraging motility of the rumen, which might expose more VFA to the rumen wall, due to increased mixing of rumen contents, to increase absorption of VFA and raise pH (Allen, 1997).

The necessity of sufficient fiber in high grain diets to combat excessive acid accumulation causes difficulties in meeting the energy requirements of high producing ruminants, such as lactating dairy cows and finishing beef cows. However, failing to meet the fiber requirement predisposes ruminants to metabolic disturbances, such as acidosis, bloat, laminitis, and reduced milk fat (Kleen et al., 2003). Considering whether a ration supplies sufficient NDF alone is not enough to determine its effects on animal health. Consuming NDF from finely chopped vs. coarsely chopped forage also predisposes ruminants to digestive disturbances (Fahey and Berger, 1988), which may be due to a decrease in chewing time, subsequently decreasing the volume of saliva available to buffer fermentation acids in the rumen (Woodford and Murphy, 1988).

Beauchemin et al. (1994) reported that milk production increased when cows were fed coarsely chopped alfalfa hay (10 mm) vs. finely chopped alfalfa hay (5 mm; 26.8 vs. 25.9 kg/d) in low forage diets, but decreased when coarsely chopped alfalfa vs. finely chopped alfalfa was provided (22.3 vs. 23.9 kg/d) in high forage diets. Rumination time increased by approximately 30 min/d for cows fed diets high in coarsely chopped forage. Increased rumination promotes

salivation, which contains phosphate and bicarbonate buffers that counteract excessive fermentation in the rumen (Bailey and Balch, 1961), and can increase milk production because decreased ruminal pH, which occurs during incidences of SARA, reduces DMI and ultimately, milk production (Kleen et al., 2003).

Krause et al. (2002a) showed that cows fed a high grain diet containing coarsely chopped alfalfa silage spent an average 165.6 min/d longer ruminating, and 208.5 min/d longer chewing compared with finely chopped alfalfa silage. Further, the cows fed coarsely vs. finely chopped silage had higher mean ruminal pH values (6.03 vs. 5.81, respectively) and lower ruminal VFA concentrations (146.7 vs. 156.3 mM, respectively). These data indicate that feeding coarsely chopped forage improves health of the cow by reducing the risk of developing SARA. However, milk production and composition was not affected by silage chop length (Krause et al., 2002b).

Lammers et al. (1996) developed a method to evaluate forage particle distribution, which is important to determine the amount of physically effective NDF in diets. Mertens (1997) suggested using physically effective NDF as a measurement to evaluate the capacity of a diet to supply adequate forage to a ruminant, taking into consideration both chemical and physical aspects of the diet and its influence on rumination and chewing. Methods for determining physically effective fiber vary in the literature (Lammers et al., 1996; Mertens, 1997; Kononoff et al., 2003; Einarson et al., 2004), and these methods were evaluated by Yang and Beauchemin (2006) to determine the most appropriate approach to evaluate physically effective NDF. They concluded that the sum of the proportion

of the ration on the 19 and 8 mm sieves (Lammers et al., 1996) and multiplying it by the concentration of NDF was the most appropriate approach to calculate physically effective NDF in rations.

Swain and Armentano (1994) investigated the effects of feeding a low forage diet supplemented with non-forage fiber sources (**NFFS)** vs. a high forage diet with similar NDF contents. While NFFS raised milk fat yield by approximately 22% compared with the low forage diet, the high forage diet raised milk fat yield by approximately 36%. Milk production was not different between NFFS and the low forage diet, although it tended to be higher for cows fed high vs. low forage (29.3 vs. 27.4 kg/d). Mooney and Allen (1997) reported that NDF from whole lint cotton seed was more physically effective (increased chewing time) than short cut alfalfa silage but less than long cut alfalfa silage. Zhang et al. (2010) reported that DMI and milk yield increased by about 14 and 10%, respectively, when dried distillers grains with solubles (**DDGS**) partially replaced forage in the diet although chewing time was decreased. These studies show that NFFS are less effective at stimulating chewing, but that whole lint cottonseed and DDGS can be effectively used to partially replace forage in the ration in order to increase DMI and subsequently, productivity.

1.2.3 Absorption of acid from the rumen

Lactic acid and VFA are the primary end products of starch fermentation in the rumen. Some ruminal microbes are capable of utilizing lactic acid, keeping the concentration relatively low in the rumen under normal conditions (Krause and Oetzel, 2006). Volatile fatty acids are used as an energy source by the animal and must be removed from the rumen for use by the animal in metabolic

processes (Bergman, 1990). Absorption, neutralization by salivary buffers, and passage through the digestive tract remove 96% of acid from the rumen, with absorption accounting for the most removal, at approximately 53% (Allen, 1997). Therefore, a thorough understanding of how VFA are absorbed will provide insights on physiological factors that regulate ruminal pH.

Finishing beef and lactating dairy cattle have high energy demands, and as such are fed highly fermentable diets, which is the most efficient way to provide energy, but puts the animal at risk for metabolic disorders. Allowing proper adaptation time for highly fermentable diets greatly reduces the risk because it allows for epithelial cell proliferation (Sakata and Tamate, 1978) as well as up-regulation of certain genes that code for transport proteins in the ruminal epithelia (Leonhard-Marek et al., 2010; Kiela et al., 2007), which will be discussed later in this review.

Dirksen et al. (1985) reported that ruminal papillae reach maximal surface area 6 to 8 weeks after being fed a highly fermentable diet. Bannink et al. (2008) showed that cows fed high concentrate diets after parturition had a greater rate of epithelial cell proliferation compared with cows that were gradually adapted to a high energy diet. Increased ruminal fermentation results in greater VFA production; therefore, these results indicate that VFA stimulate proliferation of epithelial cells. Greater surface area might provide the VFA more opportunity to diffuse out of the rumen.

Volatile fatty acids have a stimulatory effect on proliferation of the cells in the colon, which is exposed to a similar environment as the rumen, with butyrate

having the largest effects (Blottiere et al., 2003). Similarly, infusion of butyrate has been shown to stimulate epithelial cell proliferation in the rumen (Sakata and Tamate, 1978). Sutton et al. (2003) reported that VFA production in the rumen is different between cows fed a normal vs. a low fiber diet. However, the forage to concentrate ratio does not appear to affect papillae surface area, because maximum papillae surface area is reached pre-partum when a relatively low energy diet is provided (Penner et al., 2011). It is possible that animals that are resistant to acidosis have a greater capacity for absorption of VFA due to a larger surface area of ruminal papillae. However, papillae surface area alone probably does not explain the differences in individual susceptibility to acidosis, as research has shown that activity of membrane transporters increases with increasing VFA concentration without a change in papillae surface area (Penner et al., 2011). For example, absorption of electrolytes (Gabel et al., 1993) and 3-O-methyl-alpha-D-glucose (Gabel and Aschenbach, 2002) was reduced after 48 h of feed withdrawal without significant changes in epithelial cell surface area.

Additionally, metabolism of VFA by the ruminal epithelium also contributes to absorption by reducing the amount of VFA in the epithelium and subsequently increasing the concentration gradient for more rapid diffusion of VFA into ruminal epithelial cells (Penner et al., 2011).

Past research has shown that ruminants have a great range of variation in susceptibility to acidosis. For example, Brown et al. (2000) showed that steers vary greatly in their response from a carbohydrate challenge following a period of fasting; ranging from no response in some animals to severe acidosis resulting in

death in others. This extreme variation between individuals might explain why SARA is so difficult to control in industry.

Penner et al. (2009b) investigated the nature of this phenomenon by administering a glucose drench to induce SARA in sheep. Similar to the Brown et al. (2000) study, some of the sheep simply did not respond to the acidosis challenge. The ruminal epithelium of the non-responsive sheep had greater capacity for bicarbonate independent uptake of butyrate, which is indicative of passive diffusion into the epithelium. Further, plasma beta-hydroxy butyric acid (**BHBA**) was higher in the non-responsive sheep, which they attributed to an increased rate of ketogenesis from butyrate in the ruminal epithelium (Sehested et al., 1999). As well, non-responsive sheep tended to have greater capacity of bicarbonate dependent uptake of acetate, which suggests expression or activity of a bicarbonate/acetate exchanger may be greater for acidosis resistant sheep. This study suggests that differences in VFA absorption may be the reason for variation in acidosis susceptibility.

1.2.4 Other factors involved in regulation of ruminal pH

Although absorption of VFA through the rumen accounts for the majority of acid removal, other factors involved with regulating rumen pH cannot be discounted. The passage of VFA and protons into the omasum and further parts of the digestive tract accounts for approximately 7% of acid removal from the rumen (Allen, 1997).

Aschenbach et al. (2009) showed that acetate uptake can occur by bicarbonate/acetate exchange, as well as a bicarbonate independent nitrate sensitive mechanism that has not yet been further characterized. Acetate is the

biggest component of the VFA profile in the rumen, and thus it is likely that acetate/bicarbonate exchange accounts for a considerable source of bicarbonate rumen, but more research is needed to be sure.

Other factors that contribute to ruminal pH regulation include ammonia, which can act as a minor buffer, rumen osmolarity (Allen, 1997), and blood flow (Dobson, 1984).

1.3 Transport and Metabolism of VFA in the Ruminal Epithelium

Absorption of VFA through the rumen is dependent on uptake of VFA into epithelial cells and subsequent transport into the blood. Normal ruminal pH is 6.0 – 6.2, which means that the majority of VFA are in the dissociated form. Volatile fatty acids associated with a proton are permeable to the cell membrane and can thus pass via simple diffusion, while dissociated VFA can only enter the epithelial cells via facilitative diffusion. Further, as intracellular pH is 7.4, VFA entering cells via simple diffusion will be likely dissociated immediately in the cell. In order to combat falling intracellular pH, a number of transport proteins are present in the ruminal epithelium, which will be discussed in detail in this section.

1.3.1 Simple Diffusion

1.3.1.1 Sodium/Proton Exchangers

A total of nine NHE isoforms have been found in mammalian tissue (Zachos et al., 2005), with four known to be expressed by ruminal epithelium (Graham et al., 2007). Sodium absorption in the colon of rats is stimulated by presence of VFA (Krishnan et al., 1999; Kiela et al., 2001), which might be attributed to increased expression or activity of sodium proton exchangers (**NHE**). This may apply to ruminal tissue as well due to a similar environment as the large intestine. Gabel et al. (1991) proposed the presence of an apical NHE in the

ruminal epithelium, which was confirmed by Muller et al. (2000) in an in vitro study using cultured ruminal epithelial cells. They reported that treating the cultured cells with butyrate in the absence of bicarbonate decreased intracellular pH, but cells recovered in 10 min. Treating the cells with butyrate and NHE inhibitors EIPA and HOE-694 blocked recovery of cells from depressed pH by 62 and 69%, respectively.

Etschmann et al. (2006) investigated the role of NHE1, NHE2, and NHE3 on the recovery of intracellular pH of cultured rumen epithelial cells, and found that NHE1 and NHE3 are responsible for removing a significant amount of protons from epithelial cells in the absence of bicarbonate, while NHE2 removes small amounts. Graham et al. (2007) investigated the presence of NHE1, NHE2, NHE3, NHE4, and NHE8 mRNA and the role of NHE1 and NHE2 in rumen epithelium. All but NHE4 were expressed by ruminal epithelial cells. NHE1 was primarily found on the lumen facing membrane of the stratum granulosum, but was also present in the stratum spinosum and basale, but not the stratum corneum. NHE2 is located laterally in the stratum basale, spinosum, and granulosum.

To the author's knowledge, the location of NHE3 has not yet been characterized in ruminal epithelium, but is expressed on the membrane of intestinal epithelial cells in rat (Bookstein et al., 1994). Graham et al. (2007) suggested that apically located NHE proteins promote formation of undissociated VFA, which would allow faster diffusion of VFA into the epithelium.

To the author's knowledge, no studies have evaluated the other NHE isoforms in ruminants, with the exception of NHE8 (Graham et al., 2007). Of

these, NHE5 is present only in brain and sperm (Zachos et al., 2005) and therefore would not be expected to be present in the rumen, NHE6 is involved with recycling endosomes, and NHE7 is found in the trans-Golgi network (Zachos et al., 2005). Finally, NHE8 and NHE9 are related to NHE7, but their cellular localization has yet to be determined (Zachos et al., 2005). Although the research regarding NHE2 suggests that laterally located NHE may be of little importance in maintaining intracellular pH of rumen epithelial cells or VFA absorption (Etschmann et al., 2006), further research is needed.

1.3.1.2 Role of Na^+/K^+ ATPase in function of NHE

Albrecht et al. (2008) identified that the Na^+/K^+ ATPase pump is expressed highest in the basal plasma membrane of the stratum basale, although it is also present at lesser intensity in the stratum granulosum and spinosum. Inhibition of the pump using ouabain resulted in decreased activity of the NHE proteins, indicating that the Na^+/K^+ ATPase pump is important to maintain the function of NHE by removing sodium from the cell to maintain a concentration gradient.

This might indirectly affect VFA absorption, because maintaining activity of NHE would allow protons to be secreted into the rumen, promoting formation of undissociated VFA (Graham et al., 2007), which could then diffuse into the epithelium via simple diffusion. The VFA would then dissociate in the epithelial cell, requiring further removal of protons.

1.3.2 Bicarbonate exchangers in the rumen epithelium

In the rumen, bicarbonate exchangers export bicarbonate from the epithelium to the rumen in exchange for dissociated VFA (Connor et al., 2010).

Blocking NHE using amiloride or ouabain reduces VFA absorption in the colon of guinea pigs, which may be due to an interaction between the NHE and VFA/bicarbonate antiporters in the epithelium (von Engelhardt et al., 1993). Reducing the activity of NHE would cause protons to accumulate in the cell, increasing the need for bicarbonate and subsequently, reducing the availability of bicarbonate to be exported. Previous research has shown that VFA absorption is associated with appearance of bicarbonate in the rumen (Gabel et al., 1991).

Kramer et al. (1996) found that increasing chloride concentration reduced VFA absorption in sheep, which might be due to competitive inhibition as both chloride and dissociated VFA are transported via a non-specific bicarbonate exchanger. However, even if there are 2 separate bicarbonate exchangers for chloride and VFA, infusion of chloride to the rumen would decrease VFA uptake by the epithelium because more bicarbonate would be needed for chloride transfer. Bilk et al. (2005) reported that two anion exchangers found in intestines, down-regulated in adenoma (**DRA**) and putative anion exchanger, isoform 1 (**PAT1**) also exist in rumen epithelium. Further, Leonhard-Marek et al. (2010) reported that DRA was expressed only in epithelial tissues exposed to VFA, while PAT1 was expressed regardless of presence of VFA. These results are consistent with that of Oba et al. (2012), who reported that DRA expression increases with calf age, while PAT1 was not affected. This indicates that DRA might be responsible for VFA/bicarbonate exchange in the rumen, and PAT1, for chloride/bicarbonate exchange.

To the author's knowledge, no studies have characterized the location of DRA or PAT1 in the rumen epithelium; however, the studies conducted suggest an apical location in the stratum granulosum (Bilk et al., 2005). Previous research has found that DRA and PAT1 are located on the apical cell membrane in the intestines (Rajendran et al., 2000; Wang et al., 2001), which might apply to the rumen as well.

1.3.3 Regulation of ketogenesis

VFA production in the rumen is not equivalent to apparent VFA absorption, measured as appearance of VFA in portal blood, indicating that VFA are metabolized by ruminal epithelial cells. Approximately 45% of acetate, 65% of propionate and 85% of butyrate are metabolized by the rumen (Bergman, 1990). Propionate is also metabolized by the liver into glucose via gluconeogenesis (Danfaer et al., 1995) while acetate is used for lipogenesis and is found in the blood at higher concentrations compared with the other VFA (Kristensen et al., 1998). In contrast, butyrate is extensively metabolized by the ruminal epithelium to form BHBA via the ketogenesis pathway (Sehested et al., 1999). As such, the portal appearance of VFA is not a good indicator of VFA absorption (Kristensen et al., 1998).

Onset of ketogenesis in young lambs is not regulated by ruminal VFA concentration, but rather, seems to be regulated by age of the animal (Lane et al., 2002). Lane et al. (2000) reported that BHBA production in milk-fed lambs was proportional to expression of 3-hydroxy-3-methyl-glutaryl coenzyme A synthase (**HMGCS2**) activity, but not to that of aceteoacetyl CoA thiolase (**ACAT**), the first enzyme in the ketogenic pathway, which indicates that HMGCS is the rate

limiting enzyme for ruminal ketogenesis. These results are in agreement with those of Dashti and Ontko (1979), who showed that HMGCS2 is also the rate limiting enzyme for hepatic ketogenesis.

1.3.3.1 3-Hydroxy-3-methyl-glutaryl coenzyme A synthase

The HMGCS enzyme is located in two compartments of the cell: the cytoplasm and the mitochondria. Regardless, HMGCS catalyzes the reaction that condenses acetoacetyl CoA and acetyl CoA to form hydroxy-methyl glutaryl coenzyme A (**HMGC**). In the cytosol, HMGC goes on to form mevalonate, and ultimately cholesterol, while in the mitochondria, it goes on to form acetoacetate (Hegardt, 1999). Gil et al. (1986) sequenced cytosolic HMGCS, while Gil-Gomez et al. (1993) sequenced mitochondrial HMGCS, and found that these enzymes are encoded by two separate genes. This review will focus on mitochondrial HMGCS (**HMGCS2**), as it is involved with ketogenesis.

In hepatocytes, long term regulation of HMGCS2 is accomplished at the transcriptional level (Hegardt, 1999). Expression of HMGCS2 is up-regulated in rats by starvation or feeding high fat diets (Serra et al., 1993). It is unlikely that these factors would affect the expression of HMGCS2 in ruminal epithelial cells although expression of HMGCS2 in the liver of ruminants may be regulated similarly. The rumen absorbs high amounts of butyrate which is converted to BHBA (Sehested et al., 1999). Thus, it is possible that butyrate uptake by the ruminal epithelium stimulates expression of HMGCS2. However, Lane et al. (2000) reported that BHBA production increased regardless of whether lambs were fed milk or solid feed, which indicates that age rather than VFA concentration influences expression of HMGCS2 in the rumen.

1.3.4 Monocarboxylate transporters

Fourteen isoforms of monocarboxylate transporters (**MCT**) have been identified (Halestrap and Meredith, 2004) and of them, only MCT isoform 1 (**MCT1**) through MCT isoform 4 (**MCT4**) have been shown to transport protons (Halestrap and Price, 1999). MCT1, MCT2, and MCT4 are present throughout the GI tract (Halestrap and Price, 1999; Halestrap and Meredith, 2004; Kirat et al., 2007; Connor et al., 2010) while MCT3 is only present in the retina (Halestrap and Price, 1999). Compared with other isoforms, MCT1 plays the biggest role in transport of monocarboxylates such as ketones, lactate, or VFA in the GI tract. It is important to the function of the ruminal epithelium because of its involvement with removal of dissociated VFA and VFA metabolites from the cells (Connor et al., 2010).

MCT1 is localized to the basolateral side of the stratum basale in the ruminal epithelium of sheep (Muller et al., 2002), which was confirmed by Graham et al. (2007) in cattle. The location of MCT1 indicates that its function is to transport monocarboxylates into the blood but does not play a role in uptake of these compounds by the epithelium. Muller et al. (2002) used cultured ruminal epithelial cells to demonstrate that inhibiting the function of MCT1 of epithelial cells preloaded with ketone bodies and lactate depresses intracellular pH, and in the absence of these inhibitors, intracellular pH was able to recover, indicating its importance to maintenance of cellular pH by excreting these compounds into the blood. Further, Kirat and Kato (2006) found that MCT1 is important to VFA transport from the cecum of cattle. Because the cecum and rumen are similar

environments, it is likely that MCT1 also plays a role in exporting VFA from the rumen epithelium, although further study in vivo is necessary.

Koho et al. (2005) evaluated protein expression of MCT1, MCT2, and MCT4 in the rumen of reindeer and found that while MCT1 and MCT4 are expressed in ruminal tissue, MCT2 was only expressed in small intestine and liver. Contrary to these findings, Graham et al. (2007) was able to detect the presence of MCT1 and MCT2 mRNA, while MCT4 mRNA was not detected in bovine rumen. Further, the MCT2 protein was detected in the rumen although only weak staining throughout all layers of the rumen epithelium except the stratum corneum was detected. It is important to note that the NCBI Primer Blast Program showed that the reverse primer for MCT4 used in the study of Graham et al. (2007) had an incorrect nucleotide in the sequence which might explain why its mRNA was not detected. Discrepancies between these studies may also be due to the species used as a ruminant model.

Kirat et al. (2007) evaluated MCT4 in the gastrointestinal tract of cows and reported that it is located on the apical side of the plasma membrane in the stratum corneum and stratum granulosum. They suggested that MCT4 plays a role in uptake of monocarboxylates (mainly VFA, and to a lesser extent, lactate) by the ruminal epithelium. Following uptake, VFA may be exported into the blood by MCT1 located on the basolateral membrane. Similarly, as propionate is metabolized to lactate by the ruminal epithelium (Bergman et al., 1999), MCT1 might also play a role in excretion of lactate from the epithelium into the blood.

1.5 Summary

Although producers have been trying to control SARA by measures such as allowing proper time for adaptation to a high energy diet and providing adequate fiber in the ration, SARA is still a prevailing problem in industry (Stone, 2003). This may be due to the fact that rations are formulated for an average animal; however, due to the huge variation in individual susceptibility to acidosis, a ration for an average animal is not appropriate for all animals.

Grain processing techniques might contribute to incidences of SARA in industry. Kernel uniformity varies greatly, particularly for barley grain, which poses a problem for processing using roller mills. Wide roller settings allow smaller kernels to pass through undamaged, limiting their digestibility; however, narrow roller settings cause shattering of larger kernels, which may contribute to digestive disturbances (McAllister et al., 2011). As such, it is important to consider improving feed processing techniques as well. Nutrient digestibility was improved for beef cattle when barley grain was precision processed (McAllister et al., 2011), but it is not known how dairy cattle respond to precision processing.

Based on the results of Penner et al. (2009), ruminants that are resistant to SARA may have greater passive diffusion of butyrate into the ruminal epithelial cells and greater expression or activity of HMGCS2 due to greater plasma BHBA concentration in AR sheep. In addition, DRA expression or activity may be higher due to the tendency for greater bicarbonate dependent uptake of acetate by AR sheep. Further, greater absorption of butyrate into the epithelium may up-regulate the NHE proteins to decrease the proton load in the rumen. It is possible that increased production of ketone bodies by the rumen may up-regulate MCT1, as it

plays a role in exporting ketones into the blood. Similarly, an increased uptake of VFA might also up-regulate MCT1 due to its role in exporting VFA into the blood. As such, further investigation of these genes may shed light on the nature of variation in susceptibility to SARA.

The objective of this thesis work is to investigate techniques to improve productivity of ruminants by evaluating methods to better manage and prevent acidosis. We hypothesized that precision processing barley grain would improve productivity of dairy cows. We also hypothesized that expression of genes involved with intracellular pH recovery and VFA metabolism in ruminal epithelial cells is different between acidosis resistant vs. susceptible animals.

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2.0 STUDY 1: PRECISION PROCESSING BARLEY GRAIN DID NOT AFFECT RUMEN pH OR PRODUCTIVITY OF LACTATING DAIRY COWS

2.1 Introduction

Grain processing is necessary to make nutrients accessible to the microbial population in the rumen (Wang and McAllister, 2000). Barley grain fed whole to cattle is often swallowed without damage to the pericarp, limiting its digestibility (Beauchemin et al., 1994). Mathison (1996) reported that feeding whole vs. dry rolled barley decreases DM digestibility by approximately 16% and starch digestibility by about 37% for beef cattle. Feeding whole barley reduces digestibility of starch by about half for animals fed high forage diets (Campling, 1991).

However, the physical characteristics of barley grain can vary greatly making it difficult to optimally process in a manner that ensures uniform fermentability. Current industry practices usually utilize a narrow setting on a roller mill to ensure all grain particles are damaged. However, this shatters the larger particles, producing excessive fines. This increases surface area of the grain available for microbial attachment, increasing ruminal fermentation which can contribute to digestive disturbances, such as ruminal acidosis (Owens et al., 1997). Wang et al. (2003) reported that steers fed extensively processed barley gained 27% less weight compared with steers fed less extensively processed barley which may be attributed to the 18% lower DMI for steers fed extensively

processed barley grain. In contrast, a wide roller setting allows smaller grain kernels to pass through undamaged, limiting their digestibility and ultimately decreasing productivity of animals (McAllister et al., 2011).

Yang et al. (2000) determined that optimum processing index (**PI**, volume weight of barley grain after processing expressed as a percentage of volume weight of barley before processing) for dairy cows is approximately 64%. However, the grain used in their study was from one source and below industry standard. Commercially, grain producers mix light and heavy barley grain to make the light grain more marketable, which results in a large variation in particle size (Wang and McAllister, 2000). This may create difficulties with processing all grain kernels optimally and decrease productivity of animals. In fact, previous research has shown that precision processing (processing grain based on kernel size) improved the nutrient digestibility without affecting rumen pH in beef steers (McAllister et al., 2011). However, the effects of precision processing on productivity of lactating dairy cows have not been evaluated.

Therefore, the objective of this study was to investigate whether precision processing grain with a large range of bushel weight would improve productivity of lactating dairy cows.

2.2 Materials and Methods

2.2.1 Diets, Animals, and Experimental Design

This study was conducted at the University of Alberta Dairy Research and Technology Centre (Edmonton, AB Canada) from September to November, 2010. All procedures were approved by the Faculty Animal Policy and Welfare

Committee at the University of Alberta and animals were cared for in accordance with the guidelines of the Canadian Council of Animal Care (Ottawa, Ontario, Canada).

The four treatments assigned were light barley grain (52.8 kg/hL) dry rolled at a narrow roller setting (**LB**), heavy barley grain (68.6 kg/hL) dry rolled at a wide roller setting (**HB)**, a mixture of LB and HB in equal parts (precision processed, **PP**), and equal parts heavy and light barley mixed followed by dry rolling at a single narrow roller setting (industry standard, **CON**). Experimental diets consisted of 40% barley grain, 40% barley silage, and 20% of a supplement premix (Table 2-1).

Compared with HB grain, LB grain had a greater proportion of whole kernels (7.8 vs. 5.4%, respectively; $P = 0.01$), a greater proportion of kernels on the 1.18-mm screen (62.7 vs. 46.8%, respectively; $P = 0.01$) and greater processing index (**PI**, volume weight of grain after processing expressed as a percentage of volume weight of grain before processing; 80.2 vs. 77.1%, respectively; $P = 0.05$), while HB grain had a greater proportion of kernels on the 3.35-mm and 2.36-mm screens (9.9 vs. 1.8%, $P = 0.01$ and 31.6 vs. 24.9%, $P = 0.01$, respectively) but no difference in the proportion of fines ($P > 0.05$, Yang et al. 2012, Table 2-2). Geometric mean was higher for HB grain size compared with that of LB (2.74 vs. 2.42-mm, respectively; $P = 0.01$; Yang et al. 2012). Compared with PP grain, CON grain had a greater proportion of whole kernels (9.9 vs. 7.1%, respectively; $P = 0.02$), a greater proportion of kernels retained on the 3.35-mm and 2.36-mm sieves (9.7 vs. 7.2%, $P = 0.01$ and 37.4 vs. 34.6, $P =$

0.05; respectively), and more fines (13.8 vs. 9.1%, respectively; $P = 0.02$), while PP had greater more kernels retained on the 1.18-mm sieve (49.2 vs. 39.1%, respectively; $P = 0.01$, Yang et al. 2012). Geometric mean was similar between PP and CON grain size ($P > 0.05$), but PI was higher for CON compared with PP (81.4 vs. 76.6%, respectively; $P = 0.01$, Yang et al. 2012).

Twenty multiparous lactating Holstein cows, including 8 ruminally cannulated cows, were assigned to treatments in a 4 x 4 Latin square design balanced for carry over effects. Eight of the 20 cows were ruminally cannulated prior to the study and equally distributed among the four treatments (2 per treatment) to assess digestibility of the diets and their effect on rumen fermentation. Periods were 21 days long and consisted of an 18 day diet adaptation period followed by a 3 day (72 h) sample collection period.

Cows were fed at 105% of expected intake. Feed was offered once daily at 8AM. Weight of feed offered and refused was recorded daily throughout the study. Feed and orts samples were collected daily during the collection period, composited, and divided into two subsamples used for determination of DM content and for particle size distribution measurement using the Penn State Particle Separator (Lammers et al., 1996). The former subsamples were stored for proximate analysis.

Cows were weighed after the morning milking but prior to feeding on 2 consecutive days before the start of the study, and at the end of each period. Body condition score was measured immediately prior to the study and at the end of

each period using the 5 point scale where 1 = thin and 5 = fat (Wildman et al., 1982).

2.2.2 Ruminal Measurements

Rumen pH was monitored in the ventral sac of the rumen using the Lethbridge Research Centre Ruminal pH Measurement System (**LRCpH;** Dascor, Escondido, CA**)** as described by Penner et al. (2006). Loggers were placed into the rumen on d 18, and rumen pH was collected every 30 s for a 72 h period on d 19 to 21. Minimum, mean, and maximum pH values and duration and area below pH 5.8, and acidosis index (area < pH 5.8 / DMI) were determined for each animal.

Ruminal fluid was sampled from 5 locations in the rumen, composited, and filtered through a perforated screen (Peetex, pore size = 355 µm) every 9 h during the 72 h collection period beginning at 1PM on d 19. Ruminal fluid filtrate (15 mL) was centrifuged at 3,000 × *g* at 4ºC for 20 min and stored at -20ºC until analysis.

Ruminal fluid samples were analyzed for VFA profile using gas chromatography. Samples were injected by an auto sampler (Model 8200, Varian Incorporated; Walnut Creek, CA) into a Stabilwax-DA column (30 m x 0.53 mm i.d. x 0.5 µm film, Restek Corporation; Bellefonte, PA) on a Varian Gas Chromatographer (Model 3400). The samples were run at a split vent flow of 20 mL/min with a column temperature of 90ºC for 0.1 min, then increased to 170ºC by increments of 10ºC/min and finally held for 2 minutes at 170ºC. The injector temperature was 170ºC, and the detector temperature was 190ºC. Peak integration was evaluated using Galaxie Software (Varian Incorporated). Samples were

assayed in duplicate. Ruminal ammonia-nitrogen concentration was determined using the colorimetric procedure described by Fawcett and Scott (1960) and absorbance was measured at 600 nm using a SpectraMax 190 plate reader (Molecular Devices Corp., Sunnyvale, CA).

2.2.3 Nutrient digestibility measurement

Feces were collected from the rectum at the same time-points as ruminal fluid and 100 g from each collection point was composited by period and cow.

Composited feed, orts, and fecal samples were dried at 55ºC in a forced air oven for 72 h and ground to pass through a 1 mm screen (Thomas-Wiley, Philadelphia, PA).

Analytical DM content was determined by drying feed, orts, and fecal samples in an oven at 135ºC for 2 h (AOAC, 2002; method 930.15), and ash content was subsequently obtained through combustion at 600ºC for 2 h (AOAC, 2002; method 930.05). The CP concentration was determined using a TruSpec Analyzer (FP-2000, Leco Instruments Inc., St. Joseph, MI). Starch content was determined by hydrolysis in an enzymatic method as described by Karkalas (1985) following gelatinization with NaOH; glucose concentration was subsequently measured using an oxidase/peroxidase enzyme (P7119, Sigma) and dianisidine dihydrochloride (F803, Sigma). Absorbance was measured with a plate reader (SpectraMax 190, Molecular Devices Corp., Sunnydale, CA) at 450 nm. The NDF concentration was obtained using heat stable amylase and sodium sulfite according to the method described by Van Soest et al. (1991). Indigestible NDF concentration was determined by incubating feed, ort, and fecal samples in the rumen of a dry cow for 120 h in nitrogen free polyester bags (5 × 10 cm, pore

size = 50 µm; R510, Ankrom Technology, Macedon, NY), and apparent total tract digestibility was determined using indigestible NDF as an internal control (Cochran et al., 1986).

2.2.4 Milk Collection and Analysis

Cows were milked twice daily in their stalls at 4AM and 3PM. Samples were taken at each milking time during the collection period, preserved with potassium dichromate and stored at 4ºC until they were sent to the Central Alberta Milk Testing Laboratory (Edmonton, Alberta, Canada). Milk was analyzed for CP, fat, lactose, SCC and MUN using infrared spectroscopy (AOAC, 2002; method 972.16, Milk-O-Scan 605, Foss North America, Brampton, Ontario, Canada).

2.2.5 Statistical Analysis

Data were analyzed using the PROC MIXED procedure of SAS (version 9.2 SAS Institute Incorporated; Cary, NC) according to the following model:

$$Y_{ijk} = \mu + P_i + C_j + T_k + e_{ijk}$$

where Y_{ijk} is the dependent variable, μ is the overall mean, P_i is the random effect of period, C_j is the random effect of cow, T_k is the fixed effect of treatment, and e_{ijk} is the residual error. Orthogonal contrasts were used to compare least square means of LB vs. HB and PP vs. CON. Significance is declared at $P \leq 0.05$ and tendencies are discussed at $0.05 < P \leq 0.10$.

2.3 Results

2.3.1 HB vs. LB

2.3.1.1 Intake, Digestibility and Sorting Index

Intake of DM, OM, CP, and NDF were not different between HB and LB treatments, but starch intake tended to be higher for HB vs. LB (5.83 vs. 5.33

kg/d, respectively; $P = 0.07$, Table 2-3). Digestibilities of DM, OM, CP, starch, and NDF were similar for HB vs. LB.

Sorting behavior was not affected by HB vs. LB in this study (Table 2-4). Cows consistently sorted against 8-mm particles regardless of treatment, and did not sort for or against 19 or 1.18-mm particles or fines.

2.3.1.2 Rumen pH and Metabolites

There were no differences in minimum, mean, or maximum rumen pH between HB and LB treatments, or in duration or area below pH 5.8 (Table 2-5). Total rumen VFA concentration, molar proportion of VFA, and rumen ammonia-nitrogen were not different between HB and LB.

2.3.1.3 Milk Yield and Composition

Milk yield and milk fat, protein, and lactose content were not different between HB and LB (Table 2-6). Cows fed the LB diet had higher MUN compared with HB (11.6 vs. 10.7 mg/dL, respectively; $P < 0.01$). Cows fed HB had higher SCC compared with those fed LB (110 vs. 83.7 10^3/mL, respectively; $P = 0.02$).

2.3.2 PP vs. CON

2.3.2.1 Intake, digestibility and sorting index

Intake of DM, OM, CP, and starch were not different between PP and CON treatments, while NDF intake was slightly higher for PP vs. CON (9.73 vs. 8.80 kg/d, respectively; $P = 0.04$, Table 2-3).

Sorting behavior was not affected by PP vs. CON in this study (Table 2-4). Cows consistently sorted against 8-mm particles regardless of treatment and did not sort for or against 19 or 1.18-mm particles or fines.

2.3.2.2 Rumen pH and Metabolites

There were no differences in minimum, mean, or maximum pH values between PP and CON, or in duration below pH 5.8 (Table 2-5). Total rumen VFA concentration, molar proportion of VFA, and rumen ammonia nitrogen were not different among treatments.

2.3.2.3 Milk Yield and Composition

Milk yield and milk fat, protein, and lactose content were not different between PP and CON (Table 2-6). Cows fed the PP diet had higher MUN compared with those fed CON (11.0 and 10.4 mg/dL, respectively, $P = 0.02$). Diet treatment did not affect SCC with regard to PP vs. CON treatments.

2.4 Discussion

Both chemical and physical characteristics of barley can affect dairy cow performance and ruminal fermentation. Silveira et al. (2007a) showed that cows fed Xena vs. Dillon barley produced more milk and attributed it to a difference in starch content of the grain (58.7 vs. 50.0% DM, respectively). In a subsequent study, they observed that cows fed Xena vs. Dillon had a higher duration of pH < 5.8 and lower milk fat (Silveira et al., 2007b). Yang et al. (2000) reported that milk yield increased from 25.6 to 30.8 kg/d as PI decreased from 82 to 64% when dairy cows were fed lower quality barley (57 kg/hL). McGregor et al. (2007) used high quality barley grain in their study (68 kg/hL) to evaluate the effects of finely vs. coarsely rolling on dairy cow productivity, and reported that dairy cow performance was not affected.

These studies indicate that animal responses to extent of processing may be affected by the quality of barley grain prior to processing. However, some barley grains are commercially available after high and low quality grains are

mixed to create a more marketable product (Wang and McAllister, 2000), which results in a huge variation in kernel size within a lot.

Variation in kernel uniformity causes difficulty with precisely processing grain, because it can result in either under-processed small particles, potentially decreasing the availability of nutrients and causing a reduction in productivity (Valentine and Wickes, 1980), or over-processed large particles, resulting in more rapid fermentation of grain and putting the animal at greater risk for digestive disturbances such as rumen acidosis (Laksesvela, 1982), which has been shown to depress milk fat and DMI, contributing to reduced milk yield (Bauman and Griinari, 2003). Therefore, we expected that precision processing barley grain would improve productivity of lactating dairy cows.

Contrary to our hypothesis, we did not see effects of precision processing on milk yield. A previous study using the same sources of barley grain reported that ruminal fermentation was not affected in beef cattle (McAllister et al., 2011), which was consistent with our findings. However, DM, CP, and ADF digestibilities were improved for PP vs. CON and DMI was higher for the PP vs. CON and LB vs. HB treatments in the beef study. This may increase ADG, ultimately resulting in a shorter time for the animals to reach market weight.

The differences among the findings between the dairy and beef studies are likely due to the difference in the amount of grain in the rations. The animals on the beef trial were fed a diet containing 67% grain (McAllister et al., 2011), which is much greater than the 40% grain diet fed to the dairy cattle in this study. Beef cattle cannot tolerate as low of a processing index (more extensive processing) as

that of dairy cattle because of the greater amount of grain in beef cattle rations (Yang et al., 2000). Hironaka et al. (1992) reported that ADG was higher for beef cattle fed barley grain steam rolled to a PI of 82 vs. 74 or 92%, which suggests the optimal PI for a beef animal is higher than that of a dairy animal. Therefore, the PI in this study was probably closer to that of an optimal PI for beef vs. dairy cattle.

Although treatment did not affect milk yield in the current study, we found that MUN was slightly higher for cows fed LB vs. HB and PP vs. CON barley grain. The MUN concentration increases proportionally as the protein to energy ratio increases (Hof et al., 1997). In this study, the amount of protein in the treatment rations was similar, but the amount of fermentable energy available to the ruminal microbes was expected to differ among treatments. The LB grain likely fermented more slowly in the rumen due to less extensive grain processing, suggested by a higher PI for LB vs. HB grain (80.2 vs. 77.1%, McAllister et al., 2011), more whole kernels compared with the heavy diet (7.8 vs. 5.4%, respectively; Yang et al., unpublished data) and a tendency for less starch intake for cows fed LB. These factors may have lowered fermentation in the rumen for the LB vs. HB diet, which could decrease the amount of N incorporated into microbial protein for LB.

The CON grain had a higher PI compared with PP grain. However, high PI for CON may not reflect less extensive processing for the current study, as CON also had more fines compared with PP (13.8 vs. 9.1%, respectively; Yang et al., unpublished). The fines may have actually raised PI by filling in the spaces

between processed grain kernels. Excessive fines increase the surface area available for microbial attachment, which would increase ruminal fermentation. An increase in ruminal fermentation is generally associated with a decrease in ruminal pH. However, we did not see subsequent treatment effects on ruminal pH in this study, and one of the possible reasons is that animals consumed sufficient physically effective fiber in the ration to maintain relatively high ruminal pH regardless of dietary treatment.

Yang et al. (2000) reported that milk protein increased linearly from 3.15% (PI = 81%) to 3.34% (PI = 55.5%) with more extensive processing, and attributed it to a greater rate of ruminal fermentation by cows fed the more extensively processed diet, which provided the ruminal microbes with more energy to allocate for protein synthesis (Theurer et al., 1999). Contrary to these findings, we did not find treatment effects on milk protein in this study, which is consistent with the findings of McGregor et al. (2007). However, McGregor et al. (2007) reported that MUN was higher for more extensively processed barley (13.6 mg/dL, PI = 69%) compared with coarsely rolled barley (14.0 mg/dL, PI = 83%), which is opposite to our findings. This might be attributed to slightly greater starch content in the diet containing coarsely rolled barley grain vs. finely rolled barley grain (251 vs. 244 g/kg DM, respectively) in that study. Similarly, in our study there was greater starch content and intake for HB vs. LB, which might have also played a role in increasing ruminal fermentation. The CON vs. PP diet had slightly higher starch content; however, there were no significant differences in intake although it was numerically higher for CON vs. PP.

It is important to note that PI alone may not be a reliable method to assess the extent of grain processing; PI was higher for CON compared with PP grain although it seems that processing was actually more extensive due to a greater amount of fines for CON. Mathison et al. (1997) observed that "slightly rolled" and "medium rolled" barley grain were similar in weight per unit volume (60.0 vs. 58.9 kg/hL, respectively), but that "slightly rolled" grain had more undamaged grain kernels than "medium rolled" grain (64 vs. 27.0%), which is consistent with our results indicating that using only PI as a measurement for extensity of grain processing may not be reliable if the grain kernels are highly variable in size. Supplementing PI with particle size distribution analysis may be a better indicator of extent of grain processing.

2.5 Conclusion

Feeding PP vs. CON and LB vs. HB grain increases MUN, which suggests that rumen fermentation is greater for CON and HB diets. However, there were no treatment effects on rumen pH or dairy cow productivity. The current study did not provide evidence to support that precision processing improves dairy cow productivity.

2.6 Acknowledgements

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Table 2-1.Ingredients and chemical composition of experimental diets

	Heavy	Light	PP	CON
Ingredients, % DM				
Heavy barley grain (HB)	40.0	-	-	-
Light barley grain (LB)	-	40.0	-	-
Processed before mixing LB and HB	-	-	40.0	-
Processed after mixing LB and HB	-	-	-	40.0
Barley Silage	40.0	40.0	40.0	40.0
Protein Mix[1]	20.0	20.0	20.0	20.0
Chemical Composition				
DM, %	50.3	50.3	50.3	50.3
OM, % DM	88.5	87.3	89.7	89.0
CP, % DM	17.1	17.3	16.8	16.9
Starch, % DM	24.4	22.2	22.1	24.9
NDF	37.0	37.7	39.3	37.4
Processing Index[2]	80.2	76.4	76.3	82.9

[1]Contained 9.78% Wheat DDGS, 54.2% Beet pulp, 19.6% Corn gluten meal, 2.17% Calcium diphosphate, 1.09% Magnesium oxide, 6.52% Limestone, 6.52% Salt, 0.09% 222 (ADE), 0.04% 999 (Se), 0.04% 777 (TM Pack)

[2]Processing index is the volume weight of barley after processing as a percent of its volume weight prior to processing

Table 2-2. Particle size distribution and process index of rolled barley grains (Yang et al., 2012)

Item	Dry-rolled barley[1]					*P*-value	
	HB	LB	PP	CON	SE	HB vs. LB	PP vs. CON
Particle size, %							
Whole kernel	5.4	7.8	7.1	9.9	0.61	0.01	0.02
3.35 mm sieve	9.9	1.8	7.2	9.7	0.56	0.01	0.01
2.36 mm sieve	31.6	24.9	34.6	37.4	0.90	0.01	0.05
1.18 mm sieve	46.8	62.7	49.2	39.1	0.93	0.01	0.01
<1.18 mm	11.6	10.7	9.1	13.8	1.13	0.55	0.02
Geometric mean, mm	2.74	2.42	2.79	2.77	0.060	0.01	0.79
Process index, %[2]	77.1	80.2	76.6	81.4	0.94	0.05	0.01

[1] HB = precision processed heavy barley, LB = precision processed light barley, PP = HB and LB mixed equal proportions, CON = heavy and light barley mixed equal parts and processed at a single, narrow roller setting

[2]Processing index is the volume weight of barley after processing as a percent of its volume weight prior to processing

Table 2-3. Effects of processing barley grain according to grain size on nutrient intake and digestibility

	Diet					*P*-value	
	Heavy (n=2)	Light (n=2)	PP (n=2)	CON (n=2)	SE	Light vs. Heavy	PP vs. CON
Intake, kg/d							
DM	23.9	24.0	24.7	23.5	1.07	0.92	0.30
OM	21.2	21.0	22.2	21.0	0.95	0.86	0.23
CP	4.09	4.16	4.16	3.98	0.18	0.74	0.36
Starch	5.83	5.33	5.47	5.86	0.25	0.07	0.15
NDF	8.84	9.06	9.73	8.80	0.40	0.63	0.04
Digestibility, %							
DM	51.4	48.4	53.6	52.8	3.79	0.23	0.73
OM	55.4	52.3	57.3	57.1	3.56	0.16	0.90
CP	47.7	50.1	52.8	51.6	4.59	0.45	0.70
Starch	91.2	91.6	92.9	93.7	1.13	0.76	0.53
NDF	39.8	35.2	43.7	40.9	5.33	0.25	0.48

Table 2-4. Effects of processing barley grain based on size on sorting index

	Diet					*P*-value	
	Heavy (n=5)	Light (n=5)	PP (n=5)	CON (n=5)	SE	Heavy vs. Light	PP vs. CON
19-mm	105	102	104	106	2.40	0.20	0.26
8-mm	88.9	83.5	81.7	85.7	9.25	0.17	0.31
1.18 mm	101	100	102	102	0.97	0.57	0.97
Pan	104	102	105	104	1.65	0.20	0.42

Table 2-5. Effects of processing barley grain based on size on rumen pH and metabolites

	Diet					*P*-value	
	Heavy (n=2)	Light (n=2)	PP (n=2)	CON (n=2)	SE	Heavy vs. Light	PP vs. CON
Rumen pH							
Nadir	5.42	5.39	5.43	5.26	0.18	0.89	0.39
Mean	6.25	6.13	6.20	6.07	0.13	0.46	0.44
Maximum	6.97	6.86	6.90	6.85	0.07	0.25	0.59
Duration < pH 5.8, min	205	334	330	384	131	0.39	0.71
Area < pH 5.8, pH x min	54.1	173	98.7	134	78.9	0.27	0.73
Total VFA, mM	126	118	126	126	5.37	0.21	0.96
Molar Proportion, %							
Acetate	61.6	61.9	62.2	62.0	0.63	0.73	0.79
Propionate	22.0	20.9	21.1	20.8	0.66	0.29	0.78
Isobutyrate	0.90	0.93	0.94	0.98	0.03	0.49	0.37
Butyrate	12.1	12.6	12.0	12.5	0.22	0.12	0.16
Isovalerate	1.39	1.47	1.59	1.58	0.05	0.21	0.90
Valerate	1.49	1.54	1.53	1.55	0.04	0.41	0.71
Caproate	0.54	0.63	0.65	0.65	0.06	0.24	0.94
Rumen NH3-N, mg/dL	6.79	6.73	6.49	7.45	0.63	0.92	0.13

Table 2-6. Effects of processing barley grain based on size on milk yield, composition, body weight, and body condition score

	Diet					*P*-value	
	Heavy (n = 5)	Light (n = 5)	PP (n=5)	CON (n=5)	SE	Heavy vs. Light	PP vs. CON
Yield; kg/d							
Milk	28.3	28.8	28.6	28.9	1.83	0.37	0.57
Fat	1.11	1.13	1.10	1.10	0.05	0.33	0.82
Protein	0.97	1.00	0.97	0.99	0.04	0.23	0.38
Lactose	1.25	1.29	1.26	1.29	0.09	0.11	0.29
Composition							
Fat; %	3.94	3.90	3.88	3.84	0.13	0.60	0.57
Protein; %	3.45	3.45	3.41	3.42	0.10	0.90	0.90
Lactose; %	4.36	4.40	4.36	4.36	0.07	0.14	0.90
MUN; mg/dL	10.7	11.6	11.0	10.4	0.34	< 0.01	0.02
SCC; 10^3/mL	110	83.7	95.9	96.5	19.9	0.02	0.95
Body weight; kg	652	652	649	655	16.9	0.99	0.14
ADG; kg/d	0.56	0.51	0.46	0.58	0.14	0.75	0.50
BCS	2.93	2.94	3.02	3.00	0.14	0.89	0.69
ΔBCS; /d	0.05	-0.03	0.13	0.16	0.07	0.36	0.65

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3.0 STUDY 2: THE RELATIONSHIP BETWEEN RUMINAL ACIDOSIS RESISTANCE AND EXPRESSION OF GENES INVOLVED IN REGULATION OF INTRACELLULAR pH AND BUTYRATE METABOLISM OF RUMINAL EPITHELIAL CELLS IN STEERS[1]

3.1 Introduction

Subacute ruminal acidosis (**SARA**) is a metabolic disorder that greatly impacts the dairy industry, causing great economic losses for the producer (Stone, 2004). This disorder usually is not associated with acute clinical signs (Nagaraja and Lechtenberg, 2007), making it difficult to define and detect on farms. However, SARA is associated with liver abscesses (Nagaraja and Lechtenberg, 2007), laminitis (Nocek, 1997), decreased appetite and depressed milk fat (Kleen et al., 2003), all of which may result in substantial economic losses for the dairy industry. The majority of past research has focused on nutritional management such as fermentability of the diet and physical effectiveness of fiber. While nutritional management practices can reduce the incidence of SARA, some animals are more susceptible to a high grain diet than others. Brown et al. (2000) showed that the severity of acidosis varies among steers fed a common high grain diet, which may hold true for lactating dairy cows fed a high grain diet.

Ruminal pH is maintained by a balance between acid production by microbes in the rumen and its removal by absorption through the ruminal epithelial cells, neutralization with salivary buffers, and passage to the lower

[1] A version of this chapter has been accepted for publication. Schlau, N., L.L. Guan and M.Oba. 2012. The relationship between ruminal acidosis resistance and expression of genes involved in regulation of intracellular pH and butyrate metabolism of ruminal epithelial cells in steers. J. Dairy Sci., in press.

digestive tracts (Allen, 1997). Accumulation of VFA in the rumen causes pH depression and SARA. Allen (1997) estimated that approximately 37% of protons are neutralized in the rumen by salivary buffers while about 7% of protons disappear from the rumen by passage to the lower digestive tracts, leaving over half of the protons to be removed by absorption through the ruminal epithelial cells or neutralization by buffers excreted by the epithelial cells. Penner et al. (2009a) demonstrated that ruminal epithelial cells from acidosis resistant sheep had greater capability to uptake VFA in vitro, indicating that the rate of VFA absorption may partly affect the extent of resistance to rumen acidosis in vivo.

Therefore, the relationship between ruminal pH and expression of genes coding VFA transporters and a key enzyme involved in energy metabolism in ruminal epithelial cells warrants investigation. The objective of this study was to determine whether there are differences in expression of genes involved in VFA absorption and energy metabolism in ruminal epithelial cells between acidosis resistant and acidosis susceptible animals.

3.2 Materials and methods

Animals used in this study were cared for in accordance with the guidelines of the Canadian Council on Animal Care (Ottawa, Ontario, Canada). All procedures were approved by the University of Alberta Research Centre Animal Care Committee.

3.2.1 Screening Study

Seventeen ruminally cannulated steers (539 ± 49.5 kg; mean ± SE) were used as a model of ruminants experiencing SARA for this study. The steers were housed in individual pens bedded with wood shavings, and fed a diet consisting of

85% grain (Table 3-1) ad libitum. Ruminal pH was measured in the ventral sac every 30 s continuously for 3 d using the pH measurement system evaluated by Penner et al. (2009b). Minimum, mean, and maximum pH were determined, as well as duration and area below pH 5.8. These data were used to determine acidosis index (area under pH 5.8 divided by DMI; Penner et al., 2009c), and the 3 steers with the lowest and the 3 with the highest values were selected as acidosis resistant (**AR**) and acidosis susceptible (**AS**) animals, respectively, and used in the subsequent study on the following day.

3.2.2 Sample Collection and Analysis

Three AR and 3 AS steers were force-fed a common diet (Table 3-1) at 60% of their average DMI through ruminal cannulas over the 3 d immediately prior to the study; all leftover rations were placed into the rumen through ruminal cannulas at 30 min after feeding to induce SARA. Data and samples were collected during the 6-h postprandial period.

3.2.2.1 Ruminal pH Measurement

Ruminal pH was measured in the ventral sac every 30 s for the 6-h data collection period using the system evaluated by Penner et al. (2009b). Minimum, mean, and maximum pH values duration and area below pH 5.8, area < 5.8 and acidosis index (area < pH 5.8 / DMI) were determined for each steer.

3.2.2.2 Ruminal Fluid Collection

Ruminal fluid was collected from 5 locations in the rumen immediately prior to feeding and every 2 h for the subsequent 6-h period, and combined and strained through a perforated screen (Peetex, pore size = 355 µm). Samples were centrifuged at 3,000 *g* for 20 min at 4ºC, and the supernatant was stored at -20ºC for subsequent analysis.

Ruminal fluid samples were analyzed for VFA profile using gas chromatography. Samples were injected by an auto sampler (Model 8200, Varian Incorporated; Walnut Creek, CA) into a Stabilwax-DA column (30 m x 0.53 mm i.d. x 0.5 µm film, Restek Corporation) on a Varian Gas Chromatographer (Model 3400). Samples were run at a split vent flow of 20 mL/min with a column temperature of 90ºC for 0.1 min, then increased to 170ºC at a rate of 10ºC/min, and held for 2 minutes at 170ºC. The injector temperature was 170ºC, and the detector temperature was 190ºC. Peak integration was evaluated using Galaxie Software (Varian Incorporated). All samples were assayed in duplicate.

3.2.2.3 Blood Collection

Blood was collected from the jugular vein through a catheter immediately prior to feeding and every 2 h for the subsequent 6-h period into tubes containing sodium heparin (Fisher Scientific Company; Nepean, Ontario, Canada). Blood samples were centrifuged at 3,000 *g* at 4ºC for 20 min immediately after collection, and plasma was harvested and stored at -20ºC until analysis.

Plasma samples were analyzed for glucose concentration using a glucose oxidase/peroxidase enzyme (Sigma; St. Louis, MO) and dianisidine dihydrochloride (Sigma) procedure. Absorbance was determined using a SpectraMax 190 plate reader (Molecular Devices Corp.; Sunnyvale, CA) at a wavelength of 450 nm. Plasma BHBA was quantified by oxidizing BHBA to acetoacetate using 3-hydroxybutyrate dehydrogenase (Roche, Mississauga, ON, Canada) and measuring the reduction of NAD^+ to NADH in a 0.2 M Tris buffer/NAD solution (Sigma) using a SpectraMax 190 plate reader at a wavelength of 340 nm. Commercial kits were used to determine concentrations of

plasma NEFA (Wako Chemicals USA, Incorporated; Richmond, VA), insulin (Coat-a-Count kit Diagnostic Products Corporation; Los Angeles, CA), and serum amyloid A (**SAA,** Tridelta Development Ltd., Greystones Co., Wicklow, Ireland).

3.2.2.4 Ruminal Papillae Collection

Steers were treated with Liquimycin LA 200 (2 mg per 100 kg of BW, Pfizer Animal Health, New York, NY) 1 d prior to the sample collection date. Approximately 30 ruminal papillae were biopsied immediately prior to feeding and an additional 30 papillae every 2 h for the subsequent 6-h period. The papillae were rinsed with phosphate buffered saline, and stored at -20ºC in RNA-Later (Ambion, Incorporated; Foster City, CA) solution until analysis.

3.2.2.5 RNA Extraction

Total RNA was extracted from the ruminal papillae using the Trizol extraction method (Invitrogen; Burlington, ON, Canada) as described by Chomczynski and Sacchi (1987) and purified using Qiagen RNeasy MiniElute Cleanup Kit (Qiagen; Toronto, Ontario). The RNA concentration of the extract obtained was determined using a NanoDrop (NanoDrop 2000C Spectrophotometer, NanoDrop Technologies; Wilmington, DE) at absorbances of 260 and 280 nm. The A260/280 ratio of the samples was at least 1.93.

3.2.2.6 Reverse Transcription

RNA samples were diluted to 100 ng/µL, and subsequently treated with DNase I (Invitrogen; Carlsbad, CA) and RNAse OUT (Invitrogen). First strand cDNA was synthesized using Superscript II (Invitrogen).

3.2.2.7 Primer and Probe Design

Primers and probes were designed using Primer Express software (Applied Biosystems; Foster City, CA) and verified for specificity using the

National Center for Biotechnology Information (**NCBI)** database. The target genes, housekeeping genes, primer and probe sequences, and NCBI accession numbers are shown in Table 3-2.

3.2.2.8 Quantitative Real Time PCR

Genes encoding monocarboxylate cotransporter, isoform 1 (**MCT1**), down regulated in adenoma (**DRA**), putative anion transporter, isoform 1 (**PAT1**), sodium hydrogen exchanger, isoforms 1, 2, and 3 (**NHE1**, **NHE2**, **NHE3**, respectively), and Na^+/K^+ ATPase pump (**ATP1**; see Table 3-2) were evaluated for their expression in ruminal epithelial cells via quantitative real time PCR, using TaqMan gene expression assay with The StepOne Plus Real Time PCR System (Life Technologies; Carlsbad, CA). The program consisted of a 95ºC pre-incubation for 10 min, followed by 40 cycles of 15 s at 95ºC and 1 min at 60ºC. All samples were analyzed on one plate per gene, using 40 ng of cDNA per reaction. Samples prior to feeding were assayed in triplicate while samples from 2, 4, and 6 h after feeding were analyzed in quadruplicate.

The expression of each targeted gene was evaluated using the comparative cycle threshold (C_T) method and normalized using three housekeeping genes, ribosomal protein large, P0 (**RPLP0**), β-actin (**ACTB**), and glyceraldehyde-3-phosphate dehydrogenase (**GADPH**) according to the method described by Vandesompele et al. (2002).

3.2.3 Statistical Analysis

Body weight, DMI, and pH data were analyzed using the PROC TTEST procedure of SAS (version 9.2 SAS Institute Incorporated; Cary, NC). The other data were analyzed using the PROC MIXED procedure of SAS (version 9.2, SAS

Institute Incorporated; Cary, NC) with time as a repeated measure and animal as the experimental unit according to the model below, using the variance/co-variance structure of best fit as described by the Akaike Information Criterion.

$$Y_{ij} = \mu + G_i + H_j + GH_{ij} + e_{ij},$$

where Y_{ij} is the dependent variable, μ is the overall mean, G_i is the effect of *i*th group, H_j is the effect of *j*th time point, and and e_{ij} is the error term.

If significance was found, LSMEANS were determined and the PDIFF procedure was used with the Bonferroni correction to determine differences between means. The largest SE values are reported where applicable. Significance was declared at $P < 0.05$ and trends were declared at $0.05 < P < 0.10$.

3.3 Results and Discussion

3.3.1 Screening Study

Mean ruminal pH, duration and area pH below 5.8 ranged from 5.44 to 6.13, 243 to 1291 min, and from 34 to 621 pH × min, respectively. Acidosis index for 17 steers ranged from 4.0 to 96.5 pH × min/kg.

There were no difference in DMI ($P > 0.10$; Table 3-3) between the 3 steers classified as AR and the 3 steers classified as AS; however, mean pH was higher for AR compared with AS steers (6.01 vs. 5.51, respectively, $P = 0.01$), duration pH < 5.8 was lower for AR compared with AS steers (481 vs. 1130.3 min, $P = 0.03$), area pH < 5.8 tended to be lower for AR compared with AS steers (157 vs. 535 pH × min/kg, $P = 0.01$), and acidosis index was lower for AR compared with AS steers (13.5 vs. 61.7 pH × min/kg, $P = 0.01$).

3.3.2 Ruminal pH and VFA Profile

There were no differences in BW and DMI, including the force-fed rations, between AR and AS animals ($P > 0.10$; Table 3-4). However, the

minimum (5.58 vs. 4.87; $P < 0.01$) and mean ruminal pH (6.05 vs. 5.59; $P < 0.05$) were higher for AR compared with AS animals while maximum pH values were not different between the groups. Duration (224.7 vs. 80.0 min; $P < 0.01$) and area that pH was below 5.8 (133.0 vs. 7.67 pH × min; $P < 0.01$) were higher in AS animals. Acidosis index was higher in AS animals (23.9 vs.1.40 pH × min/kg, $P < 0.05$).

Total VFA concentration was lower for AR compared with AS animals (122 vs. 164 mM, $P < 0.01$; Table 3-5). Molar proportion of propionate in ruminal fluid was lower in AR steers compared with AS (21.2 vs. 37.5 mol/100 mole, $P = 0.01$) while concentrations of acetate (56.7 vs. 49.9 mol/100 mol; $P = 0.05$) and butyrate (17.5 vs. 7.33 mol/100 mol VFA; $P < 0.01$) were higher for AR steers. There were no differences in isobutyrate, isovalerate, valerate, or caproate concentrations between AR and AS animals ($P > 0.10$).

We observed significant differences in ruminal pH and acidosis index between AR and AS in this study although ruminal pH was similar prior to feeding (Figure 3-1). Because we enforced similar DMI between AR and AS animals and both groups of animals were fed a common diet, the difference in ruminal pH cannot be attributed to dietary factors. Total VFA concentration in the rumen was lower for AR compared with AS animals, although, like pH, ruminal VFA concentration was similar prior to feeding (Figure 3-2). As such, lower postprandial ruminal pH for AR steers is likely attributed to lower VFA production, faster VFA absorption, or a combination of both factors. Although neutralization by salivary buffers and passage to the lower digestive tract also

contribute to proton removal from the rumen, these factors were not measured in this study. Allen (1997) estimated that approximately 53% of protons are removed from the rumen by VFA absorption, while about 37% are removed due to neutralization by salivary buffers, and 7% of proton removal is attributed to gut passage of VFA. We cannot exclude the possibility that the other acid removal pathways were different between AR and AS animals. However, absorption accounts for acid removal from the rumen to the greatest extent, and the expected difference in acid absorption between AR and AS animals warrants further investigation.

A marked increase in molar proportion of butyrate was observed for AR steers while the sum of molar proportion of acetate and propionate was higher for AS compared with AR steers (87.4 vs. 77.9%, $P = 0.01$). Greater butyrate production may have at least partly contributed to the higher pH observed in AR steers as stoichiometric equations indicate that fewer protons are released and less acid is produced when hexose ferments to butyrate, as opposed to acetate or propionate (Owens and Goetsch, 1988).

In a companion study, Chen et al. (2012) reported decreased bacterial density in AR steers, and differences in the diversity of bacterial communities for both epimural bacteria and bacteria from ruminal digesta. The lower bacterial density might indicate that AR produced fewer VFA compared with AS. They also found that higher population of epimural bacteria is associated with higher ruminal pH and lower VFA production in AR steers, which indicates that epimural bacteria may play a stimulatory role in VFA absorption, and increasing

number of bacteria from ruminal digesta is associated with higher proportion of butyrate in AR steers. Further studies are necessary to determine whether and how microbial or host factors affect VFA production and molar proportion of butyrate in animals fed a common diet.

3.3.3 Gene Expression

3.3.3.1 Epithelial Intracellular pH Regulation

Absorption of VFA from the rumen occurs by simple diffusion of undissociated VFA into the ruminal epithelial cells, followed by subsequent dissociation in the cell, or facilitated diffusion of dissociated VFA by transport proteins located on the membrane of the epithelial cells (Connor et al., 2010). We hypothesized that difference in expression of these transport proteins might be related to ruminal pH, and could therefore play a role in resistance to ruminal acidosis.

Graham et al. (2007) showed that MCT1 is located on the basal side of ruminal epithelial cells and is responsible for removal of protons from the epithelial cell by co-transporting dissociated VFA, lactate and ketones with H^+ into the blood (Kirat et al., 2006). Bilk et al. (2005) proposed that DRA and PAT1 are responsible for neutralizing acid in the rumen by exporting bicarbonate from epithelial cells and importing dissociated VFA. Penner at al. (2009a) observed a tendency for SARA resistant sheep to have greater capacity for bicarbonate dependent uptake of acetate, which may have been due to increased expression of DRA, PAT1, or both. Therefore, we expected to see greater expressions of MCT1, DRA, and PAT1 in AR; however, they were similar between AR and AS ($P > 0.10$; Table 3-6). Differences in VFA absorption could have also been caused

by differences in ruminal motility, surface area of ruminal papillae or blood flow rate, which would allow for greater diffusion of VFA from the rumen; however, these variables were not evaluated in the current study. Future research is necessary to determine the extent of these factors on resistance to SARA. It is also important to note the difficulty to obtain representative ruminal papillae samples due to the size of the rumen. In this study, biopsies of papillae were taken from the same location of the rumen to compare AR and AS steers, but it is not known whether these samples are representative of the whole rumen, and future studies need to address this concern.

The NHE proteins are another mechanism by which the ruminal epithelial cells maintain intracellular pH. Graham et al. (2007) showed that NHE1, NHE2, NHE3, and NHE8 are present in ruminal epithelial cells. NHE1 and NHE3 are located on the apical side of the epithelial cell and imports Na^+ to the cell and exports H^+ to the rumen while NHE2 imports Na^+ to the cell but exports H^+ to the extracellular space (Connor et al., 2010). We expected to find lower expression of NHE1 and NHE3 in AR, because they would result in protons returning to the ruminal lumen, and greater expression of NHE2 because it would result in removal of a proton from the system. We found that expression of NHE1, NHE2 and NHE3 were consistently higher in AR compared with AS, but only NHE3 was significantly different ($P < 0.01$). Although greater abundance of mRNA does not necessarily mean greater protein production or its activity unless transcription limits protein synthesis, long term regulation of NHE3 is achieved through

changes in transcription of the gene (Zachos et al., 2005), and mRNA abundance of this gene likely reflect the protein synthesis.

Graham et al. (2007) proposed that apically located NHE proteins reduce extracellular pH, and promote uptake of undissociated VFA by epithelial cells. Greater uptake of undissociated VFA via simple diffusion into ruminal epithelial cells of AR might ultimately result in increasing expression of NHE3 to prevent reductions in intracellular pH caused by release of protons from dissociation of VFA inside the cell.

Ruminal pH can differ according to the location it is measured. Although we observed higher pH in the ruminal digesta for AR, it is possible that pH near the epithelial cells might have been lower due to increased NHE3 expression compared with AS. This would promote formation of undissociated VFA near the epithelia, and successive diffusion into ruminal epithelial cells. The VFA would subsequently dissociate in the epithelial cell, which might contribute to increased NHE3 expression to avoid the reduction in intracellular pH caused by excess proton accumulation, by exporting protons back into the rumen. Further studies are necessary to determine whether increased NHE3 expression is a consequence of high ruminal pH, or a cause.

Krishnan et al. (1999) found that presence of VFA stimulates absorption of sodium in the colon of rats and that VFA with longer carbon chain lengths have greater effects on sodium absorption, which was demonstrated again by Kiela et al. (2001). Greater sodium absorption may be due to NHE proteins that exchange sodium and protons to regulate intracellular pH, and these findings are consistent

with the greater NHE3 expression that we observed for the AR steers. Greater molar proportion of butyrate in AR might have contributed to higher expression of NHE3. Further, butyrate is high in proliferative effects in ruminal epithelial cells (Sakata and Tamate, 1978), which may have increased the surface area of ruminal papillae and subsequently increased absorption of VFA from the rumen. However, contrary to these data, Laarman et al. (2012) observed that calves fed milk replacer and calf starter increased molar proportion of butyrate, but decreased expression of NHE3 compared with calves fed milk replacer only. In contrast, expression of MCT1 was greater for calves fed milk replacer and starter in the Laarman et al. (2012) study, which indicates that the ruminal epithelial cells of young ruminants may have relied on MCT1 to a greater extent than that of NHE3 to remove protons from the cells. However, the exact mechanism is not known and warrants further investigation.

3.3.3.2 Epithelial Cell Metabolism

Albrecht et al. (2008) demonstrated the importance of the Na^+/K^+ ATPase to the function of NHE proteins. Because AR animals had greater expression of NHE3 compared with AS, we hypothesized that there must be greater expression of the Na^+/K^+ ATPase in AR animals to remove Na^+ from the epithelial cell. However, the current study found no difference in expression of this gene ($P >$ 0.10).

Butyrate is extensively metabolized by ruminal epithelial cells to BHBA (Sehested et al., 1999). Lane et al. (2002) showed that 3-hydroxy-3-methylglutaryl-CoAsynthase (**HMGCS**) is the rate limiting enzyme in hepatic ketogenesis, and proposed that this holds true for ruminal epithelial cells. Penner

et al. (2009a) observed greater plasma BHBA concentration in SARA resistant sheep, which indicates that expression or activity of HMGCS2 in the ruminal epithelial cells might be greater for SARA resistant sheep, although it was not measured. As such, we expected that HMGCS2 would be also expressed higher in AR steers. However, we did not observe a difference in HMGCS2 expression ($P > 0.10$) between AR and AS animals. In agreement with our findings, Lane et al. (2002) showed that presence of VFA in the rumen does not influence the expression of genes that regulate ketogenesis in growing lambs.

3.3.4 Plasma Metabolites and Hormones

Plasma glucose, insulin, and BHBA, and NEFA concentrations were not different between AR and AS steers ($P > 0.10$; Table 3-7). We expected plasma BHBA to be higher in AR steers due to an increased rate of ketogenesis which Penner et al. (2009a) found in their study. Although concentrations of butyrate and BHBA are not necessarily same as their production, it is probable that greater absorption of butyrate saturates ketogenesis pathways because BHBA does not increase proportionally with absorption of butyrate (Krehbiel et al., 1992, Rémond et al., 1993), which may explain why there was no difference between plasma BHBA concentration in AR and AS steers. Plasma SAA concentration was not affected by acidosis susceptibility, although it was 58.5% numerically higher in AR compared with AS (128 vs. 74.9 ± 23.5 μg/mL, respectively; $P = 0.18$).

3.3.5 Implications

The current study reports several interesting preliminary findings about the differences between AR and AS steers. However, the results need to be

interpreted with caution. Because we needed to evaluate a few extreme animals in ruminal pH responses to a high grain diet for the current study, we had only 3 AR and 3 AS steers, which may not have sufficient statistical power to detect significant differences in some response variables. In addition, although we tried to avoid confounding effects of dietary factors by feeding a common diet at pre-determined intake level, rates of VFA production and VFA absorption were not measured for this study. As such, the specific causes for lower VFA concentration in AR compared with AS steers could not be identified. Further research is warranted to confirm our preliminary findings and identify specific mechanisms affecting the extent of resistance to SARA.

3.4 Conclusion

The AR steers had lower total VFA concentration and higher molar proportion of butyrate in ruminal fluid, and greater NHE3 expression in ruminal epithelial cells compared with AS steers. These findings suggest that higher ruminal pH in AR might be partly attributed to increased VFA absorption via simple diffusion or lower VFA production. Further research on ruminal morphology and VFA absorption and subsequent responses in ruminal epithelial cells is needed to improve the understanding as to why SARA occurs in some animals, but not others, fed a common diet.

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Table 3-1. Ingredient and chemical composition of the diet

Ingredient	**% DM**
Sun cured alfalfa pellet	10.0
Mineral and vitamin mix[1]	5.0
Barley grain, dry rolled	56.7
Oats grain, dry rolled	28.3
Nutrient Composition	
DM	92.2
Ash	6.5
CP	17.6
NDF	28.7
Starch	38.6

[1]Contained 6.00% Ca, 0.49% P, 1.60% Na, 0.65% Mg, 0.65% S, 0.20% K, 13.0 mg/kg I, 220.0 mg/kg Fe, 242.0 mg/kg Cu, 815.0 mg/kg Mn, 11.0 mg/kg Co, 1220.0 mg/kg Zn, 6.00 mg/kg Se, 440.0 mg/kg monensin Na, 90 kIU/kh vitamin A, 13.3 kIU/kg vitamin D_3, 0.40 kIU/kg vitamin E

Table 3-2. Primer and probe sequences and National Center for Biotechnology (NCBI) accession numbers for quantitative real time PCR analysis

Gene name	Category	Accession number	Primer and probe sequences
3-hydroxy 3-methylglutaryl coenzyme A synthase isoform 2(HMGCS2)	Ketogenesis	NM_001045883	Forward: CCT GCT GCA ATC ACT GTC ATG Reverse: TCT GTC CCG CCA CCT CTT C Probe: TTG CAG AGC CCT TTC
Sodium/potassium ATPase pump, α 1 (ATP1)	Energy metabolism	NM_001076798	Forward: CAT CTT CCT CAT CGG CAT CA Reverse: ACG GTG GCC AGC AAA CC Probe: TGT AGC CAA CGT GCC AG
Sodium/hydrogen antiporter, isoform 1 (NHE1)	VFA absorption	NM_174833	Forward: GAA AGA CAA GCT CAA CCG GTT T Reverse: GGA GCG CTC ACC GGC TAT Probe: AAG TAC GTG AAG AAG TGT CT
Sodium/hydrogen antiporter, isoform 2 (NHE2)	VFA absorption	XM_604493	Forward: TTG TGC GAT GAC CAT GAA TAA GT Reverse: TGA TGG TCG TGT AGG ATT TCT GA Probe: CGT GGA AGA GAA CGT G
Sodium/hydrogen antiporter, isoform 3 (NHE3)	VFA absorption	AJ131764.1	Forward: AGC CTT CGT GCT CCT GAC A Reverse: TGA CCC CTA TGG CCC TGT AC Probe: TGC TCT TCA TCT CCG
Putative anion transporter, isoform 1 (PAT1)	VFA absorption	BC_123616	Forward: GGG CAC TTC TTC GAT GCT TCT Reverse: GTC GTG GAC CGA GGC AAA Probe: TCA CCA AGC AGC ACC T
Down regulated in adenoma (DRA)	VFA absorption	NM_001083676.1	Forward: TGC ACA AAG GGC CAA GAA A Reverse:GCT GGC AAC CAA GAT GCT ATG Probe: TGC CTT CTC CTC CTT C
Monocarboxylate cotransporter, isoform 1 (MCT1)	VFA absorption	NM_001037319	Forward: CGC GGG ATT CTT TGG ATT T Reverse: GTC CAT CAG CGT TTC AAA CAG TAC Probe: TTT TGG GTG GCT CAG C
Ribosomal protein large, P0 (RPLP0)	Housekeeping gene	NM_001012682	Forward: AGG GCG TCC GCA ATG TT Reverse: CGA CGG TTG GGT AAC CAA TC Probe: CCA GCG TGT GCC TG
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Housekeeping gene	NM_001034034	Forward: TGC CGC CTG GAG AAA CC Reverse: CGC CTG CTT CAC CAC CTT Probe: CCA AGC GTG TGC CTG
B-Actin (ACTB)	Housekeeping gene	NM_173979.3	Forward: CCT GCG GCA TTC ACG AA Reverse: GCG GAT GTC GAC GTC ACA Probe: CTA CCT TCA ATT CCA TCA TG

Table 3-3. DMI and pH measurements of acidosis susceptible (n = 3) and resistant (n = 3) steers in the screening study

Variable	Resistant	Susceptible	SE	*P*
DMI, kg	10.4	9.0	1.29	0.45
Mean ruminal pH	6.01	5.51	0.105	0.01
Duration pH < 5.8, min	481	1130	181.8	0.07
Area pH < 5.8, pH × min	157	535	105.4	0.01
Acidosis index, pH × min/kg	13.5	61.7	8.16	0.01

Table 3-4. Comparison of BW, DMI, and pH measurements between acidosis resistant and susceptible steers

Variable	Resistant	Susceptible	SE	*P*
BW, kg	515	499	44.5	0.75
DMI, kg	5.84	5.63	0.446	0.72
Ruminal pH				
Nadir	5.58	4.87	0.114	< 0.01
Mean	6.05	5.59	0.104	0.01
Maximum	6.55	6.47	0.225	0.79
Standard deviation	0.25	0.55	0.092	0.05
Duration < pH 5.8, min	80	225	18.2	< 0.01
Area < pH 5.8, pH × min	8	133	20.1	< 0.01
Acidosis index, pH × min/kg	1.4	23.9	4.28	0.03

Table 3-5. Comparison of ruminal VFA profile between acidosis resistant and susceptible steers

Variable	**Resistant**	**Susceptible**	**SE**	***P***
Total VFA, m*M*	122	164	5.3	< 0.01
Acetate, mol/100mol	56.7	49.9	1.68	0.05
Propionate, mol/100mol	21.2	37.5	2.60	0.01
Isobutyrate, mol/100mol	0.76	0.70	0.123	0.71
Butyrate, mol/100mol	17.5	7.3	1.22	< 0.01
Isovalerate, mol/100mol	1.61	0.96	0.259	0.15
Valerate, mol/100mol	1.96	2.30	0.221	0.34
Caproate, mol/100mol	0.31	1.32	0.364	0.12

Table 3-6. Comparison of mRNA abundance of genes involved in intracellular pH regulation and VFA metabolism between acidosis resistant vs. susceptible steers.

Gene	Resistant	Susceptible	SE	*P*
MCT1	1.67	1.25	0.441	0.53
PAT1	1.64	1.48	0.353	0.77
DRA	2.42	1.46	0.437	0.19
NHE1	1.20	0.82	0.151	0.15
NHE2	1.31	0.87	0.285	0.33
NHE3	1.38	0.50	0.083	< 0.01
HMGCS2	1.95	1.69	0.433	0.70
ATP1	1.73	1.80	0.273	0.87

Table 3-7. Comparison of plasma blood metabolite and hormone concentration between acidosis resistant vs. susceptible steers.

Variable	**Resistant**	**Susceptible**	**SE**	***P***
Glucose, mg/dL	81.1	76.2	4.20	0.45
BHBA, mg/dL	22.1	15.9	2.32	0.14
NEFA, mEq/L	114	77	18.5	0.24
Insulin, µIU/dL	90.2	64.2	24.4	0.49
Serum Amyloid A, µg/mL	128	74.9	23.5	0.18

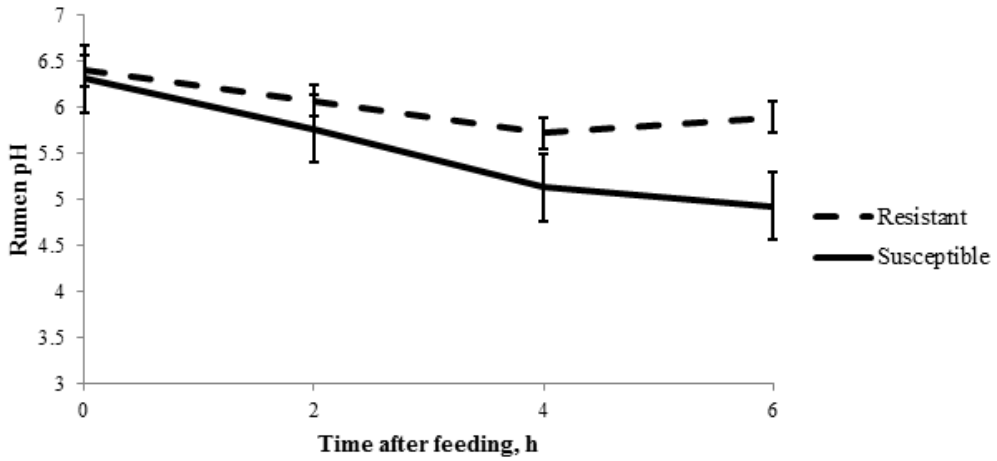

Figure 3-1. Comparison of ruminal pH between acidosis resistant and acidosis susceptible steers after feeding. Group effect, $P < 0.05$; hour effect, $P < 0.01$; group × hour interaction, $P = 0.02$.

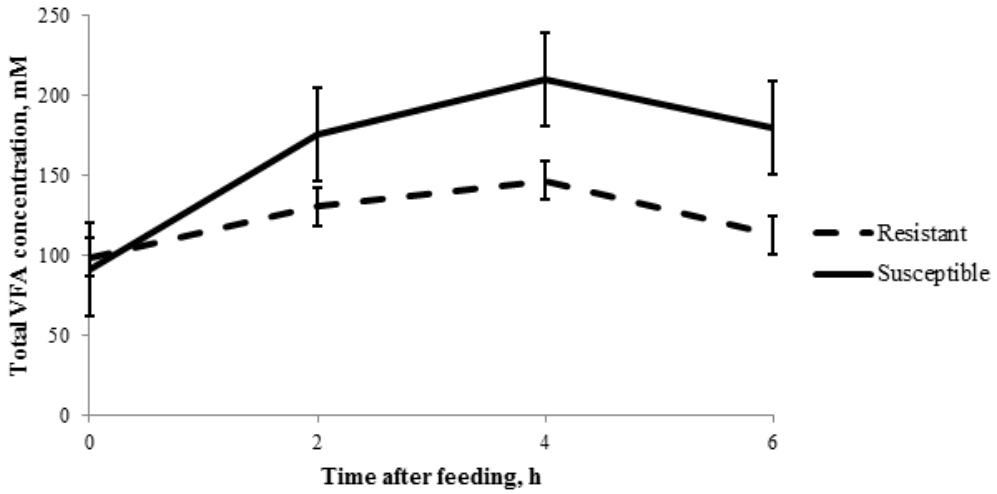

Figure 3-2. Comparison of total rumen VFA concentration between acidosis resistant and acidosis susceptible animals after feeding. Group effect, $P = 0.005$; hour effect, $P < 0.001$; group × hour interaction, $P = 0.02$.

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4.0 GENERAL DISCUSSION

4.1 Summary of research findings

Study 1 evaluated the effects of precision processing (**PI**, processing based on grain size) on ruminal fermentation and productivity of lactating dairy cows. Previous research has shown the importance of properly processing grain for ruminant productivity (Valentine and Wickes, 1980), which has led to research regarding the effects of kernel uniformity and precision processing on ruminant health. Commercially, producers mix light and heavy barley grain to make a more marketable lot; however, this can result in a high variation in kernel size (Wang and McAllister, 2000). Because of this, it is difficult to optimally process barley grain to ensure uniform fermentability in the rumen. In Study 1, animals were fed barley grain processed using four different methods - light barley grain dry rolled at a narrow roller mill setting (**LB**), heavy barley grain dry rolled at a wide roller mill setting (**HB**), LB and HB mixed equal parts (**PP**), and light and heavy barley mixed equal parts and rolled at a single narrow roller mill setting (**CON**). All other constituents of the diets were similar among treatments. A similar study was conducted using beef steers, which found that precision processing improved nutrient digestibility without affecting rumen fermentation (McAllister et al., 2011). However, in Study 1, precision processing did not affect ADG, DMI, ruminal fermentation, milk or milk component yields, although there was a slight treatment effect on MUN. The MUN concentration was slightly higher for cows fed LB vs. HB and PP vs. CON. This indicates that ruminal fermentation may have been slightly higher for HB vs. LB and CON vs. PP, providing more energy to the rumen microbes to allocate toward protein synthesis.

There were no treatment effects on ruminal pH in Study 1, which indicates the cows might have consumed sufficient physically effective fiber to counteract excess acid production. Alternatively, cows used in Study 1 might be resistant to acidosis, making it difficult to detect treatment effects on ruminal pH. However, it is unlikely that all cows were acidosis resistant as we saw a high variation in susceptibility to acidosis in the screening study for Study 2, which is consistent with previous studies by Brown et al. (2000) and Penner et al. (2009). Therefore, it is more appropriate to conclude that precision processing does not improve dairy cow productivity. The discrepancies between the dairy and beef studies might be attributed to a greater amount of grain in the ration for the beef study.

The objectives of Study 2 were to characterize animals that are resistant to sub-acute ruminal acidosis (**SARA**) and identify genes involved in VFA metabolism and regulation of intracellular pH (**pH_i**) to better understand the physiology that makes some animal more acidosis resistant than others. Animals were fed the same diet at similar DMI so the results would not be confounded by differences in DMI and diet fermentability. We found that mean ruminal pH was greater for acidosis resistant (**AR**) vs. acidosis susceptible (**AS**), while VFA concentration was lower for AR vs. AS. Sub-acute ruminal acidosis is caused by a rise in intra-ruminal VFA concentration (Krause and Oetzel, 2006). This statement appears to be confirmed by Study 2; we did find that AS had significantly more VFA in the rumen after feeding compared with that of AR, which is likely related to the difference in rumen pH. Expression of NHE3 was higher in AR steers which may promote formation of undissociated VFA near the

ruminal epithelial cells, resulting in an increase in the rate of simple diffusion of VFA into the epithelium.

4.2 Questions to be addressed in future studies

4.2.1 The role of VFA production and absorption on resistance to acidosis

The AR steers had greater molar proportion of butyrate compared with that of AS over the 6-h sampling period (Figure 4-1). Greater butyrate production by AR may have also contributed to their higher ruminal pH, as stoichiometric equations indicate that fewer protons are released and less acid is produced when hexose ferments to butyrate (Owens and Goetsch, 1988). Further, butyrate is more lipophilic relative to acetate and propionate, which means that it more readily diffuses into ruminal epithelial cells (Bergman, 1990) which may have also contributed to lower VFA concentration in the rumen of AR steers. Penner et al. (2009) reported that AR sheep had greater uptake of butyrate via bicarbonate independent pathways, which can be attributed to greater simple diffusion of butyrate from the rumen. Chen et al. (2012) reported that butyrate concentration was positively correlated with the copy number of ruminal content bacteria of AR steers, which suggests that the population, activity, or a combination, of butyrate producing bacteria is greater for AR. Future studies may measure parameters related to butyrate production, such as populations of butyrate producing bacteria or expression of enzymes related to synthesis of butyrate, such as butyrate kinase (Yarlett et al., 1985).

Aschenbach et al. (2009) showed that acetate can be absorbed by ruminal epithelial cells in exchange for bicarbonate. Penner et al. (2009) showed that AR sheep had a tendency for greater uptake of acetate by bicarbonate dependent

mechanisms, which may indicate greater expression of a bicarbonate/acetate exchanger for AR sheep. Bilk et al. (2005) proposed that DRA, PAT1, or both, might act as non-specific bicarbonate exchangers in the rumen, and may be involved in removal of dissociated VFA from the rumen.

In a review of the literature, Leonhard-Marek (2010) reported that DRA was not expressed in cultured ruminal epithelial cells when VFA were not present, which indicates that VFA concentration affects DRA expression. However, Laarman et al. (2012) showed that DRA expression was not different between calves with lower vs. higher VFA concentrations. Oba et al. (2012) showed that DRA concentration increases with calf age; however, the VFA concentration was not measured in that study. In Study 2, VFA concentration did not influence expression of PAT1 (Figure 4-2), which was consistent with the results from these previous studies. However, DRA expression was not affected by VFA concentration, either (Figure 4-3). Thus, the question still remains as to what affects DRA expression. Exposing calves to increasingly acidotic diets and measuring VFA concentration and gene expression, particularly of DRA, may help to shed light on its role in the rumen epithelium. Another possibility is to follow calves' development into adulthood. If increasing VFA concentration in the rumen results in greater expression of DRA, calves with lower ruminal pH values may become acidosis resistant in adulthood.

Further, as in vitro measurements cannot completely mimic the complexity of an in vivo environment, future studies may focus on measuring absorption in vivo by evacuating the rumen contents, blocking the esophageal and

omasal openings to the rumen and infusing a solution of known VFA concentrations as described by Kristensen et al. (2000) and measuring disappearance of VFA over time.

4.2.2 NHE3: Cause or effect of ruminal acidosis

Study 2 showed that AR had greater proportion of butyrate in the rumen compared with AS. This suggests that AR had greater diffusion of undissociated VFA into the epithelium because butyrate more readily diffuses into epithelial in the undissociated form compared with acetate or propionate (Bergman et al., 1990). Once inside the cell, VFA would subsequently dissociate increasing the concentration of protons that need to be removed. This may have further contributed to the increased NHE3 expression for AR steers in Study 2. Expression of NHE3 is increased with molar butyrate proportion in our study (Figure 4), supporting the findings of Kiela et al. (2001).

Expression of NHE3 increases as acidosis index decreases (Figure 4-5). It seems that AR steers are using NHE3 to counteract falling pH in the rumen epithelium due to a relatively greater rate of diffusion of VFA into the cells compared with AS. The excretion of protons back into the rumen might lower pH near the epithelial surface, which would further promote formation of undissociated VFA which would maintain the concentration gradient. It is not clear, however, whether this mechanism is the cause or consequence of acidosis resistance. Future studies may sequence the NHE3 gene to compare between AR and AS to investigate for the presence of SNP. If the sequence is not different between AR and AS animals, it is likely that greater expression of NHE3 is a consequence of acidosis resistance. However, if a specific SNP is identified for

AR animals, its impacts on regulation of NHE3 at the transcriptional, translational or protein activity level warrant further investigation.

A major limiting factor of Study 2 is the small sample size. The regressions shown by Figure 4-4 and Figure 4-5 only have a sample size of 6 animals, which represent extreme animals only. Future studies should incorporate more animals for stronger statistical power. It was necessary to partially evacuate the rumen in order to biopsy ruminal papillae, which may have disturbed the microbial populations. Therefore, sampling at only one time point may be beneficial. As there was no interaction between group and time for NHE3 expression, it seems evaluating a time effect is not necessary. The greatest numerical difference in NHE3 expression between AR and AS animals occurred at the 2-h time point (Figure 4-6); therefore, sampling 2-h after feeding and incorporating more animals, which would include a group with intermediate acidosis indexes, will provide data to better assess the relationship between NHE3 gene expression and VFA concentration. It would be expected that animals with intermediate acidosis indexes fall between AR and AS in regards to mRNA abundance of NHE3 and VFA concentration, which could be determined by the proposed study.

4.2.3 Is susceptibility to ruminal acidosis determined by animal or microbial factors?

Chen et al. (2012) assessed bacterial populations between AR and AS steers and found that AR had decreased bacterial density. This raises the question as to whether the animal, the microbes, or a combination of the two are related to acidosis resistance. One possibility for future research is to determine whether the

density of protozoa is different between AR and AS animals. Protozoa have the ability to store starch, which reduces the amount available for bacterial growth (Mendoza et al., 1993). They also engulf the ruminal bacteria, which can reduce the bacterial population in the rumen as well (Mendoza et al., 1993).

It would be interesting to see the effects of exchanging rumen contents between AR and AS, which might provide evidence for whether animal factors or microbial factors determine acidosis susceptibility. However, because ruminal microbes are primarily anaerobic, the act of exchanging the contents may result in exposure to oxygen which would kill the microbes. Allowing time for the microbes to recover from the exchange would confound the results because factors of the host, such as eating behavior, might affect the microbial population and so it would not be possible to tell what caused the change in microbial population, if a change occurred, or why a change did not occur. Rather, identifying major bacteria that are different between the groups and inoculating susceptible animals with resistant animals' bacteria may provide insight to the role of the microbes in acidosis resistance.

4.2.4 Effects of acute phase proteins

We expected to see a greater acute phase protein response in AS compared with that of AR due to greater severity of acidosis, which we thought would elicit a greater acute phase response. Contrary to our expectations, there was no difference in plasma serum amyloid A (**SAA**) concentration between AR and AS (Figure 4-7).

It is interesting to note, however, that plasma SAA was numerically higher in AR compared with that of AS. Chen et al. (2012) reported that toll like receptor

(**TLR**) 4 was elevated in the rumen of AR steers, which is consistent with our results as TLR-4 is involved in the recognition of endotoxin (Mani et al., 2012). Previous research shows that onset of acidosis, and therefore, an increase in ruminal acid concentration, causes an inflammatory response. However, our data suggest that an increase in ruminal acid is not the cause of the inflammatory response, but rather the diffusion of acid into the epithelium.

Another possible explanation for these results is that rate of passage of particles from the rumen to the lower GI tract is greater for AR vs. AS. Although ruminal LPS concentration increased when SARA was induced in dairy cows by decreasing physically effective fiber (Khafipour et al., 2009a) and by increasing grain concentration (Khafipour et al., 2009b), an acute phase response only occurred with a grain challenge (Khafipour et al., 2009b). The authors attributed this to the passage of starch to the lower GI tracts and subsequent fermentation in the large intestine, which may be more permeable to endotoxin compared with that of the rumen.

Therefore, it is possible that less starch was fermented in the rumen of AR vs. AS steers, which would contribute to lower VFA production as well, due to a greater rate of passage for AR. Factors such as intake/rate of intake, grain processing, amount of physically effective fiber and ruminal motility influence the rate of passage (Allen, 1997). Because animals were force fed within 30 min, DMI and rate of intake should not differ in our study. Further, both AR and AS were fed the same diet, so characteristics of the ration would not likely affect rate of passage in this case. As such, greater ruminal motility for AR vs. AS might

have increased the rate of passage and starch fermentation in the large intestine for AR. The greater starch fermentation in the large intestine of AR animals might have resulted in translocation of endotoxin across the intestinal wall and ultimately a greater acute phase response. However, it is important to note that these data need to be interpreted with caution as the difference between AR and AS steers in SAA concentration was not statistically significant.

Future studies may measure lipopolysaccharide (**LPS**) concentration in the rumen, large intestine, feces, and blood to compare between AR and AS to evaluate where LPS may be trans-located to stimulate an acute phase response following a grain challenge. Measurements of ruminal motility and rate of passage may provide additional insights. Further, assessing barrier integrity of the rumen vs. large intestine might indicate where translocation occurs. One such method to do so is to block the omasal orifices of the rumen as described by Kristensen et al. (2000), and infuse a marker such as Cr-EDTA solution into the rumen, and measure its concentration in the urine after a period of time (DeMeo et al., 2002). Concentration in the urine should be relatively low because Cr-EDTA is a large molecule and should not be able to diffuse out of an intact rumen. If there is no difference in Cr-EDTA in the urine, a subsequent experiment would then infuse Cr-EDTA without blocking the omasal orifices and measure its concentration in the urine. If there is a difference in urine Cr-EDTA, it may be attributed to damage to the intestinal epithelium.

4.2.5 Systemic effects of acidosis

A limiting factor of Study 2 is that only ruminal parameters were measured. Future studies in this area should assess systemic parameters as well. For example, blood flow to the rumen increases after feeding, which helps to increase the rate of absorption of ruminal metabolites (Dobson, 1984). In Study 2, mRNA abundance of NHE3 was highest for resistant animals at 2-h after feeding. Interestingly, Barnes et al. (1983) evaluated blood flow to the rumen of sheep prior to feeding until 4-h after feeding and found that blood flow to the ruminal epithelium was highest 2-h after feeding. Therefore, future studies should assess blood flow to the rumen, as acidosis resistant animals may have higher blood flow to the rumen compared with that of susceptible animals.

Study 2 also raised the question as to whether acidosis resistance is beneficial, as we saw numerically higher plasma SAA in the resistant steers. The lower VFA concentration in the rumen of resistant steers may be due to faster rate of passage to the lower GI tract, which may have also increased plasma SAA. Khafipour et al. (2009a,b) observed an acute phase response only when SARA was induced using a grain challenge vs. insufficient physically effective NDF. They attributed this to fermentation of starch in the large intestine and subsequent translocation of endotoxin across the intestinal barrier. This may indicate that AR steers had a greater rate of passage compared with AS, resulting in a higher amount of starch fermenting in the large intestine and ultimately a greater acute phase response. In addition, the lower VFA concentration in the rumen of acidosis resistance steers might be attributed to rapid absorption of VFA, which may affect acid base balance of the blood. Blood pH is tightly regulated and as such, minor

incidences of metabolic acidosis such as those causes by SARA should not drastically alter it. However, other factors may indicate a disturbance in acid-base balance, such as a greater concentration of NH_4 in urine (Terao and Tannen, 1980). Therefore, measuring parameters such as NH_4 in urine may be a better indicator of systemic acidosis compared with blood pH.

4.3 Conclusions

Sub-acute ruminal acidosis causes substantial economic losses to the dairy industry each year, but it is very difficult to detect it. Further research is important to better understand the physiology of acidosis and what causes individual variation in susceptibility to this disorder. Heritability of acidosis resistance can also be evaluated in order to develop breeding programs to improve herd health. Identifying parameters that are correlated with ruminal pH reduction and that can be measured easily, quickly, and inexpensively such as blood metabolites or genetic markers would help producers evaluate the susceptibility of their animals to acidosis. This would allow producers to formulate diets providing optimal fermentable energy (e.g. higher fermentable diet for acidosis resistant animals). This would maximize productivity of the animals without increasing the occurrence of SARA, and thus improve animal welfare and profit.

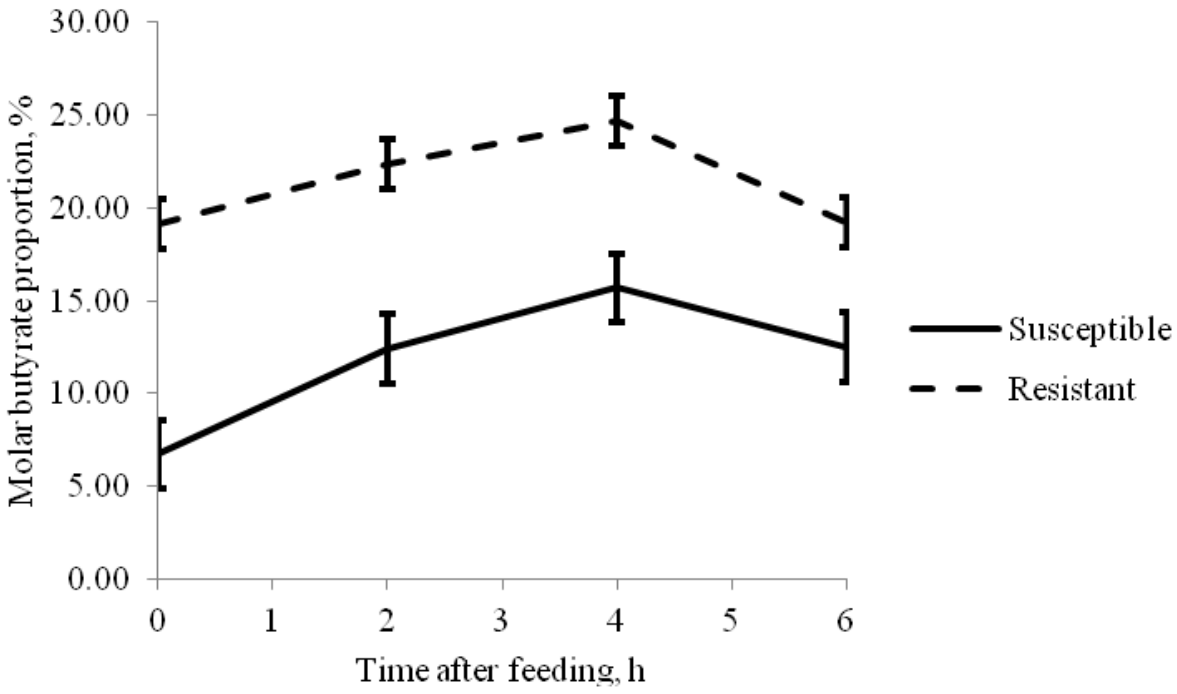

Figure 4-1. Comparison of molar butyrate proportion between acidosis resistant and susceptible steers after feeding. Group effect, $P < 0.01$, hour effect, $P < 0.05$, group × hour interaction, $P = 0.02$.

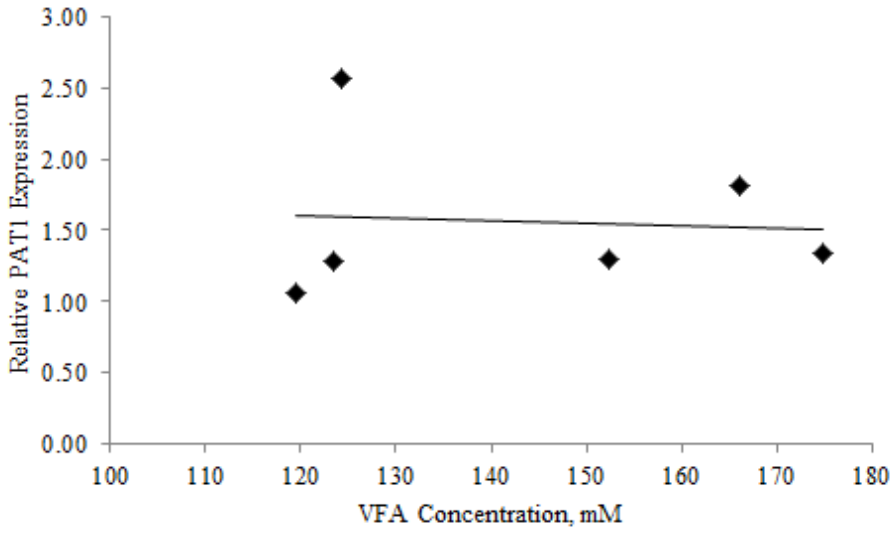

Figure 4-2. Relationship of PAT1 expression to VFA concentration in the rumen. Regression is PAT1 expression = -0.0081 × VFA concentration, mM + 1.8167 (n = 6, *P* = .88, r^2 = -0.24)

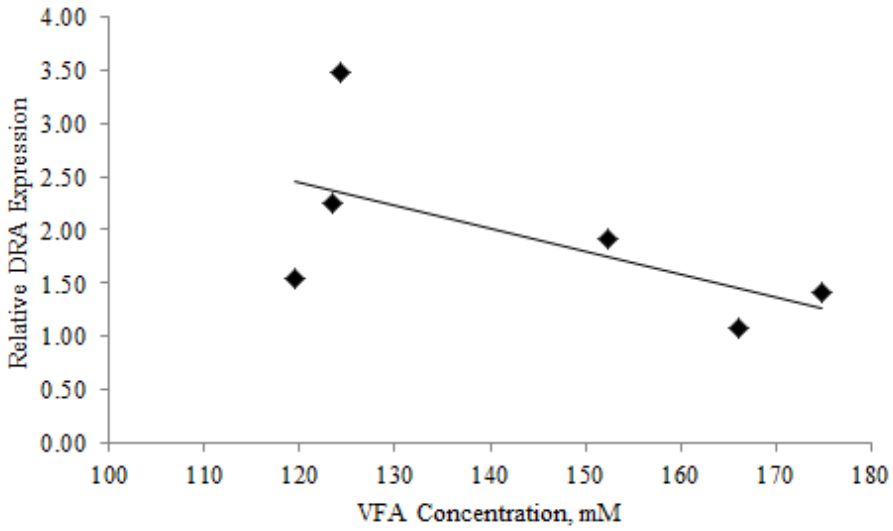

Figure 4-3. Relationship of DRA expression to VFA concentration in the rumen. Regression is DRA expression = -0.0215 × VFA concentration, mM + 5.0291 (n = 6, *P* = 0.20, r^2 = 0.21)

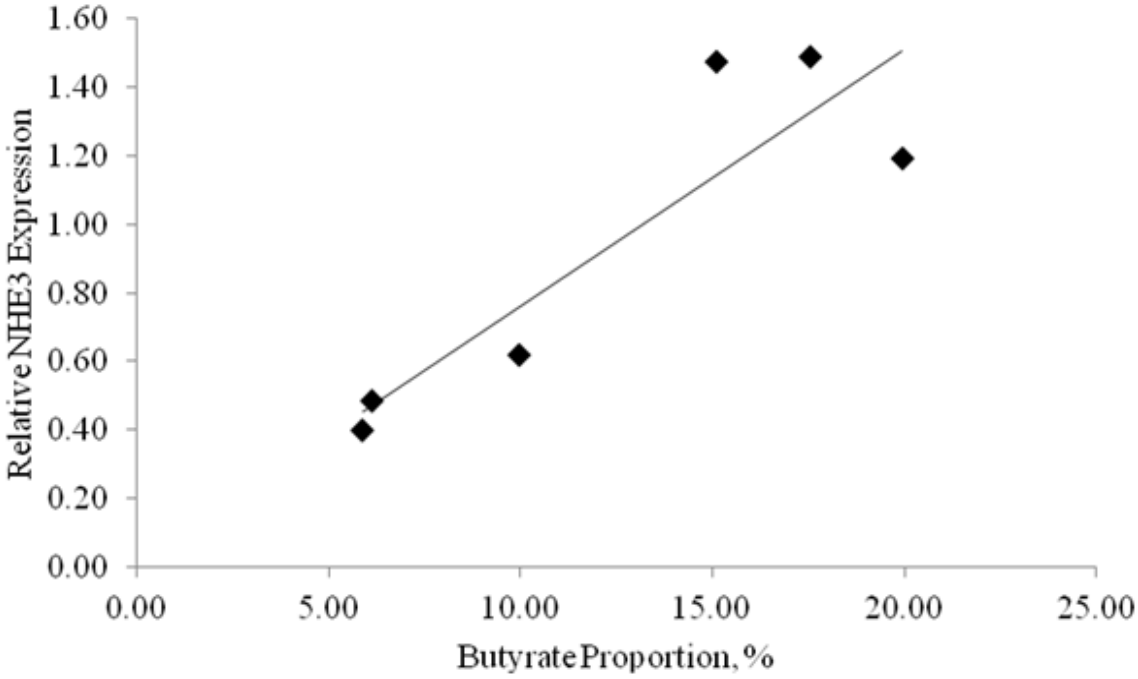

Figure 4-4. Relationship of NHE3 expression to molar butyrate proportion in the rumen. Regression is NHE3 expression = 0.0747 × butyrate proportion, % + 0.0148 (n = 6, $P = 0.02$, $r^2 = 0.74$)

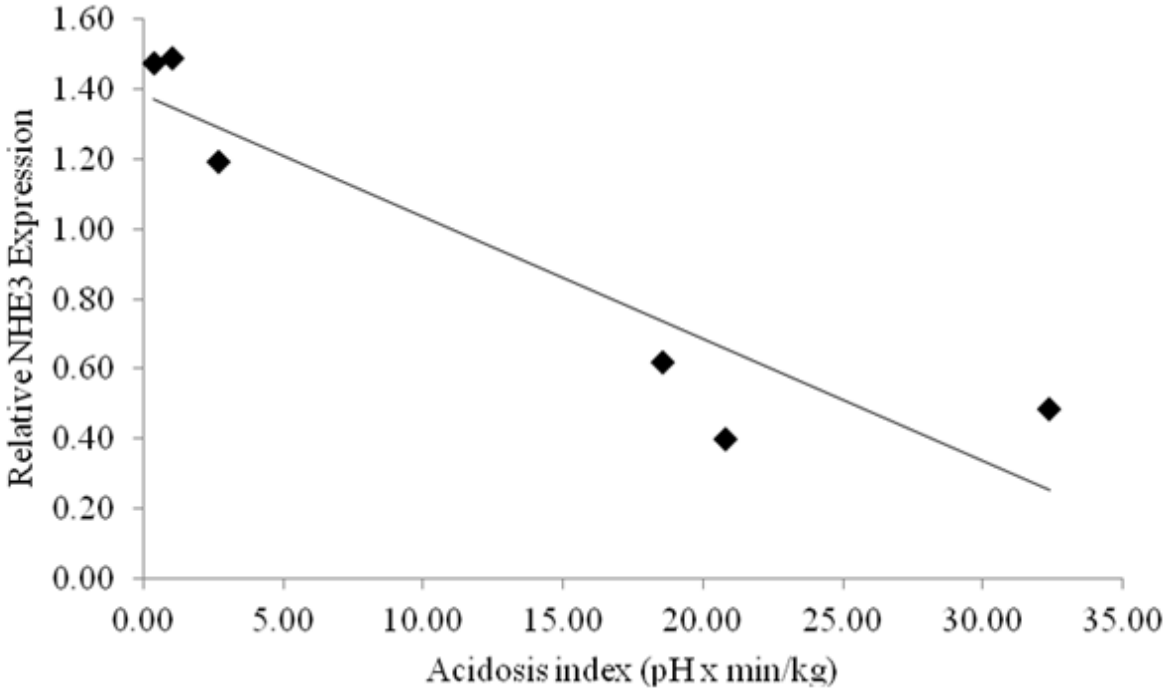

Figure 4-5. Relationship of NHE3 expression to acidosis index. Regression was NHE3 expression = - 0.0351 × acidosis index, pH × min × kg^{-1} + 1.3873, (n = 6, $P < 0.01$, adjusted $r^2 = 0.82$)

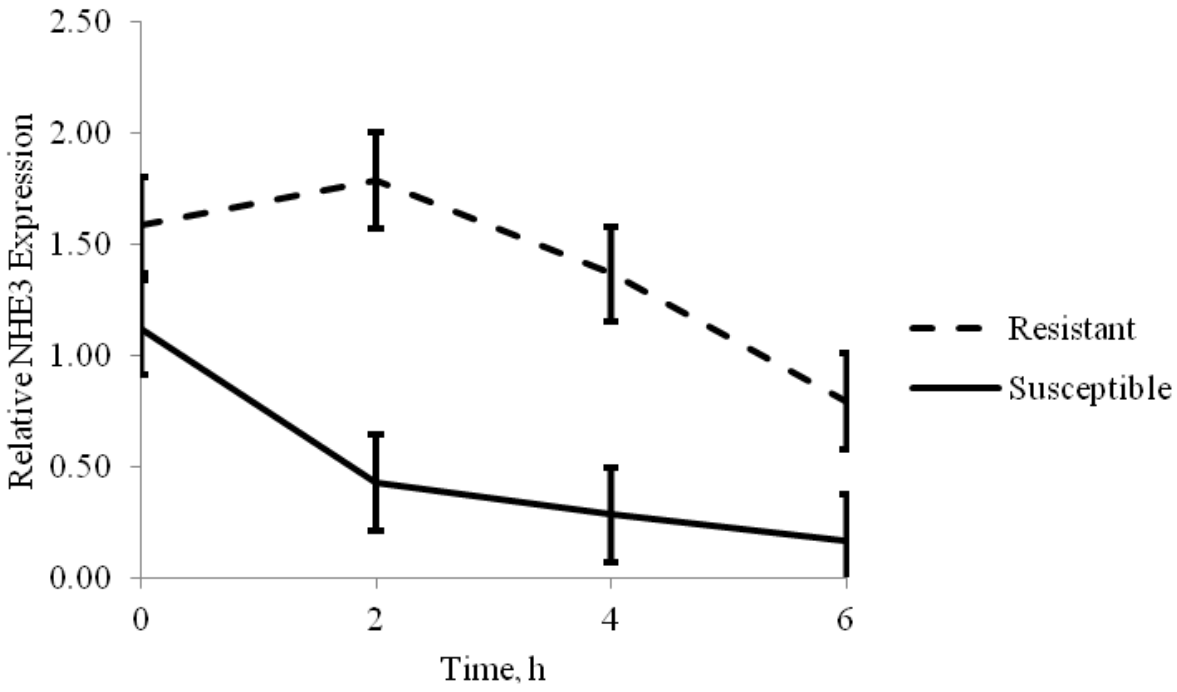

Figure 4-6. Comparison of NHE3 expression between acidosis resistant and susceptible animals after feeding. Group effect $P < 0.005$, hour effect $P = 0.03$, group × hour interaction $P = 0.43$

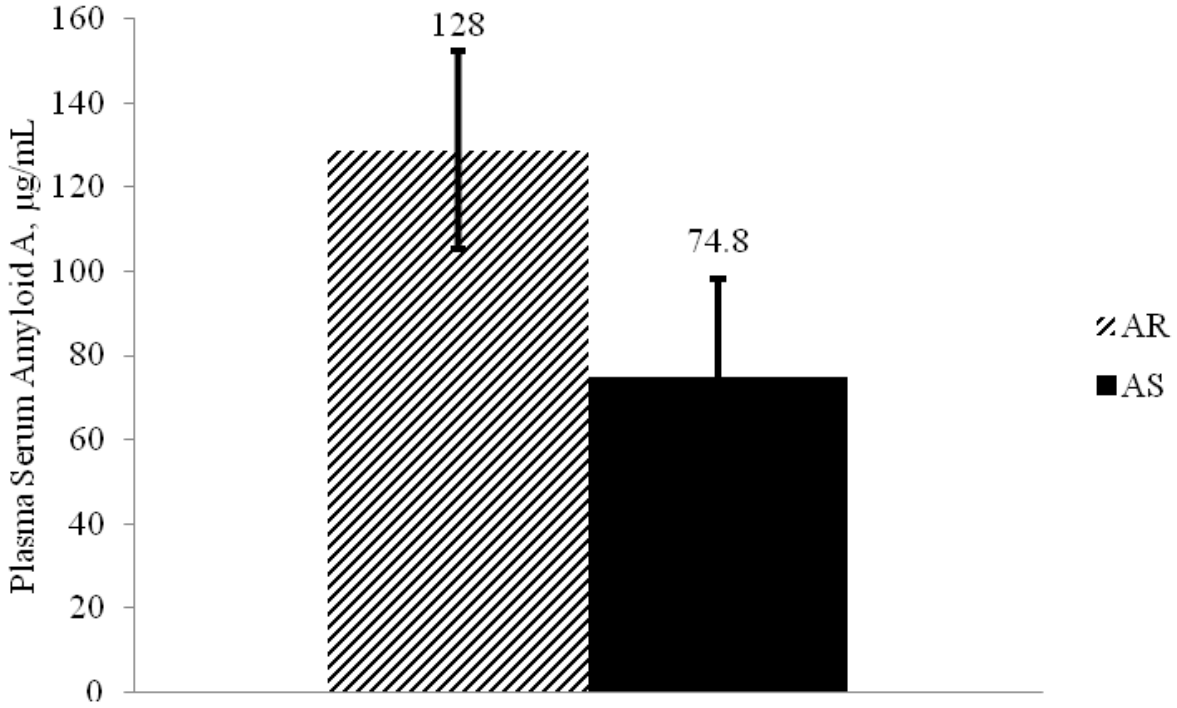

Figure 4-7. Comparison of plasma serum amyloid A concentration between AR and AS animals. Group effect $P = 0.18$, hour effect $P = 0.14$, group × hour interaction $P = 0.81$

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